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MOLECULAR MECHANISMS OF HUMAN γδ T CELL ACTIVATION AND TUMOR CELL RECOGNITION

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MECANISMOS MOLECULARES DE ATIVAÇÃO DAS CÉLULAS T γδ HUMANAS NA SUA INTERAÇÃO COM CÉLULAS TUMORAIS

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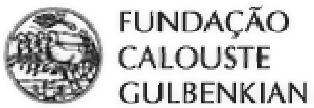

Para os meus pais,

Cesário e Filomena

Somewhere, something incredible is waiting to be known.

Carl Sagan

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1. Correia DV*****, d'Orey F*****, Cardoso BA, Lança T, Grosso AR, deBarros A, Martins LR, Barata T, Silva-Santos B. **Highly active microbial phosphoantigen induces rapid yet sustained MEK/Erk- and PI-3K/Akt-mediated signal transduction in anti-tumor human γδ T-cells.** PLoS One. **2009** ; 4(5):e5657. *co-first authors.

2. Gomes AQ*****, Correia DV*****, Grosso AR, Lança T, Ferreira C, Lacerda JF, Barata T, Gomes da Silva M, Silva-Santos B. **Identification of a panel of ten cell surface protein antigens associated with immunotargeting of leukemias and lymphomas by peripheral blood gamma-delta T cells.** Haematologica. **2010** ; 95(8):1397-404. Epub 2010/03/12. *co-first authors.

3. Correia DV, Fogli M, Hudspeth K, da Silva MG, Mavilio D, Silva-Santos B. **Differentiation of human peripheral blood Vδ1^{+} T cells expressing the natural cytotoxicity receptor NKp30 for recognition of lymphoid leukemia cells.** Blood. **2011** ; 118(4):992-1001. Epub 2011/06/03.

4. Lança T, Correia DV, Moita CF, Raquel H, Neves-Costa A, Ferreira C, Ramalho JS, Barata JT, Moita LF, Gomes AQ, Silva-Santos B. **The MHC class Ib protein ULBP1 is a nonredundant determinant of leukemia/lymphoma susceptibility to gammadelta T-cell cytotoxicity**. Blood. **2010** ; **115**(12): 2407-11.

5. Goncalves-Sousa N, Ribot JC, deBarros A, Correia DV, Caramalho I, Silva-Santos B. **Inhibition of murine gammadelta lymphocyte expansion and effector function by regulatory alphabeta T cells is cell-contact-dependent and sensitive to GITR modulation**. Eur J Immunol. **2010** ; **40**(1): 61-70.

6. Gomes AQ, Correia DV, Silva-Santos B. **Non-classical major histocompatibility complex proteins as determinants of tumour immunosurveillance.** EMBO Rep. **2007** ; **8**(11): 1024-30.

7. Hudspeth K, Fogli M, Correia DV, Mikulak J, Roberto A, Silva-Santos B. **Engagement of NKp30 on Vδ1 T-cells induces the production of CCL3, CCL4 and CCL5 and suppresses HIV-1 replication.** Submitted manuscript.

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ABBREVIATIONS

γc	Common Cytokine Receptor Chain
mAb(s)	Monoclonal antibody /antibodies
ADCC	Antibody-dependent cell cytotoxicity
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
APC(s)	Antigen presenting cell(s)
APC	Allophycocyanin
Akt	Protein kinase B
β2m	β2-microglobulin
Bcl-2	B-Cell Lymphoma 2
BCR(s)	B cell receptor(s)
BrHPP	Bromohydrin pyrophosphate, also known as “Phosphostim”
CaMK	Ca^{2+}- calmodulin dependent kinase
CBA	Cytometric Bead Array
CD	Cluster of Differentiation
CD3	CD3 molecule, (CD3-TCR complex)
CD4	Monomeric IgG superfamily protein expressed on $CD4^+$ T cells
CD8	Homo or Heterodimeric protein co-receptor expressed on $CD8^+$ T cells
CD25	Interleukin-2 receptor α
CD28	CD28 molecule
CDR(s)	Complementarity-determining region(s)
CFSE	Carboxyfluorescein succinimidyl ester, a fluorescent cell staining dye
CLL	Chronic Lymphocytic Leukemia
CT	Cancer-testis
CTL(s)	Cytotoxic T lymphocyte(s)
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DAG	Diacylglycerol
DC(s)	Dendritic cell(s)
DLBCL	Diffuse large B cell lymphoma
DNAM-1	DNAX accessory molecule-1
DNA	Deoxyribonucleic Acid
EBV	Epstein-Barr virus

Erk	Extracellular-signal-regulated kinase
FcRγ	Fc fragment of IgG, receptor (CD16)
FCS	Foetal Calf Serum
FITC	Fluorescein
FL	Follicular lymphoma
GM-CSF	Granulocyte–macrophage colony stimulating factor
HDMAPP	Hydroxy-dimethyl-allyl-pyrophosphate
HLA	Human Leukocyte Antigen
HMBPP	(E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
HSP(s)	Heat shock protein(s)
HCMV	Human cytomegalovirus
ICAM-1	Intercellular Adhesion Molecule 1
ICOS	Inducible T-cell co-stimulator
IEL(s)	Intraepithelial lymphocyte(s)
IFN	Interferon
Ig	Immunoglobulin
IL-2	Interleukin-2
IL-2R	Interleukin-2 Receptor
iNKR	Inhibitory Natural Killer cell-associated receptor
IP3	Inositoltriphosphate
IPP	Isopentenyl pyrophosphate
ITAM(s)	Immunoreceptor tyrosinebased activation motif(s)
ITIM(s)	immunoreceptor tyrosine-based inhibitory motif(s)
KIR(s)	Killer-cell immunoglobulin-like receptor(s)
LAT	Linker of Activated T cells
Lck	Lymphocyte-specific protein tyrosine kinase
LIR	Leukocyte Ig-like receptors
LPS	Lipopolysaccharide
LTi	Lymphoid tissue inducer
MACS	Magnetic-activated cell sorting
MAGE	Melanoma antigen family
MAPK	Mitogen-activated protein kinase
MCA	Methylcholanthrene
MEP	2-C-methyl-D-erythritol 4-phosphate

MFI	Median Fluoresence Intensity
MHC	Major Histocompatibility Complex
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B
mRNA	Messenger RNA
NCR	Natural cytotoxicity triggering receptor
NFAT	Nuclear Factor of Activated T Cells
NF-κB	Nuclear Factor Kappa B
NHL	Non-Hodgkin's lymphoma
NK	Natural Killer
NKR(s)	Natural Killer cell–associated receptor(s)
NKT	Natural Killer T
NOD	Non-obese diabetic
OKT3	anti-CD3 antibody, clone OKT3
PAMP(s)	Pathogen associated molecular pattern(s)
PHA	Phytohemagglutinin
PI3K	Phosphatidylinositol 3-kinase
PB	Peripheral Blood
PBL(s)	Peripheral Blood Lymphocyte(s)
PBMC(s)	Peripheral Blood Mononuclear Cell(s)
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PD-1	Programmed cell death protein 1
PLCγ1	Phospholipase Cγ1
PE	Phycoerythrin
Pen/Strep	Penicillin Streptomycin
PerCP	Peridinin chlorophyll
PerCP -Cy5.5	Peridinin chlorophyll-Cy5.5
PKCθ	Protein kinase Cθ
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptors
PTK	Protein tyrosine kinases
RAG	Recombination activating gene
Rag	Recombination activating gene
Ras	RAt Sarcoma

RNA	Ribonucleic Acid
RPMI	Roswell Park Memorial Institute cell culture medium
RT-qPCR	Real-Time –quantitative Polymerase Chain Reaction
SCID	Severe Combined Immunodeficiency
SLP 76	Lymphocyte cytosolic protein 2
SOCE	Store-operated calcium entry
STAT	Signal transducer and activator of transcription
Syk	Spleen tyrosine kinase
TAA(s)	Tumor-associated antigen(s)
TAP	Transporter, ATP-binding cassette
TCM	Central Memory T cell
TCR(s)	T Cell Receptor(s)
TEIPP	T-cell epitopes associated with impaired peptide processing
TEM	Effector Memory T cell
TIL(s)	Tumor infiltrating lymphocyte(s)
TGF-β	Transforming Growth Factor Beta
Th1	T helper cell type 1: defined by their ability to produce Inteferon gamma
TLR(s)	Toll-like receptor(s)
TNF	Tumor Necrosis Factor
TNFR	Tumor necrosis factor receptor
TLR(s)	Toll-like receptor(s)
TSA(s)	tumor-specific antigen(s)
UCB	Umbilical cord blood
ULBP(s)	UL16 binding protein(s)
UV	Ultraviolet
ZAP70	zeta-chain (TCR) associated protein kinase 70kDa

SUMÁRIO

Os animais vertebrados mandibulados possuem um sistema imunitário que inclui várias populações de linfócitos capazes de reconhecer e eliminar células tumorais, e que constituem a base da imunoterapia contra o cancro.

Os linfócitos T γδ representam cerca de 1-15% dos linfócitos no sangue periférico humano (PBL), e constituem a maioria das células T nos tecidos epiteliais de indivíduos saudáveis. Tem sido demonstrado que os dois principais tipos de células γδ humanas, Vδ1$^+$ e Vδ2$^+$, apresentam uma forte citotoxiciade contra células tumorais de diversas origens celulares, as quais reconhecem independentemente da apresentação de antigénios por moléculas do complexo major de histocompatibilidade (MHC).

As estratégias atuais de imunoterapia para o cancro utilizando células T γδ baseiam-se na exclusiva reactividade das células Vγ9Vδ2, o subtipo de células γδ dominante no sangue periférico, para antigénios de origem não peptídica, denominados prenil-pirofosfatos (fosfoantigénios). No entanto, os mecanismos moleculares responsáveis pela activação das células Vγ9Vδ2 por fosfoantigénios não são ainda bem conhecidos. Neste estudo, caracterizámos os eventos celulares e moleculares despoletados nas células Vγ9Vδ2 pelo (E)-4-hidroxi-3-metil-but-2-enil pirofosfato (HMB-PP), o fosfoantigénio natural mais potente identificado até hoje. Comparámos os efeitos moleculares produzidos após a estimulação por HMB-PP, com os que são produzidos por sinalização clássica, através do complexo do receptor de células T (TCR), induzida por tratamento com o anticorpo monoclonal anti-CD3ε (OKT3). O HMB-PP activou as vias de sinalização molecular MEK/Erk e PI-3K/Akt, tão rapidamente e eficientemente quanto o OKT3, e induziu um padrão transcricional praticamente idêntico nas células Vγ9$^+$. Adicionalmente, as actividades das vias de sinalização MEK/Erk e PI-3K/Akt mostraram-se indispensáveis para os efeitos celulares do HMB-PP, incluindo a activação, proliferação e citotoxicidade anti-tumoral das células T γδ. Estas actividades foram inibidas após o bloqueio do TCR Vγ9$^+$ por anticorpos específicos, durante o período de estimulação. Assim, os nossos dados caracterizam detalhadamente o HBM-PP como agonista do TCR Vγ9Vδ2.

A elucidação dos mecanismos moleculares responsáveis pela ativação das células T Vγ9Vδ2, permitiu a dissecação subsequente dos mecanismos implicados no

reconhecimento das células tumorais. Foram analizadas várias linhas celulares tumorais hematológicas, identificadas como suscetíveis ou resistentes às células T Vγ9Vδ2 ativadas (tratadas com HMB-PP), para identificação de potenciais determinantes de reconhecimento tumoral, e de biomarcadores que poderão ser úteis em ensaios clínicos de cancro baseados em células T Vγ9Vδ2. Realizámos um estudo abrangente de transcritómica em linhas celulares e em amostras primárias de leucemia linfoblástica aguda e linfoma não-Hodgkin, utilizado microarrays de cDNA e PCR quantitativo em tempo real. Identificámos um painel de 10 genes codificando proteínas membranares de superfície, diferencialmente expressos entre tumores hematológicos resistentes versus sensíveis às células T Vγ9Vδ1. Dentro desta lista, 3 genes (*ULBP1, TFR2* e *IFITM1*) foram associados a uma maior suscetibilidade à citotoxicidade das células T Vγ9Vδ2, ao passo que outros 7 (*CLEC2D, NRP2, SELL, PKD2, KCNK12, ITGA6* e *SLAMF1*) estavam sobre-expressos nos tumores resistentes, sugerindo que os tumores hematológicos apresentam um repertório altamente variável de proteínas superficiais que podem influenciar o seu reconhecimento pelas células Vγ9Vδ2. Entre os potenciais biomarcadores, demonstrámos que o ULBP-1 é um determinante não-redundante do reconhecimento de tumores hematológicos pelas células T Vγ9Vδ2. Adicionalmente, observámos uma frequente diminuição da expressão de ULBP1 em amostras primárias de doentes com linfoma ou leucemia, a qual foi associada a resistência à citotoxicidade de células T Vγ9Vδ2 *in vitro*.

Com o objetivo de contrariar potenciais mecanismos de escape ao sistema imunitário (tais como a perda da expressão de ULBP-1), testámos uma série de compostos ativadores de células T, no sentido de aumentar a citotoxicidade das células γδ contra os tumores resistentes. A fitohemaglutinina (phytohemagglutinin ; PHA), um mitogénio extraído de plantas, combinada com IL-2, induziu a diferenciação de um novo subtipo de células $V\delta2^{(-)}$ $V\delta1^{+}$ do sangue periférico, altamente citolítico e que expressa receptores de citotoxicidade natural (NCRs). Assim, a expressão dos recetores NKp30, NKp44 e NKp46 pode ser seletivamente induzida em células $V\delta1^{+}$, através de sinais mediados pela cinase AKT, despoletados sinergisticamente por citocinas da família γc (IL-2 ou IL-15) e estimulação via TCR. Experiências de ganho ou perda de funções específicas demonstraram que o recetor NKp30 é o NCR mais

importante para o reconhecimento de células de leucemia. As células NKp30^{+} Vδ1^{+} constituem, pois, uma nova e indutível população de células citotóxicas especializadas, cujo potencial imunoterapêutico contra o cancro deve ser avaliado em futuros ensaios clínicos.

Palavras-chave: linfócitos T $\gamma\delta$; células T Vγ9Vδ2; células T Vδ1^{+}; fosfoantigénios; recetores de citotoxicidade natural; leucemia; linfoma; imunoterapia contra o cancro.

SUMMARY

The immune system of jawed vertebrates includes various lymphocyte populations capable of recognizing and eliminating tumor cells, which constitutes the basis of cancer immunotherapy.

γδ T lymphocytes are innate-like cells that account for 1-15% of human peripheral blood lymphocytes (PBL), and represent the majority of T cells in epithelial tissues of healthy individuals. Moreover, it is well established that both $V\delta1^+$ and $V\delta2^+$ γδ T cell subsets are endowed with strong, MHC-unrestricted cytotoxicity against tumor cells of diverse tissue origin.

The unique responsiveness of Vγ9Vδ2 T cells, the dominant subset of γδ PBLs, to non-peptidic prenyl-pyrophosphate antigens (phosphoantigens), constitutes the basis of current γδ T cell-based cancer immunotherapy strategies. However, the molecular mechanisms responsible for phosphoantigen-mediated activation of Vγ9Vδ2 T cells have remained unclear. We have here characterized the cellular and molecular events triggered in Vγ9Vδ2 T cells by the most potent natural phosphoantigen yet identified, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP). We compared the molecular signatures produced by HMB-PP stimulation, with those of canonical T cell receptor (TCR) complex signaling, induced by anti-CD3ε monoclonal antibody (OKT3) treatment. HMB-PP activated the MEK/Erk and PI-3K/Akt signaling pathways as rapidly and efficiently as OKT3, and induced an almost identical transcriptional profile in $V\gamma9^+$ T cells. Moreover, MEK/Erk and PI-3K/Akt activities were indispensable for the cellular effects of HMB-PP, including γδ T cell activation, proliferation and anti-tumor cytotoxicity, which were also abolished upon antibody blockade of the $V\gamma9^+$ TCR during the stimulation period. Thus, our data provided a detailed characterization of HMB-PP as a putative Vγ9Vδ2 TCR agonist.

The elucidation of the molecular mechanisms responsible for Vγ9Vδ2 T cell activation, permitted the subsequent dissection of the mechanisms involved in tumor cell recognition. Several hematological tumor cell lines, identified as susceptible or resistant to fully-activated (HMB-PP-treated) Vγ9Vδ2 T cells, were screened for the identification of potential determinants of tumor cell targeting, and biomarkers that could

be useful for Vγ9Vδ2 T cell-based cancer clinical trials. We performed a comprehensive transcriptomics study using cDNA microarrays and quantitative real-time PCR, in acute lymphoblastic leukemia and non-Hodgkin's lymphoma cell lines and primary samples. We identified a panel of 10 genes encoding cell surface proteins that were statistically differentially expressed between "γδ-susceptible" and "γδ-resistant" hematopoietic tumors. Within this panel, 3 genes (*ULBP1*, *TFR2* and *IFITM1*) were associated with increased susceptibility to Vγ9Vδ2 T-cell cytotoxicity, whereas the other 7 (*CLEC2D*, *NRP2*, *SELL*, *PKD2*, *KCNK12*, *ITGA6* and *SLAMF1*) were enriched in resistant tumors, suggesting that hematological tumors display a highly variable repertoire of surface proteins that can impact on Vγ9Vδ2 T cell-mediated immunotargeting. Among the candidate biomarkers, we established ULBP-1 as a non-redundant determinant of Vγ9Vδ2 T cell recognition of hematological tumors. Furthermore, we observed a frequent down-modulation of ULBP1 expression in primary samples from leukemia and lymphoma patients, which associated with resistance to Vγ9Vδ2 T cell cytotoxicity *in vitro*.

Aiming to overcome potential immune escape mechanisms (such as loss of ULBP-1 expression), we screened a series of T cell-activating compounds in order to elicit γδ T cell-mediated killing of resistant tumors. Phytohemagglutinin (PHA), a plant-derived mitogen, combined with IL-2, induced the differentiation of a novel, highly cytolytic subset of $V\delta2^{(-)}$ $V\delta1^{+}$ PBLs expressing natural cytotoxicity receptors (NCRs). Thus, NKp30, NKp44 and NKp46 could be selectively upregulated in $V\delta1^{+}$ cells by AKT-dependent signals provided synergistically by γ_c cytokines (IL-2 or IL-15) and TCR stimulation. Specific gain-of-function and loss-of-function experiments demonstrated that NKp30 makes the most important contribution to leukemia cell recognition. Thus, $NKp30^{+}$ $V\delta1^{+}$ T-cells constitute a novel, inducible and specialized killer lymphocyte population whose potential for cancer immunotherapy should be evaluated in future clinical trials.

Keywords: γδ T lymphocytes; Vγ9Vδ2 T cells; $V\delta1^{+}$ T cells; phosphoantigens; natural cytotoxicity receptors; leukemia; lymphoma; cancer immunotherapy.

1 GENERAL INTRODUCTION

1.1 T CELLS IN TUMOR SURVEILLANCE

1.1.1 The tumor immunosurveillance theory

Cell transformation may occur as a result of the normal cell metabolism. The fundamental mechanisms of cellular division and DNA replication carry the inherent danger that the replication machinery will inevitably make mistakes, compromising the integrity of the genome. Added to this, all cells are exposed to harmful environmental or biological agents (such as UV radiation, viruses, toxic elements, etc.), that will damage their DNA. Cancer is a essentially a genetic disease that arises when the natural tumor suppressor mechanisms fail to prevent cell transformation and uncontrolled growth[1].

The ideia of a tumor suppressor immune mechanism was conceived by Paul Ehrlich, 102 years ago. Ehrlich suggested that cells of the immune system are able to eliminate tumors, much like they destroy pathogens or pathogen-infected cells[2]. A cancer immunosurveillance theory was formulated in the 1950s by McFarlane Burnet and Lewis Thomas, who proposed that lymphocytes act as sentinels in recognizing and eliminating continuously arising, nascent transformed cells[3,4].

Tumor immunology research intensified in the mid-1990s after the observation that transplanted tumors grew more robustly in mice treated with neutralizing monoclonal antibodies specific for interferon-γ (IFN-γ), a key molecular component of the immune system[5]. Indeed, immunodeficient mice that lacked either IFN-γ responsiveness or an intact T cell compartment were more susceptible to methylcholanthrene (MCA)-induced sarcoma formation[6-8]. Since then, extensive research has established cancer immunosurveillance as an important host protection process that maintains regular cellular homeostasis in mice[9-11]. Moreover, several human and mouse tumor antigens that are recognized by the host immune system have been identified[12,13]. It is now widely accepted that the immune system can act as a tumor-suppressor mechanism at different levels: i) it can protect the host from virus-induced tumors by eliminating or suppressing viral infections; ii) it can prevent the establishment of an inflammatory environment conducive to tumorigenesis by eliminating the pathogens that cause it; and importantly, iiii) the immune system can specifically identify and eliminate tumor cells in

certain tissues on the basis of their expression of tumor-specific antigens (TSAs) or tumor-associated antigens (TAAs ; reviewed in[14]). Thus, the immune system has evolved as a complex mechanism able to recognize, not only "host versus exogenous pathogens", but also to distinguish between "self versus altered self"[15].

1.1.2 **The immune response against tumors**

The immune response is broadly classified into either the innate (antigen-nonspecific response), or the adaptive (antigen-specific response). Leukocytes of the innate immune system serve as sentinels for detecting general signs of danger. These cells are usually equipped with pattern recognition receptors (PRR) leading to a fast and unspecific activity against groups of pathogens. As so, these innate myeloid cells (Phagocytes, Mast cells, Basophils, Eosinophils) and innate-like lymphocytes (NK cells, NKT cells and $\gamma\delta$ T cells) constitute the first line of defense against pathogens and/or transformed cells[16,17].

Innate responses can also modulate the adaptive branch of the immune system[18]. The adaptive system is constituted by B cells and $\alpha\beta$ T lymphocytes, which react specifically to each individual pathogen. Adaptive immunity is triggered later and usually generates memory cells that persist in the organism and can be reactivated upon a second encounter with the same antigen. The cells of the adaptive immune system express a comprehensive repertoire of cell surface antigen-specific immunoglobulin receptors - B cell receptors (BCR) for B cells; and T cell receptors (TCR) for T cells - that can collectively recognize millions of distinct antigens[19,20].

The ability to recognize and eliminate transformed cells is common to several lymphocyte subsets of both the innate and adaptive systems[14,21,22]. Specifically, genetic, immunochemical or functional ablations of NK cells, NKT cells, $\gamma\delta$ T cells or $\alpha\beta$ T cells all lead to increased susceptibility of the host to tumors[21,23]. Immunodeficient mice lacking either IFN-γ responsiveness or recombination activating gene-2 ($Rag2^{-/-}$, failing to generate T, B, and Natural Killer T lymphocytes) develop more spontaneous neoplasia upon aging and are more susceptible to MCA carcinogen-induced sarcomas compared with wild-type mice[9].

1.1.3 The Cancer Immunoediting hypothesis

The immune system acts as an extrinsic tumor suppressor, but paradoxically can also promote cancer initiation, promotion, and progression[23,24]. In fact, a significant proportion (40%) of MCA sarcomas derived from immunodeficient Rag2$^{-/-}$ mice were spontaneously rejected when transplanted into naive syngeneic wild-type mice, whereas all MCA sarcomas derived from immunocompetent wild-type mice grew progressively when transplanted into naive syngeneic wild-type hosts[9]. Thus, tumors formed in the absence of an intact immune system are, as a group, more immunogenic than tumors that arise in immunocompetent hosts. The immune system not only protects the host against tumor formation, but also edits tumor immunogenicity. These new data prompted a refinement of the cancer immunosurveillance concept and led to the formulation of the cancer immunoediting hypothesis, which stresses the dual host-protective and tumor-sculpting actions of immunity on developing tumors. We now view cancer immunoediting as a dynamic process composed of three distinct phases: elimination, equilibrium, and escape[14].

1.1.3.1 Antigen recognition by T cells

The fundamental property of T cells is the expression of a T cell receptor. T cells use their signature TCR to recognize antigens and initiate cellular immune responses. The specificity of most T cell responses is conferred by the $\alpha\beta$ TCR; an alternative $\gamma\delta$ TCR with limited diversity is present on about 1% to 5% of peripheral T cells, and on the majority of intraepithelial T cells. TCRs are formed by two transmembrane glycoproteins, each composed of one extracellular variable and constant domain joined by a hinge region to the transmembrane domain[25]. The diversity of the TCR is primarily found in the variable region, which is formed at the DNA level by the juxtaposition of V, D, and J gene segments (β or δ chain) or V and J gene segments (α or γ chains). These gene segments are assembled by RAG-dependent somatic recombination, generating millions of different TCR gene combinations. The addition or removal of nucleotides at joining sites, further contributes to the V–(D)–J junctional-region complexity[25,26]. Different aminoacid sequences will create distinct molecular surfaces at the TCR

complementarity-determining regions (CDRs), generating TCRs with very different binding specificities.

αβ TCRs bind to short fragments of antigens that have been broken down and loaded onto Major Histocompatibility Complex (MHC) molecules (also known as Human leukocyte antigens, HLA)[20,27]. MHC molecules are highly polymorphic, with multiple alleles of several genes giving rise to the protein products. Antigen processing and presentation for subsequent T cell recognition by the αβ TCR can occur through different pathways. On one hand, all nucleated cells, including tumor cells, have the capacity to directly present endogenous antigens to $CD8^+$ cytotoxic T cells in the context of MHC class I molecules. On the other hand, professional antigen presenting cells (APCs) can present antigen bound to both MHC class I and MHC class II molecules[20]. For example, dendritic cells (DCs) can phagocytize exogenous pathogens such as bacteria, parasites, and toxins, and display the corresponding processed antigens on MHC Class II molecules, which interact with $CD4^+$ helper T cells. Binding of a TCR with its cognate peptide-MHC complex triggers T cell activation.

1.1.3.2 **T cell activation**

T cell activation typically requires signal transduction via the MAPK/ Erk and PI3 K/ Akt pathways, which relay information to Fos/Jun and NF-kB/NFAT transcription factors, respectively, and these control the expression of genes like IL-2, cyclins and Bcl2 family members (Figure 1; reviewed in[28]). In summary, for αβ T cells, TCR/CD3 ligation results in the activation of Src (p56 Lck and Fyn) protein tyrosine kinases (PTK), leading to phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) present in the CD3 subunits of the TCR/CD3 complex. Recruitment of the PTK Zap-70 to such phosphorylated ITAMS forms a signaling molecular complex, containing the proteins LAT, SLP76 and GADS. This allows the afferent TCR/PTK signal to spread into multiple distal signaling pathways[28,29]. Following TCR ligation, the phospholipase Cγ1 (PLCγ1) binds to the proximal complex where it is phosphorylated, and then hydrolyzes the membrane lipid $PI(4,5)P_2$ into inositoltriphosphate (IP3) and diacylglycerol (DAG). On one hand, DAG will activate two major pathways involving Ras and protein kinase C θ (PKCθ). The guanine-nucleotide binding protein Ras will in turn activate the MAPK

pathway, leading therefore to the phosphorylation of the extracellular signal-regulated kinase 1 (Erk1) and Erk2[30]. The second major signaling pathway initiated by DAG is mediated by PKCθ recruitment and activation at the plasma membrane level, therefore driving the localization of the nuclear factor kappa B (NFkB) to the nucleus[31].

On the other hand, IP3 generated by PLCγ1 activity will initiate intracellular calcium signals in a process called store-operated calcium entry (SOCE)[32]. This ultimately leads to the activation of several Ca^{2+} dependent signaling proteins and their target transcription factors, such as the phosphatase calcineurin and its target NFAT, Ca^{2+}-calmodulin dependent kinase (CaMK) or NFkB. In immune cells, Ca^{2+} signaling is a major second messenger thought to be involved during the establishment of several cellular functions such as cell differentiation and effector responses.

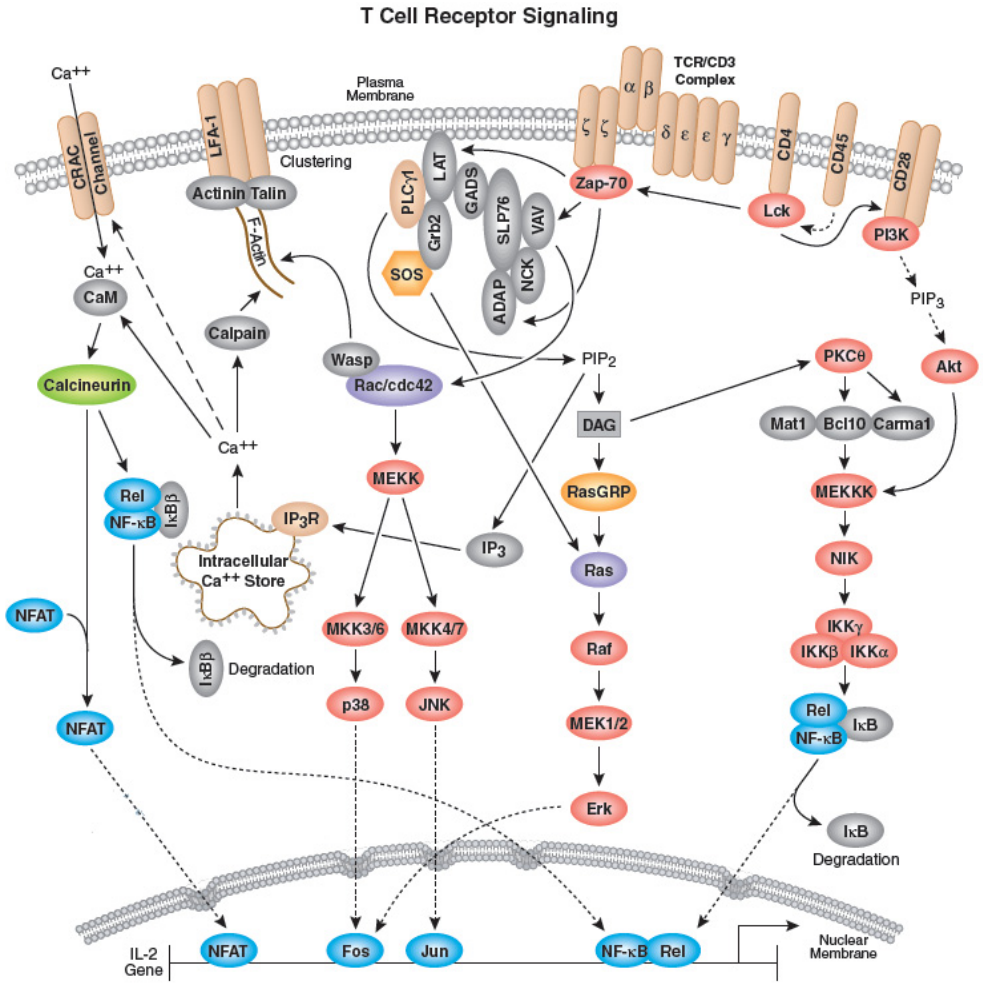


Figure 1 . T cell receptor signaling. Adapted from http://www.cellsignal.com/

While the TCR makes a key contribution to the activation of these molecular pathways, TCR signaling alone is not sufficient to release αβ T cells from their quiescent state. Co-ligation of other receptors, which provide "signal 2" (or costimulation), is usually required, such as CD28. Association of the T cell receptor of a naive T cell with MHC:antigen complex without CD28:B7 interaction results in T cell anergy[28,33]. Several other costimulatory receptors affect T cell activation, division, survival and cytokine secretion. The Immunoglobulin (Ig) superfamily of costimulatory receptors includes CD2, CTLA-4, ICOS and PD-1, among others (reviewed in[34]). The tumor necrosis factor receptor (TNFR) superfamily includes 4-1BB (CD137), OX40 (CD134), CD27 and HVEM, among others (reviewed in[35]).

1.1.3.3 αβ T cell responses

Two major subsets of αβ T cells collaborate to mediate effective immune responses. $CD4^+$ helper T cells are activated after binding a cognate peptide presented by MHC Class II molecules (HLA-DR, -DP, -DQ in humans), and provide cytokine-mediated "help" that enhances phagocyte functions, shapes B cell-mediated humoral responses, and maximizes the quality and durability of the $CD8^+$ T cell-mediated cytotoxic response. $CD8^+$ cytotoxic T lymphocytes (CTLs) are activated after binding antigen presented by MHC class I molecules (HLA-A, -B, -C, in humans) and deploy an amount of cytokines and enzymes that can effectively lyse cellular targets. These targets include host cells altered by either viral infection or oncogenic transformation.

To accomplish their function, $CD8^+$ CTL (like $CD4^+$ T cells), must migrate towards lymphoid organs, where they can interact with professional antigen presenting cells (APCs) (DCs, B cells, macrophages and γδ T cells)[36-38]. After the "priming" provided by APC-T cell interaction, $CD8^+$ CTL can expand and engage the target cells.

Due both to their exquisite antigen specificity and their capacity for a longer, more vigorous secondary response, the use of αβ T cells for the immune-mediated therapy of cancer has attracted great interest. While their need to recognize antigen in the context of MHC presentation may seem a drawback, in fact it allows for T cell recognition of epitopes derived from virtually any protein, whether it is expressed at the cell surface or inside the cell. It also allows sensitivity to even single amino acid changes in proteins,

so that αβ T cells are able respond to mutated proteins but not their normal counterparts. However, to further understand $CD8^+$ CTL antitumor responses it is necessary to introduce the concepts of tumor-specific and tumor-associated antigens.

1.1.4 Tumor antigens for αβ T cells

In 1991, the first human tumor antigen recognized by T cells, called MAGE-1, was discovered[13], and since then, hundreds of other tumor antigens have been described[39,40], and grouped in different categories (Table 1).

T cells can recognize antigens unique to tumors, the so-called tumor-specific antigens. They are found exclusively in tumors, and in many situations are mutated proteins or fusion molecules associated with malignant transformation and progression. For example, antigens expressed in tumors driven by viral infection such as EBV associated lymphoproliferative disorders, or antigens expressed in tumors created by translocations such as the bcr-abl rearrangement in chronic myelogenous leukemia (table 1). On the other hand, T cells can also recognize self antigens enriched in tumors, the so-called tumor-associated antigens. Most T cells auto-reactive to self antigens are usually deleted in the thymus (to avoid auto-immunity), but some T cells with low affinity avoid thymic deletion, and thus retain the ability to recognize tumors bearing self antigens which are expressed at higher levels than in normal tissues.

Category	Description	Examples
Viral antigens	Virus-induced tumors (e.g. EBV, HPV)	EBNA-1, E6, E7
Point mutations	Unique for each tumor	MUM-1, CDK-4, p53
Differentiation antigens	Expressed in tissue lineages	Tyrosinase, GP100
Cancer testis antigens	Largely expressed during development and cancer	MAGE, NYO-ESO-1
Cryptic epitopes	Associated with aberrant transcription or translation	RU2,GnTV,HPX42B
TEIPP	Associated with antigen processing defects	CALCA

Table 1. Categories of human tumor antigens recognized by CTL. Adapted from Lampen *et al.* 2011[41].

Differentiation antigens are expressed predominantly during fetal development and in only minimal quantities in adult tissues. Most of these antigens were originally described in melanomas and melanocytes (tyrosinase, gp100, TRP-1 and MART-1/Melan-A)[42] but others have been well described in other malignancies (CEA, Epcam, PSA).

The most frequently targeted tumor antigens in clinical trials of cancer vaccines are cancer-testis antigens (or germ cell antigens). CT antigens are proteins that represent reactivation of genes in tumors that are usually silent in adult tissues[43]. The list of CT antigens includes the MAGE, BAGE and GAGE families among other.

In any case, peptides recognized by T cells cannot always be predicted from the aminoacid sequence of the antigen. Some T cell epitopes may be generated by splicing aberrations leading to cryptic epitopes encoded by nonspliced introns, alternative open reading frames, or post-translational modification[44,45].

Finally, it was recently described a unique population of CTL that is capable of recognizing MHC-I-low tumors[46]. These CTLs displayed preferential killing for tumors with defects in the antigen processing pathway and targeted a novel category of tumor antigens[46,47]. This novel peptide repertoire emerges in MHC class I at the cell surface upon impediments in the classical processing route, for example proteasome, TAP or tapasin, implying that processing defects block the conventional tumor antigens, but stimulate alternative processing routes. This novel category of tumor antigens were designated as TEIPP (T-cell epitopes associated with impaired peptide processing)[46].

1.1.5 αβ T cell-mediated cancer immunotherapy

The identification of tumor-specific antigens boosted the investigation on tumor-specific $CD8^+$ CTL and adaptive immune responses. Although it allowed the development of tumor vaccines — which are being assayed in clinical trials (reviewed in[48]) — the overall success of such immunotherapeutic strategies has had only limited efficacy to date[48,49], as tumor mutations can increase resistance to T cell cytotoxicity or induce T cell anergy[50]. Another limiting factor is the common downregulation of tumor

antigens and/or the MHC class I presentation elements in advanced tumors, since $CD8^+$ T cell immunity is critically dependent on MHC-I-mediated antigen presentation.

1.1.6 **MHC class I-unrestricted tumor cell recognition**

Many reports have illustrated the capacity of innate unconventional lymphocytes ($\gamma\delta$ T, NK and NKT cells), to detect and destroy tumor cells independently of classical MHC presentation[11,51]. This capacity has probably evolved as a mechanism to ensure that tumors evading MHC-dependent $\alpha\beta$-T cell recognition would still be recognized and eliminated by other effector cells[52]. In contrast to antigen-specific $\alpha\beta$ T cells, NK cells, NKT cells and $\gamma\delta$ T cells can directly recognize patterns of stress-induced self molecules present in malignantly transformed cells[53,54]. Consequently, they can lyse tumor cells almost immediately upon encounter and independently of tumor-specific antigen processing[54-57].

Some of these unconventional lymphocytes were shown to recognize non-classical MHC proteins (called MHC class Ib proteins) expressed at the surface of tumor cells. MHC Ib molecules (in humans, MICA, MICB, HLA-E, -F, -G and –H proteins) are structurally related to class Ia proteins because they show a typical (α1–α2) MHC fold on a single polypeptide, which, in the case of Ib, does not pair up obligatorily with β2-microglobulin (β2m)[58]. Furthermore, although many MHC Ib genes are also located in the MHC locus, they tend (with the notable exception of MICA/B) to be oligomorphic— few alleles exist in the population— which is in marked contrast with the extensive polymorphism of class Ia. MHC I b proteins have been largely shown to bind to stimulatory or inhibitory receptors expressed on T, NK and/or NKT lymphocytes. Some MHC Ib proteins can act as ‘reporters’ of cellular transformation and trigger anti-tumor immune responses[22]. Additionally, several mechanisms have been described by which MHC Ib can activate or inhibit anti-tumor lymphocytes[22]. These unconventional cell subsets thus represent an alternative approach to cell-mediated anti-cancer immunotherapy.

1.2 NATURAL KILLER CELLS

Natural killer (NK) cells are bone marrow-derived lymphocytes, playing critical effector functions in the early innate immune response[59,60]. They continuously circulate through the blood, lymphatics and tissues, on patrol for the presence of transformed or pathogen-infected cells[61]. Even in the absence of prior sensitization, NK cells can exert potent cytolytic activity directly against their cellular targets. Activated NK cells secrete cytokines, such as IFN-γ, TNF-α and granulocyte–macrophage colony stimulating factor (GM-CSF), which activate both innate and adaptive immune cells[62].

Contrary to $\alpha\beta$ T, $\gamma\delta$ T and B cells, NK cells are regulated by a limited repertoire of germ line-encoded receptors that do not undergo somatic recombination[63]. Thus, individual NK cells lack a unique antigen recognition receptor and do not use RAG enzymes for rearrangement of their receptor genes, although transient expression of RAG proteins and even incomplete V(D)J recombination have been observed in a low frequency of NK cells during their development, probably due to early progenitors shared with T cells[60]. In any case, NK cells are present in normal numbers in mice deficient in RAG1 or RAG2[64,65]. Accordingly, and unlike T cells, most NK cells do not require the thymus for their development and, thus, exist in normal numbers in athymic nude mice[60].

Many of the NK cell receptors have opposite functions, thus the strength of each signal can finely tune NK cell cytolytic activity and cytokine production. If the target cells express ligands for NK inhibitory receptors (iNKRs), phosphatases recruited by the inhibitory receptors are brought to the synapse, where they act to dampen or even prevent NK cell activation[52]. When an NK cell encounters an abnormal cell (e.g. tumor or virus-infected) and activating signals from activating receptors (aNKRs) predominate, the NK cells can rapidly induce apoptosis of the target cell through directed secretion of cytolytic granules containing perforin and granzymes or engagement of death domain-containing receptors[62]. Although this is the prevailing view, there are probably other mechanisms involved, because some NK cells expressing activating receptors and lacking inhibitory receptors for MHC-I ligands present in target cells, still fail to lyse their targets[66,67]. Following these results, it was recently proposed an “education mechanism”

for NK cells, whereby such NK cells acquire self-tolerance. The potentially autoreactive NK cells are not clonally deleted but instead acquire a state of hyporesponsiveness to stimulation through various activating receptors[68].

Recent data suggests that the expression of inhibitory receptors specific for self-MHC confers greater responsiveness to NK cells, a property termed 'licensing'[63], which requires functional ITIMs. The potency with which NK cells reject cells with aberrant MHC class I expression appears to correlate with the number and strength of inhibitory receptor–MHC class I interactions. In other recent studies, mature NK cells have been shown to undergo a 're-education' process in the periphery, whereby functional competence is reset following adoptive transfer into a different MHC class I environment. In these studies, functional NK cells lost their effector capabilities in an environment devoid of MHC class I molecules[69,70]. Together, these results suggest that continuous engagement of inhibitory receptors with MHC class I molecules is required to determine NK cell responsiveness.

Finally, although NK cells are classified as innate immune cells, there is evidence in both mice and humans that NK cells share some attributes of the adaptive immune system with B cells and T cells. For example, it was shown that NK cells can mount a form of antigen-specific immunologic memory (e.g. a clonal expansion during infection and generation of long-lived memory cells)[60,68,71,72].

1.2.1 Natural Killer cell Inhibitory receptors

The most relevant inhibitory NK receptors recognize MHC class I molecules and ''protect'' normal cells from autologous NK cell-mediated killing (Table 2). Consequently, many cells with down-regulated MHC class I molecules (as in the case of tumor transformation or viral infection) become susceptible to NK-cell mediated killing[52,60]. As previously described, the functional response of NK cells to activating stimuli was shown to increase significantly with the number of different inhibitory receptors for self-MHC that the NK cells expressed[73,74]. Despite exhibiting greater responsiveness, NK cells with more inhibitory receptors are not autoreactive, because interactions of their inhibitory receptors with MHC class I molecules on normal cells inhibits their activity. iNKRs contain immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tail, and these motifs can recruit SH2 domain-containing protein tyrosine

phosphatase 1 (SHP1; also known as PTPN6) and SH2 domain-containing inositol 5-phosphatase (SHIP), initiating a signaling cascade that counteracts that of activating kinases[52].

In humans, the NK cell MHC-I-binding inhibitory receptors include the killer-cell immunoglobulin-like receptor (KIR; also known as CD158) family, the NKG2A/CD94 heterodimer and CD85j (ILT2, LIR1)[75]. Other inhibitory NK receptors are specific for non-MHC ligands. These receptors include, for example, CD200 (an immunosuppressive cell surface glycoprotein)[76] and CD161 (also known as NKR-P1A, that binds to CLEC2D[77-79].

1.2.1.1 **Killer Ig-like receptors (KIRs)**

Human KIRs are a family of transmembrane glycoproteins belonging to the immunoglobulin superfamily[80]. The major ligands for KIR are MHC class I (HLA-A, -B or -C) molecules, which are expressed on the surface of nearly every normal nucleated cell in the body, and are encoded by the most polymorphic genes in humans, thus defining immune 'self'. The KIR-mediated regulation of NK cells can significantly impact on their responsiveness during viral infection, cancer, haematopoietic stem cell transplantation and pregnancy[81]. On the other hand, the expression of self MHC-I-reactive KIR is also critical for the 'licensing', or 'arming' of NK cells[81].

1.2.1.2 **CD94/NKG2A**

NKG2A and NKG2C C-type lectin molecules are expressed as heterodimers coupled to CD94, forming receptors with opposite functions[82]. The inhibitory NKG2A molecule contains cytoplasmic ITIMs that recruit SHP-1 and SHP-2 tyrosine phosphatases[83]. In contrast, NKG2C is coupled through the DAP12/KARAP adaptor to a tyrosine kinase-dependent pathway, triggering NK cell effector functions[82,84]. Both receptors specifically recognize HLA-E, which presents peptides derived from the leader sequence of other HLA class I molecules[85]. In mice, CD94/NKG2 receptors are conserved and recognize the Qa1b class Ib molecule, which presents as well MHC class I-derived peptides[86]. CD94/NKG2A expression was previously shown to be

inducible in T cells stimulated with IL-12[87] or upon TCR-dependent activation under the influence of IL-15 or TGFβ[88,89]. Also, HLA-E expression by gynecological cancers restrains tumor-infiltrating CD8 T lymphocytes or NK cells[90].

GENE	SIGNALING	LIGAND
KIR2DL1 (CD158b)	2 ITIM	HLA-C group 2 ; N77/K80
KIR2DL2 (CD158b1)	2 ITIM	HLA-C group 2 ; N77/K80
KIR2DL3 (CD158b2)	2 ITIM	HLA-C group 1 ; S77/N80
KIR2DL5A (CD158f) KIR2DL5b	2 ITIM	?
KIR3DL1 (CD158e1)	2 ITIM	HLA-B alleles
KIR3DL2 (CD158k)	2 ITIM	HLA-A alleles
LIR-1/ILT2 (CD85j)	4 ITIM	HLA class I
KLRD1-KLRC1 [CD94-NKG2A (CD159a)]	1 ITIM	HLA-E
KLRG1 (Mafa)	1 ITIM	E-, N- and R - cadherins
KLRB1 (NKR-P1A, CD161)	1 ITIM	LLT1 (OCIL, CLEC2D)
SIGLEC-7 (CDw328,p75)	1 ITIM	α−2,8 disialic acid
SIGLEC-9 (CD329)	1 ITIM	α−2,8 disialic acid
IRp60 (CD300a)	1 ITIM	?
CD244 (2B4)	0 ITIM (4 ITSM)	CD48
LAIR1 (LAIR-1, CD305)	2 ITIM	Collagen
CEACAM1 (CD66a)	2 ITIM	CEACAM1 (CD66a)
4Ig-B7-H3	?	?
TIGIT	1 ITIM	PVR
CD200	1 ITIM	CD200R

Table 2. Inhibitory receptors expressed on human peripheral blood NK cells. Inhibitory receptors expressed by freshly isolated resting NK cells and their ligands are listed. KIR, NKG2A, LIR-1, KLRG1, NKR-P1, Siglec-7, and Siglec-9 are only expressed by subsets of NK cells. Adapted from Lanier *et al.* 2008[52].

GENE	SIGNALING	LIGAND
FCGR3 (CD16, FcgRIIIA)	FcεRIγ, CD3ς	IgG
NCR3 (NKp30,CD337)	FcεRIγ, CD3ς	B7-H6
NCR2 (NKp44, CD336)	DAP12	?
NCR1 (NKp46, CD335)	FcεRIγ, CD3ς	Viral hemaglutinin
KIR2DS1 (CD158h)	DAP12	HLA-C , N77/K80
KIR2DS2 (CD158j)	DAP12	?
KIR2DS4 (CD158i)	DAP12	HLA-Cw4
KIR3DS1 (CD158e2)	DAP12	?
KLRD1-KLRC2 CD94-NKG2C (CD159c)	DAP12	HLA-E
KLRK1 (NKG2D, CD314)	DAP10	MICA/B , ULBP1-6
CD244 (2B4)	ITSM, SAP	CD48
CD2	FcεRIγ, CD3ς	LFA-3 (CD58) , CD48
SLAMF7 (CRACC, CD319)	ITSM, EAT2	CRACC
SLAMF6 (NTB-A)	ITSM, SAP, EAT2	NTB-A
CD226 (DNAM-1)	Protein kinase C	PVR (CD155), CD112
CD7	?	SECTM1, Galectin
BY55 (CD160, NK1)	?	HLA-C , HLA-G
KIR2DL4 (CD158d)	FcεRIγ	HLA-G (soluble)
CD44	?	Hyaluronan
SLAMF5 (CD84)	SH2D1A/SAP	CD48
SLAMF3 (Ly9)	SAP	?
KLRF1 (NKp80, CLEC5C)	?	AICL
LFA-1 (αLβ2, CD11a/18)	?	ICAM-1-5
MAC-1 (αMβ2, CD11b/18)	?	ICAM-1, iC3b, Fibrinogen
CD11c/18	?	ICAM-1, iC3b
VLA-4 (α4β1, CD49d/29)	?	VCAM-1, Fibronectin
VLA-5 (α5β1, CD49e/29)	?	Fibronectin

Table 3. Activating receptors expressed on human peripheral blood NK cells. Activating receptors expressed by freshly isolated resting NK cells and their ligands are listed. KIR, NKG2C, and CD2 are only expressed by subsets of NK cells. Adapted from Lanier *et al.* 2008[52]

1.2.2 Natural Killer cell Activating receptors

In some cases, NK cells do efficiently recognize and kill tumors or virus-infected cells that maintain expression of inhibitory MHC class I molecules. This means that, in order to exert they cytolytic activity, NK cells require strong activating signals generated upon effector/ target cell interaction[91]. NK cells do not possess one dominant receptor, but instead rely on a vast combinatorial array of receptors to initiate effector functions (Table 3). What constitutes an activating receptor or a costimulatory molecule in NK cells is uncertain and somewhat semantic[52]. When agonist antibodies are used to cross-link the 'activating' receptors on freshly isolated primary human NK cells, none of the receptors alone, with the exception of CD16 and NKG2D, are able to elicit cytolytic activity or cytokine secretion[92]. However, when different pairs or combinations of receptors are simultaneously cross-linked, effector functions are triggered, with evidence in some cases for additive effects and in other cases synergistic actions. These observations suggest that a critical threshold of signaling that exceeds the counterbalancing influence of the inhibitory receptors must be achieved by these coactivating receptors in order for NK cells to mount a productive response.

The NK cell activating receptors primarily involved in tumor cell killing are NKp46[93,94], NKp30[95] and NKp44[96,97], collectively referred to as natural cytotoxicity receptors (NCR). Other activating receptors playing a relevant role in tumor cell lysis are NKG2D[98], 2B4 and DNAM-1[99]. In contrast to inhibitory receptors, most activating receptors are expressed by all NK cells. Furthermore, activating receptors induce diverse signaling cascades, whereas inhibitory receptors appear to use a common mechanism for inhibition. Some activating receptors are associated with ITAM-containing adapter proteins that propagate strong activation signals through recruitment of tyrosine kinases Syk and ζ-associated protein of 70 kDa (ZAP-70)[63]. Other activating receptors do not contain ITAMs or associate with different adapters.

1.2.2.1 **Natural cytotoxicity receptors**

1.2.2.1.1 NKp30

NKp30 is a type I transmembrane protein triggering receptor expressed by all resting and activated human NK cells[95]. Its transmembrane domain associates via a charged amino acid with ITAM-bearing adapters (CD3ζ and FcRγ)[100]. NKp30 is mostly restricted to NK cells although recent studies have shown protein expression on umbilical cord blood T cells after IL-15 stimulation and on endometrial epithelial cells following progesterone exposure[101,102]. Moreover, the recently identified IL-22-producing mucosal innate lymphoid cell population that shares properties with LTi (lymphoid tissue inducer) and NK cells also expresses NKp30[103,104].

Several studies have shown that NKp30 is a major activating receptor involved in tumor cell lysis. IL-2[105] or IL-21[106] induce NKp30 up-regulation, whereas TGF-β down-regulates NKp30, leading to impaired NK cell cytotoxicity[107]. Additionally, an NKp30-dull phenotype was shown to be acquired during leukemia development in acute myeloid leukemia (AML)[108,109] and breast cancer[110] patients. This down-regulation is possibly a mechanism of escape from innate immunity. Finally, NKp30 can also target immature DC and thus favor the accumulation of mature DCs[111,112].

NKp30 is encoded on chromosome 6 and has no homology with NKp44 or NKp46, which are encoded on chromosomes 6 and 19, respectively[95]. Notably, NKp30 is a pseudogene in mice, with the exception of the wild strain *Mus caroli*[113]. A functional NKp30 protein is expressed on resting peripheral chimpanzee NK cells although at low level[114].

The NKp30 gene encodes six alternatively spliced transcripts[115]. Delahaye *et al*.[116] have recently shown that different isoforms transmit distinct signals and thus mediate different cell functions; NKp30a and NKp30b are immunostimulatory isoforms that trigger Th1 cytokine production. In contrast NKp30c promotes IL-10 secretion, relaying an immunosuppressive signal through rapid phosphorylation of p38 MAP kinase. An additional layer of regulation could be provided by the existence of several glycosylation profiles of NKp30[101].

The human cytomegalovirus (HCMV) tegument protein pp65 was shown to bind NKp30, leading to a general inhibition of the NK cell ability to kill infected and tumor

cells[117]. The decrease of the activating signal was due to the CD3ζ dissociation from NKp30 upon pp65 interaction, thus acting as an immune suppression mechanism during HCMV infection.

The intracellular BAT3 protein was found to bind and activate NKp30[118]. Although BAT3 has a C-terminal nuclear localization signal and lacks a putative secretory leader peptide, it can be directed to the cell surface or even secreted after heat shock. Moreover, BAT3 was shown to be expressed on the membrane of exosomes released from immature DC[118]. Accordingly, NK-dependent lysis of immature DC was inhibited by BAT3 antiserum.

Several groups have shown the constitutive expression of NKp30 ligands on tumor cells by assessing the binding of soluble NKp30[119]. However, only one ligand, B7-H6, was demonstrated to be clearly involved in NKp30-mediated tumor cell recognition[120]. B7-H6 is a surface protein similar to other members of the B7 family. In contrast to B7.1 and B7.2, that recognize both CD28 and CTLA-4, B7-H6 is not promiscuous since it does not bind any other CD28 family members nor other NCRs[120]. Similarly to NKp30, but in contrast to other B7 members, a functional B7-H6 gene is missing in *Mus musculus*.

Strikingly, B7-H6 transcripts have not been detected in most normal adult tissues, consistent with the absence of the protein on circulating cells isolated from healthy individuals. In contrast, B7-H6 cell surface expression is observed in a restricted panel of tumor cell lines from various origins including lymphoma, leukemia, melanoma, and carcinoma as well as on primary tumor blood cells[120]. The pattern of B7-H6 expression, which appears so far to be limited to tumor cells, is another example of stress-induced self-recognition by NK cells[100]. However, in pilot experiments, treatment of some NKp30 ligand-negative tumor cells with a panel of DNA-damaging agents had no major effect on B7-H6 expression.

1.2.2.1.2 NKp44

NKp44 is a type I transmembrane protein that is noncovalently associated in the plasma membrane with a disulfide-linked homodimer of the DNAX-activation protein of 12 kDa (DAP12; also known as killer cell-activating receptor-associated protein

(KARAP))[96,97]. DAP12 is a transmembrane accessory protein that contains an ITAM, which transduces intracellular activation signals.

The NKp44 molecule (encoded by the NCR2 locus) is expressed on the surface of IL-2 stimulated, but not on resting human NK cells, and therefore is referred to as an activation-induced triggering receptor[97]. Anti-NKp44 mAb can reduce NK cell cytotoxicity toward certain tumor target cells, thereby indicating that these targets express appropriate ligands for the receptor[96]. However, the identity of NKp44 ligands on tumors is currently unknown. Viral hemagglutinins have been reported to interact with sialic acid determinants on NKp44 and another NCR, NKp46, to promote cytolysis of influenza, West Nile virus and Dengue virus-infected target cells[121-124]. Moreover, an unidentified cellular ligand for NKp44 is expressed during HIV-1 infection and is correlated with both the progression of $CD4^+$ T cell depletion and the increase of viral load. $CD4^+$ T cells expressing this putative ligand are highly sensitive to the NK lysis activity mediated by $NKp44^+$ NK cells[125].

NKp44 transcripts have only been detected in human NK cells. However, some $V\gamma9V\delta2$ $\gamma\delta$ T cell clones were found to express NKp44[97], suggesting that a subset of T cells may also express this receptor. More recently, NKp44 was also shown to be expressed in lineages of IL-22^+ cells and LTi-like cells that are distinct from conventional NK cells[126].

1.2.2.1.3 NKp46

NKp46 is the only NCR to have a functional ortologue in mice[91,127]. Similarly to NKp44, NKp46 has been shown to directly interact with viral hemagglutinin and its engagement with these structures induced NK cell activation[128]. Along this line, influenza infection has been reported to be lethal in NKp46-deficient mice[121,127], and NKp46 was shown to play an important role in the innate immune response to human influenza[129].

Although the role of NKp46 in tumor cell recognition is not clear, a recent study reported enhanced *in vivo* growth of lymphoma tumors in NKp46-deficient mice[130]. Moreover, human melanoma cell lines were shown to express (yet unidentified) ligands for NKp46[131]. In a different context, the activating receptor NKp46 is essential for the

development of type 1 diabetes[132], mediating the recognition and killing of human and murine Pancreatic β cells[133]. NKp46 is also expressed by various cell types, other than NK cells. NKp46 expression identifies a functionally distinct NKT subset in mice and humans[134], and it is also expressed in the LTi cell lineage[135]. Microbial flora drives IL-22 production by intestinal NKp46$^+$ cells that provide innate mucosal immune defense[136].

1.2.2.2 **NKG2D**

NKG2D is a C-type lectin receptor shared by NK, γδ and conventional CD8 T lymphocytes. It recognizes the MHC class I chain-related A or B (MICA/MICB) and UL16 binding proteins (ULBP1-6) expressed in humans; and RAE (retinoic acid early inducible proteins), MULT and H-60 (dominant minor histocompatibility antigen) proteins expressed in mice[98,137,138]. NKG2D ligands are not expressed by most normal tissues but are upregulated in many epithelial tumor cells, virus or bacteria-infected cells, and "stressed" cells[54,57,139].

Expression of NKG2D on the cell surface requires its association with DAP10, a transmembrane-anchored adaptor protein expressed as a disulfide-linked homodimer[52]. IL-2 stimulation increases NKG2D surface expression by increasing DAP10 mRNA and a large up-regulation of DAP10 protein synthesis. On the contrary, TGF-β1[140] or IL-21[106] treatment leads to the down-regulation NKG2D protein on human NK and CD8$^+$ T cells.

Crosslinking of the NKG2D receptor on murine NK cells triggers several effector mechanisms on NK cells (e.g. mobilization of intracellular Ca^{2+}, production of cytokines including IFN-γ, GM-CSF, TNF-α, and production of chemokines such as macrophage inflammatory protein (MIP-1β)[57,141,142]. Depending on the levels of NKG2D ligands, the stimulatory signal can override coexisting inhibitory signals provided by the same target cells[143]. However, the stimulatory signal provided by NKG2D is not entirely refractory to inhibitory signals[143]. Sutherland *et al*. observed that stimulation of human NK cells with soluble NKG2D ligands resulted in the activation of Janus kinase 2, STAT5, MAPK and PI3 kinase/Akt signal transduction pathways[144,145]. NKG2D can trigger strong cytotoxic and cytokine responses mediated by the PI3K signaling pathway, with the help of the adaptor protein Grb2. NKG2D initiates PI3K activation, via a YxxM motif in the

intracytoplasmic domain of DAP10, lead to MAPK phosphorylation and intracellular calcium release.

Triggering of the NKG2D receptor alone is sufficient to stimulate NK cell activation. By contrast, the engagement of NKG2D alone in naïve and effector CD8 αβ T cells remains unable to trigger any effector or proliferative response. A recent study suggests that this could be due, in part, to regulation of human DAP10 gene expression downstream of TCR ligation. Because DAP10 and CD28 share the same YxxM intracytoplasmic motif, NKG2D is supposed to play a similar co-stimulatory role than CD28 and thereby refine TCR activation[146,147]. In $CD8^+$ T cells and (γδ T cells[148]), NKG2D provides a co-stimulatory signal that synergizes with T cell receptor signal[142,146,149]. Hence, interactions of NKG2D with induced ligands may provide functions appropriate to the cells in which it is expressed.

A low level of MICA/B expression is maintained on the epithelial cells lining the gastrointestinal surfaces, which may be due to interactions of these cells with various environmental "stressors"[54]. Direct evidences of NKG2D contribution during anti-tumor responses have been demonstrated for NK cells and CD8 αβ T cells in mouse lymphoma model[150] and for γδ T cells in a mouse model of skin carcinomas[11].

1.3 γδ T CELLS

γδ T cells were discovered 25 years ago[151,152]. Since then, they have been intensely studied, in a global effort to unravel their intriguing features, concerning development, antigen recognition, activation and function[26,153]. γδ T cells are typically considered a "first line of defense," a "bridge between innate and adaptive responses". However, the roles of γδ T cells in immunity are not yet fully understood, mostly because of their complex biology.

Several distinct features characterize murine γδ T cells. They were shown to recognize conserved stress-induced antigens, conferring broad anti-tumor and anti-infectious reactivity. They rapidly acquire a memory/pre-activated status in "naïve"

animals (i.e. not exposed to the *bona fide* eliciting agent), and they generally locate outside classical secondary lymphoid organs. In fact, although γδ T cells represent only a minor portion of peripheral blood $CD3^+$ T lymphocytes[26], they comprise up to 50 % of T cells in epithelial-rich tissues (skin, reproductive tract and intestine)[154]. Furthermore, they express a highly restricted γδ TCR repertoire in particular tissue locations, presumably as the consequence of the coordinated acquisition of a restricted set of TCR, homing receptors and functional properties during their development. Many of these features are also shared by human γδ T cells[29].

1.3.1 γδ TCR repertoires

The γδ TCR may be rearranged with two (and three in case of humans) Dδ segments at the same time and in all possible reading frames. Some other mechanisms (insertion of N and P nucleotides or imprecise V(D)J joining) also increase the possibility to generate diversity in the γδ TCR[25,26]. While the number of available TCR Vγ and Vδ elements is small, the γδ TCR repertoire is likely to be at least as diverse as the αβ TCR or the Ig repertoire[26]. However, most murine γδ T-cell subsets have no

Species	Peripheral location	Predominant V gene segment usage	V(D)J diversity
Mouse	Adult thymus	Diverse	High
	Spleen	Vγ1 and Vγ4	High
	Lymph nodes	Diverse	High
	Epidermis	Vγ5Vδ1	Invariant
	Liver	Vγ1Vδ6.3, Vγ4 and Vγ6	Intermediate
	Gut epithelia	Vγ7Vδ4, Vγ7Vδ5 and Vγ7Vδ6	Intermediate
	Uterovaginal epithelia	Vγ6Vδ1	Invariant
	Lung epithelia	Vγ4 and Vγ6	Intermediate
Human	Thymus	Vδ1	High
	Peripheral blood	Vγ9Vδ2	Intermediate
	Spleen	Vδ1	High
	Liver	Vδ1 and Vδ3	High
	Gut epithelia	Vδ1 and Vδ3	High
	Dermis	Vδ1	High

Table 4. γδ TCR repertoires. Adapted from Bonneville *et al.* 2010[155]

junctional diversity; they have a short complementarity-determining region 3 (CDR3) and are essentially an oligoclonal cell population (Table 4). This is true for Vγ5–Vδ1 γδ T cells in the skin (dendritic epidermal T cells; DETCs), and Vγ6–Vδ1 γδ T cells of the uterine epithelia. These γδ cell subsets have been described in mice and rats but not in humans[156,157]. Throughout this thesis, we use the *V*γ gene nomenclature of Heilig and Tonegawa[26,151,157,158] for rodent γδ T cells and Lefranc and Rabbitts[159] for human γδ T cells.

In humans and non-human primates, responses to small non-peptidic metabolites known as phosphoantigens are multi-clonal yet limited to a single γδ T cell subset (Vγ9Vδ2 T cells). Numerous studies have documented peripheral TCR selection, including human diseases such as polymyositis and multiple sclerosis. However, how the selected TCRs might be functionally different is not clear, because their ligands remain unknown[160]. The preferential expression of certain TCR Vγ and Vδ elements in different anatomical sites is usually considered to reflect the importance of a particular γδ T cell subset in the local immune surveillance[26,54]. Individual γδ T cell subsets in particular tissue locations show biased use of certain TCR V gene segments and, in some cases, express 'invariant' TCRs with identical (canonical) junctional sequences. These invariant γδ T cell subsets are derived from early 'waves' of fetal γδ thymocytes, unlike peripheral subsets that express more diverse γδ TCRs.

1.3.2 **Ligand recognition by γδ T cells**

Immunologists have been searching for ligands for γδ TCR for about two decades. However, this proved to be a very difficult task, as very few peptide antigens have been found to bind γδ TCR (Table 5). It is well established that γδ T cells express adaptive antigen receptors encoded by rearranging genes. However, a paradigm is lacking for γδ T cell ligand recognition. Although γδ T cells do not specifically respond to allo-antigens and do not show MHC-restricted specificities involving conventional antigens, some γδ T cells are capable of recognizing classical MHC molecules and related cell-surface proteins, including the CD1 molecules[160]. Moreover, the crystal structure of a murine γδ TCR in complex with the T10/22 molecules has been solved[161]. Murine non-classical

MHC class I molecule T10/T22 proteins (thymus leukemia, TL, antigens) are up-regulated in activated or stressed cells. The structure showed an interesting molecular interaction, very different from those of most MHC-restricted αβ TCRs and their MHC-ligands[162]. In humans, much of the research has been concentrated on Vδ1 and Vγ9Vδ2 T cell subsets.

γδ T cell source	Protein source	Peptide origin, name (epitope)	MHC restriction	TCR-dependent
Mouse	HSP-60	Mycobacterial (180–196)	No	Yes
		E. coli (181–197)		
		Mammalian (205–221)		
	Undefined	Several, - HSP-70 BiP motif	No	Yes
	Insulin B chain	B:9-23	No	Yes
Rat	Retinal S-antigen	PDSAg (341-354)	?	?
	HLA-B	B27PD (125-138)	?	?
Human	Tetanus toxin	C. tetani (1235-1246)	HLA-DRw53	Yes
	Ig λ light chain	Processed peptide	No	Yes
	Listeriolysin O	*L. monocytogenes (470-508)*	No	Yes

Table 5. Responses of γδ T cells to specific peptides. Adapted from Born *et al.* 2011[160].

1.3.3 Vδ1 T cells

10-30% of human peripheral blood γδ T cells expresses Vδ1 in association with various Vγ elements[26,158]. Quantitative increases in circulating Vγ1 cells are observed in HIV infections and, even more strikingly, in human cytomegalovirus (HCMV) infections following renal transplantation[154]. Under physiological conditions, however, the primary localization of Vδ1-expressing γδ T cells is not the peripheral blood but rather the intraepithelial T cell compartment of the intestine, where Vδ1 cells constitute the major T cell population[26,54]. The recognition of lymphoid malignancies by human γδ T cells has

been mostly assigned to the Vγ9Vδ2 subset, whereas γδ T cells expressing the Vδ1 TCR have been implied in the defense against epithelial cancers.

1.3.4 **Vγ9Vδ2 T cells**

Vγ9Vδ2 T cells are the best characterized γδ T cell subset in humans. Although less frequent in childhood, they account for 70-95% of human adult circulating γδ T cells[163]. This expansion of peripheral blood Vγ9Vδ2 T cells after birth is associated with an early acquisition of a memory phenotype and strong cytolytic and Th1-like effector functions. The exposure to environmental and/or bacterial antigens (see below) might shape the γδ TCR repertoire during postnatal development[26,163,164].

The role of Vγ9Vδ2 T cells in immune biology and diseases have been difficultated by the fact that mice do not express homologues of Vγ9Vδ2 TCR, and there is no functional equivalent of these cells in other small laboratory animals. The only alternative model are non-human primates. Macaque Vγ or Vδ elements share up to 91% similarity in amino acid sequences to their human counterparts.

Similarly to NK cells, activation of Vγ9Vδ2 T cells does not require antigen processing and restriction by MHC molecules. In fact, most human peripheral blood γδ T cells lack MHC restriction by classical MHC class I or class II molecules[165,166]. Correspondingly, Vγ9Vδ2 usually lack CD4 and CD8 co-receptor expression and thus display a 'doublenegative' phenotype[26].

The ligands interacting with the Vγ9Vδ2 TCR on tumor cells have not been identified in detail. Besides the TCR, the cytotoxic activity of Vγ9Vδ2 T cells is influenced by the expression of several co-receptors, including the typical NK cell receptors (KIRs and KARs), explaining the stronger cytolysis of targets with reduced or missing MHC class I expression[167,168].

Vγ9Vδ2 T cells also qualify as professional APCs. They are capable of antigen uptake, processing and presentation to αβ T cells, and of induction of primary αβ T cell responses[37]. For example, a large fraction of tonsillar γδ T cells are activated and express high levels of MHC II and the CD28 ligands, CD80 and CD86[37].

The ability of Vγ9Vδ2 T cells to interact with various other immune cells can help in the course and outcome of a variety of inflammatory immune responses. Activated Vγ9Vδ2T cells produce proinflamatory cytokines (TNF-α and IFN-γ) and chemokines (MIP 1α/β and lymphotactin). In addition, it has been reported the production of fibroblast growth factor-9 (FGF-9) and connective tissue growth factor (CTGF), indicating that γδ T cells have a distinct role in local immunosurveillance and tissue repair[169,170].

1.3.5 **Structure of the Vγ9Vδ2 TCR**

Ligand recognition by γδ T cells shares more features with antigen binding of immunoglobulins than peptide/MHC recognition by αβ T cells[25]. In fact, sequence analysis of the Vγ9Vδ2 TCR showed that Vδ more closely resembles the variable immunoglobulin heavy chain domain (VH) than TCR Vα or Vβ domains[171,172]. Like the immunoglobulins, where the CDR3 of the IgH chain makes a major contribution to receptor diversity and specificity, the TCR-δ CDR3 in the γδ TCRs contributes the most to receptor diversity. Not surprisingly, in mice, TCR-δ CDR3 is solely implicated in the recognition of the T10/22 molecule, both structurally and functionally[161,162,173], and other studies provided further evidence for the importance of this particular portion of the γδ TCR in the recognition of additional ligands[174].

The aminoacid sequences of γδ TCR domains have their analogous domains in Fabs and αβ TCRs, although with some differences[26]. The CDR loop (site of antigen recognition) of the V domains of Vγ9Vδ2 TCR exhibits more protrusions and clefts compared to the flatter surfaces of αβ TCRs that bind a peptide/MHC complex and of antibodies that bind protein antigens[25]. CDR1 and CDR2 fragments are encoded by germ line V genes, whereas the CDR3 is formed by somatic rearrangement of V(D) and J fragments. Sequence diversity in antigen receptors is not evenly distributed among all six CDRs but is highly concentrated in one or two CDR3. It had been proposed that the principal antigen specificity of an immunoglobulin or TCR is derived from its most diverse CDR3 fragments (5).

1.3.6 Ligands recognized by human Vγ9Vδ2 TCR

1.3.6.1 Non-peptide ligands

Human Vγ9Vδ2 T cells can be efficiently activated by small phosphate or amine containing compounds, called phosphoantigens[175-177], as well as alkylamines from several sources[178] and aminobisphosphonates[179].

1.3.6.1.1 Phosphoantigens

Early *in vitro* studies indicated that Vγ9Vδ2 T cells strongly react in a non-MHC restricted fashion towards inactivated *Mycobacterium tuberculosis* and a variety of other microorganisms, including *Plasmodium falciparum*, *Toxoplasma gondii*, *Yersinia enterocolitica, Francisella tularensis*[165,166,175,180,181]. It was found later that the γδ T cell-stimulating moiety of microbial extracts was not protein, but rather consisted of non-proteinaceous, phosphatase-sensitive, low-molecular-weight compounds[181,182]. Different types of phosphorylated ligands were isolated from Mycobacteria, including four structurally related phosphoesters (so-called TUBag [1-4])[183] 1996). The other identified phosphate-containing antigens were isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). These molecules were collectively termed Phosphoantigens[56,183,184]. This class of compounds contains in fact several members, naturally produced or synthetic, able to slightly or heavily activate Vγ9Vδ2 T cells[185]. The most potent natural phosphoantigen identified to date is a phosphorylated intermediate of isoprenoid biosynthesis pathway, produced by Eubacteria and Protozoa but not by eukaryotes, called E-4-hydroxy-3-methylbut-2-enyl-pyrophosphate (HMBPP, also known as HDMAPP for hydroxy-dimethyl-allyl-pyrophosphate). It was first characterized in *Escherichia coli*[181,186] and plants.

1.3.6.1.2 The isoprenoid biosynthetic pathways

Isoprenoids are essential metabolites, important for cellular and intercellular biology and are produced by all living organisms. They constitute a diverse structural family, comprising, ubiquinones, sterols, terpenes, carotenoids, gibberellins, and taxoids. All of

these compounds are synthesized through the same precursors, the IPP and its isomer dimethylallyl pyrophosphate (DMAPP). IPP can be synthesized via two different biosynthetic pathways. Archaebacteria, few bacterias and most eukaryotes synthesize IPP from acetyl-CoA through the mevalonate pathway (MVA)[187]. Cyanobacteria, algae, plastids and most eubacteria (including *M. tuberculosis*), produce IPP in a different way, through a carbohydrate-based route referred to as 1-deoxy-d-xylulose-5-phosphate (MEP pathway or DOXP pathway; Figure 2). [188]. Which of these two pathways, MEP or MVA has evolved first remains unknown, since MEP only exists in bacteria and plastids where it provides most primary isoprenoids instead of the MVA used by *Archae*[188]. Both pathways can be used simultaneously by some bacterial species, but for different roles, MAP for primary metabolism and MVA for secondary metabolites[189]. Vγ9Vδ2 T cells recognize metabolites of isoprenoid synthesis that are generated by the MEP pathway in certain pathogenic microorganisms but not by the mevalonate pathway in other bacteria and mammalian cells. HMB-PP has a 1000 fold stronger stimulating activity of Vγ9Vδ2 T cells than IPP, probably due, in part, to its non-human origin[181]. The high potency of HMB-PP as stimulator of Vγ9Vδ2 T cells correlates with the γδ T cell stimulatory activity of the bacteria exploiting the MEP but not the mevalonate pathway (like *Mycobacterium tuberculosis* and *E. coli*)[190]. To a lesser extent, the synthetic bromohydrin pyrophosphate (BrHPP) is also considered as a strong activator of Vγ9Vδ2 T cells and is frequently used in experimental procedures[185].

Although highly controversial, some studies suggested an interaction between Vγ9Vδ2 TCR and phosphoantigens[179,191]. However, there is still no evidence for the existence of a cognate phosphoantigen/TCR direct interaction, since attempts to co-crystallize phosphoantigens with the Vγ9Vδ2 TCR have not succeeded[25]. More recently, the Vγ9Vδ2-selective antigens from human tumor cells were characterized as endogenous metabolites of the mevalonate pathway and most likely as IPP[184]. IPP is the final product of both biosynthesis pathways, and activates Vγ9Vδ2 T cells only in the μM range (medium activity), which, however, is not achieved in normal mammalian cells (Figure 3) . Vγ9Vδ2 T cells may sense accumulation of IPP in malignant mammalian cells[184] or cells that accumulate IPP under treatment with inhibitors of the IPP consuming enzymes of isoprenoid biosynthesis. IPP seems thus to be a sensor of cell stress.

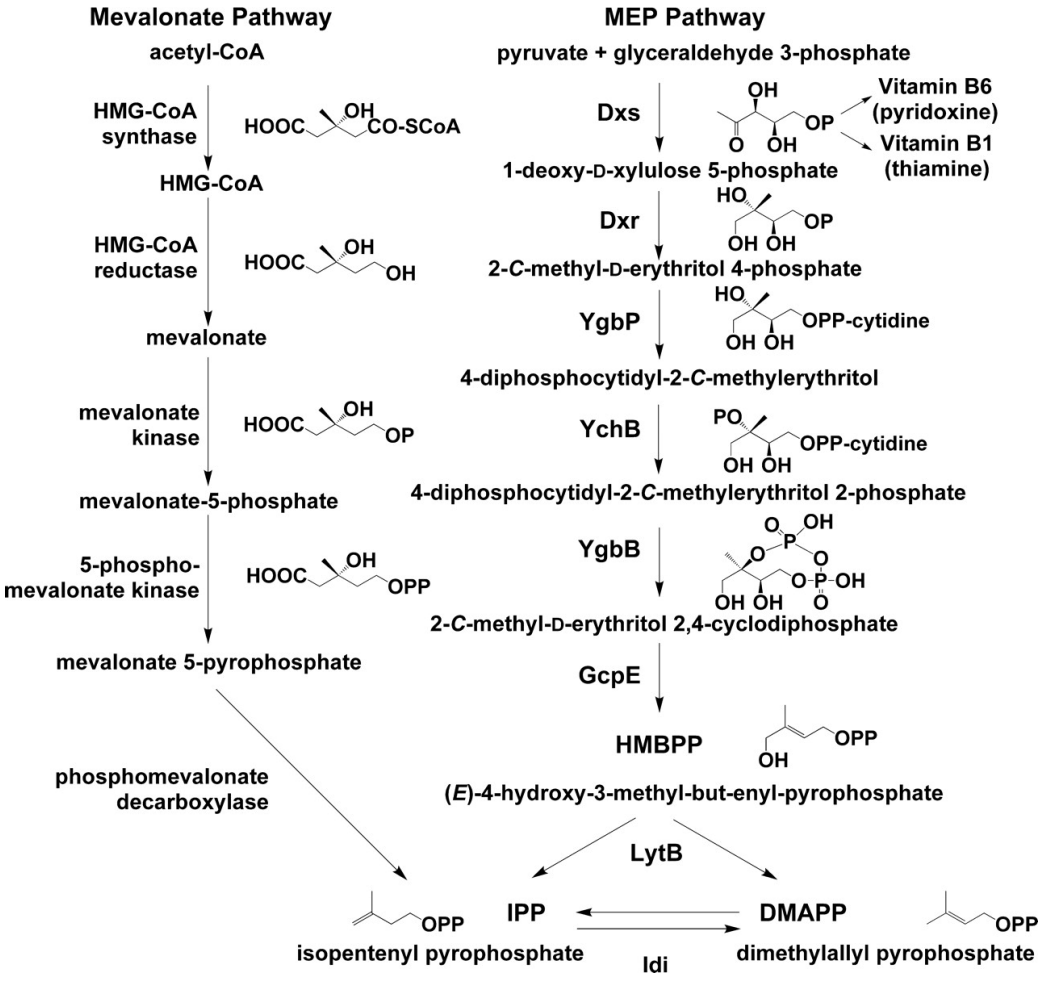


Figure 2. 2-C-methyl-D-erythritol 4-phosphate (MEP) and mevalonate pathways for isoprenoid biosynthesis. Adapted from Morita *et al.* 2007[192].

In plants and yeast, the regulation of the MVA pathway occurs at the HMGR level[193]. High levels of Farnesyl pyrophosphate (FPP), sterols or phenylalanine inhibit HMGR activity. In mammalian cells, the HMGR activity is inhibited by statins[194], by phenylalanine[195] or by a feedback inhibition with aminobisphosphonate-induced FPP accumulation[196]. The HMGR activity, and thus, the whole MVA pathway, were found increased in cancer cell lines such as leukemia, non-Hodgkin lymphoma[197], mammary and lung adenocarcinoma[198,199], in which HMGR activity is controlled by epidermal growth factor-PI3- Kinase mediated signaling.

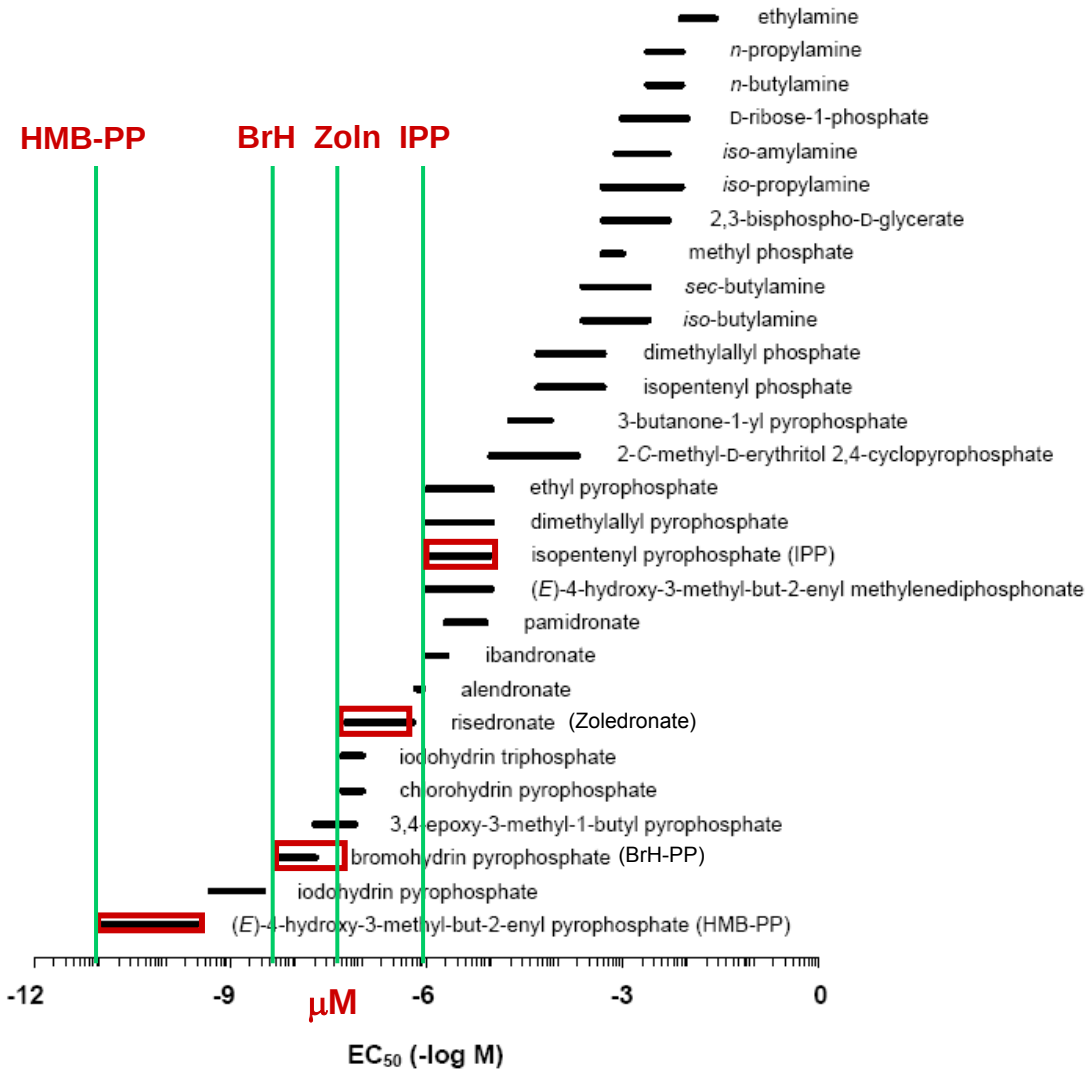


Figure 3. Bioactivities of natural and synthetic non-peptidic phosphoantigens on human Vγ9Vδ2 T cells. Adapted from Bonneville *et al.* 2005[189]

1.3.6.1.3 Aminobisphosphonates

Besides Phosphoantigens, Vγ9Vδ2 T cells are stimulated in a cross-reactive fashion by synthetic or natural alkylamines[164,200] and therapeutic aminobisphosphonates like pamidronate. In 1999, Kunzmann *et al.* discovered that several patients with multiple myeloma treated with the well-established osteoporosis inhibitor pamidronic acid presented significantly high numbers of blood-borne γδ T cells[201]. Later, it was shown that Pamidronate activates γδ T cells *in vitro* to secrete cytokines (IFNγ), proliferate and

exhibit strong cytotoxicity against various cancer cell lines[179]. Curiously, the bioactivity of aminobisphosphonates required the presence of accessory "Antigen Presenting Cells" (APCs) treated with this drug prior to the assay with the γδ T cells[202]. A wide variety of tumor cell lines pre-treated with aminobisphosphonates (especially pamidronate or zoledronate) could efficiently activate Vγ9Vδ2 T cells to proliferate and produce cytokines in a TCR-dependent manner[203]. To activate Vγ2Vδ2 T cells, aminobisphosphonates must be internalized and exert a statin-sensitive effect, namely inhibiting the endogenous MVA pathway[184]. Thus, the aminobisphosphonates cause a pharmacological inhibition of the mevalonate pathway in the treated cells, leading to IPP bioaccumulation. Aminobisphosphonates are inhibitors of the farnesyl pyrophosphate synthase (FPPS), an enzyme acting downstream of IPP synthesis along the isoprenoid pathway[184]. Accordingly, more potent aminobisphosphonates analogues for both MVA inhibition and γδ T cell activation are now available, such as zoledronate or ibandronate[204]. Non-amino bisphosphonates inhibitors for osteoporosis such as etidronate or clodronate neither inhibit the MVA pathway nor enable γδ T cell activation by etidronate- or clodronate-treated cells, respectively. Nonhuman aminobisphosphonate-treated target cells also fail to activate Vγ9Vδ2 T cells[191] suggesting implication of species-restricted molecules during this activation process.

1.3.6.1.4 Alkylamines

Similarly to aminobisphosphonates, alkylamines were shown to inhibit FPPS activity. Thus, Vγ9Vδ2 T cells should be activated through accumulation and presentation of phosphoantigens in such treated cells. Alkylamines are structurally composed of non-phosphate short alkyl chains bearing a terminal amino group. Prototypic bioactive alkylamines are ethylamine and sec-butylamine, present in wine and green tea, and produced by certain plants and bacteria. *Listeria monocytogenes, Bacterioides fragilis, Proteus morganii, Clostridium perfringes,* and *Salmonella typhimurium* produce alkylamines in concentrations able to activate Vγ9Vδ2 T cell responses[178]. Contrary to phosphoantigens, they only work in the mM range (weak activity). The activated γδ T cells then release, mainly, Th1 cytokines. For this reason it is thought that alkylamines-rich diets may contribute to prevent food allergies[194].

1.3.6.2 **Peptide Ligands**

1.3.6.2.1 Self- ligands

Most of the well-defined self-ligands of the γδ TCR belong to a group of self-molecules potentially capable of indicating cellular "stress" or activation. Accordingly, several self antigens have been found to bind Vγ9Vδ2 TCR, including heat shock protein-65 (HSP-65), phospholipid cardiolipin (CL), ULBP-4 and F1-ATP synthase[205].

Heat-shock proteins

Because of their role as sensors during cell stress or transformation, HSP-58, HSP-65 and HSP-70 were initially proposed as antigenic targets for γδ T cells. These proteins were shown to be upregulated on tumors, where γδ T cell had infiltrated, suggesting HSP65-dependent recognition of tumor cells by Vγ9Vδ2 T lymphocytes[189,206]. Recently HSP-60 was identified as a ligand for Vγ9Vδ2 TCR[174]. However, more studies are needed to access their biological role. It is possible that HSP may indirectly contribute to γδ T cell activation, e.g. through regulation of γδ T cell antigen processing or through the generation of TCR-independent costimuli such as interaction with innate receptors or induction of innate receptor-ligands.

F1-ATPase

Recently, Scotet *et al.* described the direct binding of the Vγ9Vδ2 TCR to Ecto-F1-ATPase, a form of the mitochondrial ATP synthase (ATPase), ectopically expressed at the cell membrane. This receptor was identified by screening mAbs able to inhibit the recognition of tumor cell lines by Vγ9Vδ2 T cells in vitro[207]. F1-ATPase is recognized by Vγ9Vδ2 TCR in a complex with the serum protein Apolipoprotein A1 (ApoA-1). These components seem involved in endogenous phosphoantigen presentation, considering ecto-F1-ATPase capacity to bind and present triphosphoric acid 1-adenosin-5'-yl ester 3-(3-methylbut-3-enyl) ester (Apppl)[208]. Apppl is an intracellular nucleotidic metabolite containing an isopentenyl moiety which accumulates in Aminobisphosphonate treated cells. Apppl can specifically activate Vγ9Vδ2 T cells, but not in its native form. Indeed, optimal activation by Apppl requires addition of a nucleotidic pyrophosphatase (NPP), releasing therefore IPP and AMP. In this regard, Apppl should represent an inactive

storage form of phosphoantigens which may bind to ecto-F1-ATPase before cleavage by NPP, thus generating IPP which in turn will stimulate Vγ9Vδ2 T cells[208].

However, the biological relevance of this interaction is still being addressed. It is possible that mitochondrial antigens could be an alerting signal that indicates the state and fate of the cell. On the other hand, the interaction between these two molecules could be justified by the specific microbial origin of mitochondria, carrying antigens similar to modern microbes.

ULBP-4

ULBP-4 expression is detected on the cell surface of EBV-infected cells as well as on colon, ovarian and liver cancer cells, suggesting that ULBP-4 may play a role in anti-infection and anti-tumor immunity. A recent study demonstrated that immobilized soluble ULBP-4, not only induces proliferation, cytokine production and cytotoxic activity of human ovarian and colonic carcinoma-infiltrating Vδ2 T cells *in vitro*, but importantly binds directly to soluble Vγ9Vδ2 TCR and stimulates the activation of Vγ9Vδ2 Jurkat transfectants (lacking NKG2D expression)[55]. Moreover, blocking experiments also indicate that both NKG2D and γδ TCR are involved in ULBP-4 recognition[55]. This raises questions about the already controversial hierarchy occurring between NKG2D and Vγ9Vδ2 TCR in γδ T cell activation and target recognition.

1.3.6.2.2 Non-self ligands

Tetanus toxoid, a strong immunogen derived from a protein, the tetanospasmin of *Clostridium tetani*, was the first defined antigen reported capable of stimulating γδ T cell responses[209,210]. Others that followed include Ig λ light chain[211], viral proteins such as glycoprotein I from *Herpes simplex*[212], and staphylococcal enterotoxin A[213]. More recently, the defined mycobacterial protein ESAT-6 was found to stimulate γδ T cells[214], and this may not be the only mycobacterial protein recognized by γδ T cells[215].

1.3.7 Vγ9Vδ2 T cell activation

The immunological synapse (IS) is composed of two concentric regions based on their molecular composition, where the TCR-enriched central supramolecular activation cluster (cSMAC) is surrounded by the integrin-rich peripheral SMAC[28]. Vγ9Vδ2 T cells require strong TCR engagement to fully induce the activation of their effector functions[99]. When co-cultured with the myelomonocytic THP-1 tumoral cell line, Vγ9Vδ2 T cells establish a mature IS containing TCR/CD3 complex, CD2, CD94 and NKG2D, whereas LFA-1, CD45 and the activation marker CD69 remain excluded of the central area[216]. CD6 expression was also described as an important driving force of the IS during tumor cell recognition by Vγ9Vδ2 T cells[217].

Vγ9Vδ2 and αβ TCR are differently organized on cell surface. Nanoscale imaging provided some evidence of a unique distribution of Vγ9Vδ2 TCR on cell surface of resting γδ T cells, as compared with αβ T cells[218]. Indeed, prior to phosphoantigen (namely HMB-PP) stimulation, Vγ9Vδ2 TCR are associated into scattered nanoclusters whereas αβ TCR expression is predominantly distributed into fewer but larger clusters. Vγ9Vδ2 TCR nanoclusters associate in high density nanodomains similar to those observed in αβ T cells supramolecular activation clusters. So, in agreement with the mature IS formation process described previously[28], nanoclusters may take place during preliminary steps of IS establishment - i.e. focusing of TCR and NKR (NK cell receptors) in a central synaptic area surrounded by LFA-1, CD45 and CD69 - whereas high density nanodomains, set up following phosphoantigen activation, may act as platforms for fully functional intracellular signaling and effector functions triggering.

1.3.8 Costimulation requirements of Vγ9Vδ2 T cells

Similarly to NK cells, Vγ9Vδ2 T cell activation is controlled by the engagement of numerous surface receptors with either inhibitory or activating properties. However, the integration of signals via the γδTCR and other receptors probably produces unique responses and distinctive functions. These receptors presumably ensure efficient discrimination by Vγ9Vδ2 T cells of “stress” vs. “normal” cell target through MHC, MHC-like and non-MHC ligands that are under- or over- expressed in transformed or infected

cells. Besides the T cell receptor, other molecules contribute to Vγ9Vδ2 T cell activation, including adhesion molecules, scavenger receptors, Toll-like receptors (TLR) and NKRs (Figure 4)[29]. I review here the literature on the receptors more clearly implicated on Vγ9Vδ2 T cell activation. Other costimulatory molecules have been recently reviewed by Ribot *et al,* 2011[219].

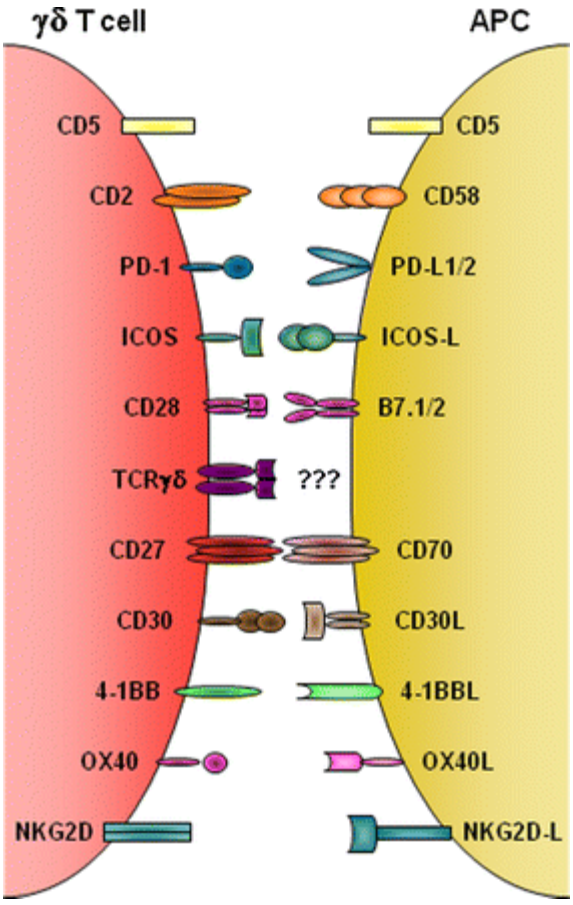


Figure 4. Prototypic costimulatory receptors of Vγ9Vδ2 T cell activation. Adapted from Ribot *et al*, 2011[219].

1.3.8.1 **CD28**

CD28, the receptor for B7.1 (CD80) or B7.2 (CD86), is the primary co-stimulatory receptor of αβ T cells. CD28 ligation is known to promote proliferation, survival and cytokine production of $CD4^+$ and $CD8^+$ T cells, and those responses are very frequently impaired in $CD28^{-/-}$ mice[220]. CD28 is upregulated upon activation in mouse γδ T cells

and it is expressed by 40-60% of freshly isolated human peripheral blood γδ cells[219]. However, resting murine γδ splenocytes and ruminant γδ T cells , mostly lack CD28, and peripheral blood γδ T cells downregulate CD28 upon activation. Although some reports suggested that CD28 costimulation promotes the proliferation of peripheral γδ T cells, other biological processes appear to be CD28-independent[219]. Thus it is not clear that, as a general rule, γδ T cells use CD28 for interaction with B7 (+) professional APCs[26,221]. However, recent data in the host laboratory suggests that B7-CD28 interactions play an important role in phosphoantigen-mediated activation of Vγ9Vδ2 T cells (J. Ribot, A. de Barros and B. Silva-Santos, unpublished observations).

1.3.8.2 **DNAM-1**

DNAX accessory molecule-1 (DNAM-1, aka CD226), a transmembrane glycoprotein tightly associated with LFA-1, is emerging as a key NK cell co-activating receptor in immunity to human cancer[222], as shown for neuroblastoma[223], ovarian carcinoma[224], and hematopoietic malignancies[225]. Both PVR and Nectin-2 represent specific ligands for DNAM-1. DNAM-1 participates in cancer cell recognition together with NCRs and, to a lesser extent, NKG2D[226]. Decreased expression of DNAM-1 has been observed on NK cells from acute myeloid leukemia patients[227,228]. In mouse, DNAM-1 is a crucial component of T cell–mediated immunological surveillance and partially contributes to NK cell–mediated lymphoma rejection[229]. In humans, Vγ9Vδ2 T cells were shown to express DNAM-1, and upon recognition of ligands expressed by hepatocellular carcinoma cells, DNAM-1 signals increase Vγ9Vδ2 T cell cytotoxicity and IFN-γ secretion[230].

1.3.8.3 **CD27**

CD27 (TNFRSF7) plays critical roles in αβ T cell activation, particularly in response to viral or tumor challenge[35]. In naïve C57Bl/6 mice, the expression levels of CD27 define two distinct and stable subsets of γδ T cells[219,231]. In the spleen, lymph nodes and various other tissues, approximately 75–90% of γδ T cells are $CD27^+$. Upon activation, these cells make IFN-γ, whereas IL-17 is only produced by their $CD27^-$ counterparts.

Interestingly, these distinct phenotypes are “pre-programmed” in the thymus, where $CD27^+$ γδ cells express genes associated with a Th1 differentiation program, while $CD27^-$ thymocytes constitutively express IL-17[231]. Ligation of both TCRγδ and CD27[231] are required for the differentiation of IFN-γ-producing γδ T cells, whereas the Th17-like pathway is preferentially driven by cytokines such as TGF-β. In the periphery, CD70-CD27 interactions provide survival and proliferative signals that control TCRγδ-driven activation. Thus, CD27 signaling activated the non-canonical NF-kB pathway and enhanced the expression of anti-apoptotic and cell cycle-related genes[219,232,233].

In humans, an average of 80% of Vγ9Vδ2 T cells express CD27[232] including both naive and central memory cells[234]. Upon activation with PMA and ionomycin, the vast majority of $CD27^+$ Vγ9Vδ2 T cells produced IFN-γ, whereas less than 1% made IL-17[232]. The administration of soluble recombinant CD70 enhanced Vγ9Vδ2 T cell proliferation, whereas anti-CD27 (or anti-CD70) mAbs reduced it. A major role of CD27 costimulation appears to be the protection from activation-induced-cell-death (AICD) following phosphoantigen stimulation[232]. Interestingly, CD70 is highly induced in Vγ9Vδ2 T cells after stimulation with phosphoantigen.

1.3.8.4 **TOLL-like receptors**

Toll-like receptors belong to the pattern recognition receptor family and recognize molecules that are broadly shared by microbial pathogens referred to as pathogen associated molecular patterns (PAMP)[235].

The role played by TLRs expressed by γδ T cells is not completely understood. However, TLR2, TLR3 and TLR5 are expressed by Vγ9Vδ2 T cells and these cells respond to the TLR2 agonist Pam3Cys and the TLR3 synthetic agonist poly(I:C)[236]. Nevertheless, the observed response requires simultaneous stimulation of the γδ T cell receptor[237-239]. These results suggest an early activation of γδ T cells in antiviral immunity. It appears that rapid activation of Vγ9Vδ2 T cells by TLR agonists occurs indirectly, e.g. in response to inflammatory cytokines released by myelomonocytic cells[240].

Scotet *et al.* recently showed that various TLR ligands engaging either TLR-3, -4, -5 and -9 triggered early activation and IFN-γ production by the majority of Vγ9Vδ2 PBMC after short-term in vitro incubation. This phenomenon was tightly dependent on type I IFN release by DC (either monocytic or plasmacytoid) in response to TLR engagement[241]. This behaviour is not restricted to γδ T cells but primarily reflects their predominant innate-like memory phenotype.

1.3.8.5 **Fc Receptors: CD16**

NK cells are able to detect IgG antibody-coated cells through the FcγRIIIA (CD16) cell surface receptor and to exert antibody-dependent cell cytotoxicity (ADCC) and cytokine production. Specifically, higher cytolytic activity and early IFN-γ production is a functional property of CD56(dim) CD16+ NK cells[242]. CD16 is coupled to the CD3ς and FcRγ signal transduction proteins bearing ITAMs.

Besides NK cells, a subset of Vγ9Vδ2 T cells has been shown to express CD16. CD16 upregulation is associated with terminal differentiation into effector cells of both αβ and γδ T cells. Interestingly, Angelini *et al*. showed that this phenotypic differentiation was associated with decreased Vγ9Vδ2 TCR signaling that paralleled enhanced CD16-mediated T cell activation[243]. The mechanisms underlying the balanced contribution of TCR versus CD16 signaling along γδ T cells functional differentiation remain unclear. Nevertheless, experiments led by Lafont *et al*. have highlighted the role played by CD16 engagement in γδ T cells. Indeed, cross-linking of CD16 on Vγ9Vδ2 T lymphocytes initiates intracellular signaling events similar, although significantly delayed, to those occurring following TCR activation. Moreover, as observed with the TCR activation process, CD16 triggered TNF-α production can be efficiently inhibited by the coincident ligation of CD94/NKG2A[244].

1.3.8.6 **Natural Killer cell-inhibitory receptors (iNKRs)**

As previously reported for NK cells, most human peripheral blood Vγ9Vδ2 T cells express several inhibitory Natural Killer receptors, including killer Ig-like receptors (KIR),

leukocyte Ig-like receptors (LIR) and lectin-like receptors (such as the NKG2A/CD94 heterodimer).

The heterodimer CD94/NKG2A is regarded as a crucial complex molecule for the inhibition of γδ T cell responses [245].. Most of these inhibitory NKR decrease the killing of target cells expressing high levels of either classical and/or non-classical MHC molecules. Due to the broad cellular distribution of some Vγ9Vδ2 TCR agonists such as IPP, which are up-regulated on transformed cells, MHC class I-specific inhibitory NKR may selectively down-regulate recognition of healthy cells by Vγ9Vδ2 CTL[11,246,247]. Accordingly, iNKR masking increases Vγ9Vδ2 T cell killing of several hematopoietic and non-hematopoietic tumors[248]. Recently, HLA-G, a non-classical MHC I molecule, was shown to impair Vγ9Vδ2 T-cell cytotoxicity by interacting with ILT2 inhibitory receptor[248,249]. Probably much more will be discovered, since non classical MHC class Ib molecules play very important roles in $CD8^+$ and NK cells[90]

1.3.8.7 **Natural Cytotoxicity Receptors: NKp44**

A recent study characterized polyclonal Vγ9Vδ2 T cells expressing NKp44, a member of the NCRs previously described in NK cells[250]. NKp44 seems involved in cytotoxicity of Vγ9Vδ2 T cells against multiple myeloma cell lines lacking expression of NKG2D ligands. However $NKp44^+$ Vγ9Vδ2 T cells could only be induced *in vitro* by stimulation with complex cytokine cocktail protocols, and the percentage of $NKp44^+$ γδ T cells in culture was very low[250]. This raises questions about the biological role of NKp44 on Vγ9Vδ2 T cells.

1.3.8.8 **NKG2D**

As described for NK cells, essentially all Vγ9Vδ2 T cells also express NKG2D on their cell surface. NKG2D is an activating C-type like lectin noncovalently associated with the adaptor molecule DAP10 (which enables signal transduction).

The expression of NKG2D ligands, ULBPs and MICA/B in humans (Rae1, H60, and MULT-1 proteins in mice), modulated in response to stress or as a result of transformation, provides a means by which γδ T cells may recognize tumors[164]. MICA/B

and ULBPs are frequently expressed on the surface of tumor cells efficiently killed by Vγ9Vδ2 T cells. Several studies have emphasized the contribution of NKG2D for tumor immunosurveillance[251], and particularly Vγ9Vδ2 T cells displayed cytotoxicity triggered through NKG2D stimulation against a wide range of tumor cell lines such as colon, colorectal and pancreatic carcinomas, melanomas[252-254], lymphomas and leukemias[255]. NKG2D$^{-/-}$ mice have impaired Immunosurveillance of epithelial and lymphoid malignancies in two transgenic models of *de novo* tumorigenesis.

As described for NK cells, some studies reported the ability of Vγ9Vδ2 T cells to trigger effector responses through NKG2D stimulation alone[254,256]. However, others have failed to show any Vγ9Vδ2 T cell NKG2D-induced activation without coincident TCR stimulation (as is described for CD8$^+$ T cells)[137,146,148]. Such discrepancies may be due to different activation status of γδ T cells as a result of different cell culture conditions. More recently, Nedellec *et al*. showed that, while NKG2D *per se* could not induce calcium flux, its co-engagement significantly augmented the intensity of TCR/CD3-mediated responses, which also translated into enhanced cytotoxic activity. By contrast, the production of IFN-γ was unaffected by NKG2D costimulation[148].

γδ T, CD8$^+$ αβ T lymphocytes and natural killer (NK) cells display different sensitivity to signals delivered via NKG2D. All the three effector cell populations activate Akt1/PKBα through the engagement of this molecule. Upon binding to leukemic cells expressing NKG2D ligands (NKG2DL), including chronic lymphocytic leukemias treated with transretinoic acid, most γδ T (>60%) and half CD8$^+$ αβ T cells (about 50%) received a survival signal, at variance with the majority of NK cells (>80%) that underwent apoptosis by day 5[257]. These biochemical data may explain, at least in part, why γδ T and CD8$^+$ αβ T cells are cytolytic effector cells more resistant to target-induced apoptosis than NK cells[257].

1.3.9 Tumor control by γδ T cells

1.3.9.1 Tumor cell recognition *in vitro*

Activated Vγ9Vδ2 T cells isolated from peripheral blood mononuclear cells of healthy donors can recognize and kill a large variety of tumor cell lines *in vitro*[258,259], including

breast cancer[260], colon and nasopharyngeal carcinomas[252,261], melanoma[262], pancreatic adenocarcinomas[262] and particularly a large number of hematopoietic cell-derived tumors[167,263], including Daudi cell line, derived from Burkitt´s Lymphomas[183,264-267]. However, the molecular basis of these two distinct susceptibilities remains unclear. It has been suggested a role for Natural Killer inhibitory receptors (CD94/NKG2A) binding to HLA-I in tumor cells[167,268-270]. An alternative explanation suggests that Vγ9Vδ2 T cells recognize tumor targets (such as Daudi, MOLT-4 and K562), through TCR interaction with phosphoantigens endogenously produced by tumor cells, and use NKR signals to fine-tune the cell activation threshold[148,192,208,271,272]. Recently, DNAM-1 and NKG2D were described as co-modulators of Vγ9Vδ2 T cell cytotoxicity against hepatocellular carcinoma[230]. Other studies have shown that heat-shock proteins[174] and ULBP-4[55] can directly bind Vγ9Vδ2 TCR and impact on tumor cell recognition, although the biological relevance of these interactions was not yet fully demonstrated.

The pattern of cytotoxicity mediated by peripheral blood γδ T cells is markedly different from that mediated by NK cells from same individuals, suggesting an influence of the γδ TCR[273]. Another presumably important anti-tumor effect is the induction of IFN-γ-producing Vγ9Vδ2 T cells *in vivo*. Multiple anti-tumor effects have been attributed to IFN-γ, including direct inhibition of tumor growth or more indirect effects such as the blocking of angiogenesis[274]. Interestingly, a significant negative correlation between the serum levels of the angiogenic factors VEGF (vascular endothelial growth factor) and IFN-γ was found in cancer patients treated with Aminobisphosphonates[275].

1.3.9.2 **Mouse tumor models**

Conventional mouse models cannot be used to explore the possible anti-tumor activity of human γδ T cells *in vivo*, due to the lack of Vγ9Vδ2 homologous TCR and thus the reactivity to phosphoantigens. However, Xenogeneic immune deficiency (SCID) mouse models of human tumors have been established, and revealed the efficacy of Vγ9Vδ2 T cells against several human tumors *in vivo*[259,261,262,276-282]. Pre-activated adoptively transferred human Vγ9Vδ2 T cells localized to tumors[261,276], increased survival, and inhibited tumor growth[261,262,276,278,280]. Vγ9Vδ2 T cells are also

active against freshly isolated tumor cells from patients with follicular B lymphoma or B-cell chronic lymphocytic leukemia (B-CLL)[283]. Similarly, a high survival rate is obtained when Vγ9Vδ2 TCR^+ tumor infiltrating lymphocyte (TILs) (expanded from human colorectal tumors in vitro) are transferred into Daudi cell-bearing BALB/c nude mice compared with the transfer of αβ TCR^+ TILs or mice without treatment[284].

1.3.9.3 **Clinical trials**

Aminobisphosphonates, such as pamidronate and zoledronate, which were originally developed as therapeutic drugs for osteoporosis but are increasingly used for cancer therapy, have also been shown to activate Vγ9Vδ2 T cells in patients. By inhibiting the enzyme farnesyl pyrophosphate synthase in the antigen presenting cells, they provoke an accumulation of natural phosphoantigens (i.e. IPP) in target cells[184,207]. Zoledronate is efficient at expanding *in vitro* γδ T cells from patients with different diseases[285]. Several clinical trials tested the efficacy of the administration of aminobisphosphonate-activated Vγ9Vδ2 T cells to patients with multiple myeloma[286], renal cell carcinoma[287] and various other tumors[288]. These studies revealed no serious treatment-related adverse effects[286,287], and demonstrated efficient expansion of Vγ9Vδ2 T cells[287], and inhibition of tumor growth[286]. Administration of aminobisphosphoantes in conjunction with IL-2 resulted in an anti-tumor partial response in patients with low-grade non-Hodgkin lymphoma and multiple myeloma[289] and hormone-refractory prostate cancer[290]. However, in these studies, some of the patients did not respond to therapy.

Alternative strategies considering the adoptive transfer of *in vitro* expanded tumor-reactive Vγ9Vδ2 T cells, were performed with different synthetic phosphoantigens to treat patients with renal cell carcinoma[287,291], and produced promising results. In pre-clinical models, HMB-PP injection in macaques induced a prolonged major expansion of circulating Vγ9Vδ2 T cells that expressed CD8 and produced perforin[292], and BrHPP induced amplification of effector-memory Vγ9Vδ2 T cells in cynomolgus monkeys[293].

A general disadvantage of autologous γδ T cell-mediated tumor immunotherapy is the frequent impaired function of γδ T cells in cancer patients. The anergy of γδ T cells has impact in the anti-lymphoma effect. This phenomenon has been described in

certain chronic infectious diseases such as HIV infection or tuberculosis, although the cause of this γδ T cell anergy is not fully understood[294,295]. Recent data obtained with other lymphocyte subsets suggests that tumor-derived PDL1/2 signals may be responsible for the inhibition of PD-1$^+$ T cells[296,297], although these findings need to be further investigated[298]

In general, the clinical trials completed to date, particularly those stimulating γδ T cells *in vivo*, have shown objective responses in the range of 10 to 33%[299]. If, in some cases, the lack of response to therapy could be attributed to deficient expansion of effector Vγ9Vδ2 T cells[289,290,300], many patients exhibiting significant and sustained *in vivo* activation and proliferation of Vγ9Vδ2 T cells also failed to respond to treatment[290]. Thus, current γδ T cell–based treatments, although feasible and safe, have obvious limitations[299].

1.4 OBJECTIVES OF THIS THESIS

The anti-tumor capacity of γδ T cells has prompted the development of clinical trials involving their stimulation through the administration of small non-peptidic phosphoantigens and bisphosphonates. Despite the promise of tumor surveillance by γδ T cells, the molecular mechanisms mediating tumor cell recognition and activation of γδ T cells remain poorly understood. In fact, the greatest enigma in γδ T cell biology is their target cell recognition, as very few tumor-associated antigens for γδ T cells have been identified.

We aimed to analyse the molecular mechanisms involved in human γδ T cell activation by phosphoantigens, and to characterize tumor susceptibility to γδ T cell-mediated killing. Our main goal was to define novel tumor antigens expressed in malignant hematopoietic cells that trigger γδ T cell cytotoxicity. Finally, we aimed at manipulating γδ T cells in order to optimize their anti-tumor function, and thus provide new fundamental knowledge to be translated into innovative immunotherapies for cancer.

2. HIGHLY ACTIVE MICROBIAL PHOSPHOANTIGEN INDUCES RAPID YET SUSTAINED MEK/ERK- AND PI-3K/AKT-MEDIATED SIGNAL TRANSDUCTION IN ANTI-TUMOR HUMAN γδ T-CELLS

2.1 ABSTRACT

2.1.1 Background / Objectives

The unique responsiveness of Vγ9Vδ2 T-cells, the major γδ subset of human peripheral blood, to non-peptidic prenyl pyrophosphate antigens constitutes the basis of current γδ T-cell-based cancer immunotherapy strategies. However, the molecular mechanisms responsible for phosphoantigen-mediated activation of human γδ T-cells remain unclear. In particular, previous reports have described a very slow kinetics of activation of TCR-associated signal transduction pathways by isopentenyl pyrophosphate and bromohydrin pyrophosphate, seemingly incompatible with direct binding of these antigens to the Vγ9Vδ2 TCR. Here we have studied the most potent natural phosphoantigen yet identified, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), produced by *Eubacteria* and *Protozoa*, and examined its γδ T-cell activation and anti-tumor properties.

2.1.2 Methodology/Principal Findings

We have performed a comparative study between HMB-PP and the anti-CD3ε monoclonal antibody OKT3, used as a reference inducer of *bona fide* TCR signaling, and followed multiple cellular and molecular γδ T-cell activation events. We show that HMB-PP activates MEK/Erk and PI-3K/Akt pathways as rapidly as OKT3, and induces an almost identical transcriptional profile in $V\gamma9^+$ T-cells. Moreover, MEK/Erk and PI-3K/Akt activities are indispensable for the cellular effects of HMB-PP, including γδ T-cell activation, proliferation and anti-tumor cytotoxicity, which are also abolished upon antibody blockade of the $V\gamma9^+$ TCR Surprisingly, HMB-PP treatment does not induce down-modulation of surface TCR levels, and thereby sustains γδ T-cell activation upon

re-stimulation. This ultimately translates in potent human γδ T-cell anti-tumor function both *in vitro* and *in vivo* upon transplantation of human leukemia cells into lymphopenic mice.

The development of efficient cancer immunotherapy strategies critically depends on our capacity to maximize anti-tumor effector T-cell responses. By characterizing the intracellular mechanisms of HMB-PP-mediated activation of the highly cytotoxic Vγ9^{+} T-cell subset, our data strongly support the usage of this microbial antigen in novel cancer clinical trials.

2.2 INTRODUCTION

2.2.1 Phosphoantigen-mediated activation of Vγ9Vδ2 T cells

The capacity to recognize and eliminate transformed cells is common to several lymphocyte subsets of both the adaptive and the innate immune systems that are being targeted in cancer immunotherapy[21,22]. One population that appears to bridge these two systems in humans is characterized by the expression of a Vγ9Vδ2 T-cell receptor and represents 1–10% of peripheral blood lymphocytes (PBL) of healthy individuals, but expands up to 30–50% upon bacterial or protozoan infection[192].

In line with the cancer susceptibility phenotype of mice devoid of γδ T-cells[11], human Vγ9Vδ2 T-cells are endowed with notable anti-tumor activity toward a large spectrum of malignant cell lines of diverse tissue origin, particularly among lymphomas and leukemias[255], but also including melanomas and carcinomas[252], and are being explored in various clinical trials[289,290]. Unexpectedly, Vγ9Vδ2 cells were shown to respond to self- and foreign *non*-peptidic low molecular weight antigens with phosphate moieties ("phosphoantigens"), in what turns out to be an exclusive property of this lymphocyte subset[56,183,184]. Indeed, no other human T-cell subset (namely Vδ1 cells), or any of the murine γδ populations, respond to phosphoantigens such as prenyl pyrophosphates[192].

From its early isolation from mycobacteria, isopentenyl pyrophosphate (IPP)[56] became the model phosphoantigen for studies on Vγ9Vδ2 activation. However, it is now clear that this class of compounds contains multiple members, either naturally occurring or synthetic, which span an extremely diverse range of bioactivities, up to 10^{10} fold

differences. To date, the natural phosphoantigen with highest bioactivity known (32 picomolar) is (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), an intermediate of the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway employed by *Eubacteria* and apicomplexan *Protozoa* but not by eukaryotes[271]. Although HMB-PP is respectively 30,000 and 100 times more potent than IPP and bromohydrin pyrophosphate (BrH-PP, also known as "Phosphostim"), most of the studies on phosphoantigens have been performed with these compounds (already applied in the clinic) due to their historical precedence[192]. Such studies revealed a very slow kinetics of activation of TCR-associated signal transduction pathways, and conflicting results regarding their potential interactions with the Vγ9Vδ2 TCR[185,244,271]. This, added to the consistent failure to demonstrate cognate interactions between Vγ9Vδ2 TCRs and phosphoantigens in acellular systems[301], has shed some skepticism regarding the action of phosphoantigens as direct TCRγδ agonists. As HMB-PP is considered for γδ T-cell-based cancer clinical trials, hoping to improve the performance of previous phosphoantigens[289,290] it is crucial to clarify its own molecular/cellular mechanisms of action, including its potential capacity to trigger *bona fide* Vγ9Vδ2 TCR signaling. Consistent with such potential, it has been recently shown that HMB-PP has the capacity to induce the formation of high-density TCR nanoclusters on the surface of human γδ T-cells[218], and a newly-developed tetramer reagent for the Vγ9Vδ2 TCR of rhesus macaques was reported to bind to HMB-PP loaded on the surface of human APCs[302].

In this study we have analyzed the intracellular effects of HMB-PP stimulation of human γδ T-cells. Our data show that HMB-PP induces the activation of MEK/Erk and PI-3K/Akt signaling pathways with similar kinetics to direct cross-linking of the TCR complex in human γδ T-cells, and requires those activities to mediate effective γδ T-cell activation, including a full repertoire of TCR-associated transcriptional signatures and the secretion of pro-inflammatory cytokines IFN-γ and TNF-α. Although TCR accessibility is required for HMB-PP activity, this phosphoantigen does not lead to ligand-induced TCR internalization, which appears to be advantageous for sustaining the cells' activation status upon re-stimulation. Finally, very low amounts of HMB-PP in conjugation with IL-2 confer human γδ T-cells with very potent anti-lymphoma/leukemia activity both *in vitro* and in a human/SCID mouse model for the transplantation of

human tumors, thus attesting the therapeutic potential of HMB-PP for cancer immunotherapy.

2.3 MATERIAL AND METHODS

Ethics statement: all experiments involving animals (rodents) were performed in compliance with the relevant laws and institutional guidelines and have been approved by the Instituto de Medicina Molecular animal ethics committee.

2.3.1 *In vitro* cultures of human peripheral blood lymphocytes

Peripheral blood was collected from anonymous healthy volunteers, diluted 1:1 (v/v) with PBS(1×) (Invitrogen Gibco) and centrifuged in LSM Lymphocyte Separation Medium (MP Biomedicals) in a volume ratio of 3:4 (3 parts of LSM for 4 of diluted blood) for 15 minutes at 1500 rpm and 25°C. The interfase containing PBMC was collected, washed in PBS (1×) and cultured at 1×10^6 cells/mL at 37°C, 5% CO2 in round-bottom 96 well plates with RPMI 1640 with 2 mM L-Glutamine (Invitrogen Gibco) supplemented with 10% foetal bovine serum (Invitrogen Gibco), 1 mM Sodium Pyruvate (Invitrogen Gibco), 50 mg/mL of penicillin/streptomycin (Invitrogen Gibco), in the presence or absence of 100 U/mL of rhIL-2 (Roche Applied Science), 1–10 nM of HMB-PP (4-hydroxy-3-methyl-but-2-enyl pyrophosphate) (a kind gift from H. Jomaa and M. Eberl), and 1-10 ug/ml of soluble anti-CD3 antibody (eBioscience, clone OKT3).

For TCR blockade, freshly-isolated PBMC were CFSE-labeled and then incubated for 6 days with anti- TcRVγ9 (Beckman Coulter, clone IMMU360) diluted 1:20 in complete medium supplemented with 1 nM HMB-PP.

For the phophoimmunoblotting experiments, MACS-isolated γδ T cells were expanded with 100 U/mL rhIL-2 for 15 days.

To study the effects of chemical inhibitors of signal transduction, the MEK inhibitor UO126 and the PI-3K inhibitor LY294002 (both from Calbiochem) were added at 10 μM for a 2-hour incubation period, and then transferred to fresh medium (without inhibitors).

2.3.2 **Magnetic cell sorting and flow cytometry analysis**

γδ T-cells were isolated (to above 95% purity) from PBMC by magnetic cell sorting via positive selection with a FITC-labeled anti-TCRγδ antibody (Miltenyi Biotec). For flow cytometry analysis (on a FACSCalibur, BD Biosciences), cells were labelled with fluorescent monoclonal antibodies: anti-CD69-PE (BD Pharmingen), anti-TcRVγ9-PC5 (Beckman Coulter) and anti-CD4-PerCP (BD Pharmingen). In all cultures the percentage of $V\gamma9^+$ T-cells was evaluated by flow cytometry. Cell proliferation was measured by following a standard CFSE staining protocol (CellTrace CFSE Cell Proliferation Kit, Invitrogen; final concentration 0.5 μM), while apoptosis was assessed by AnnexinV-FITC (BD Pharmingen) staining. Cells were counted in Mossbauer chambers using 0.4% Trypan Blue solution (Sigma-Aldrich) for viability control.

2.3.3 **Cytometric Bead Array (CBA)**

Cytokine secretion was measured using Cytometric Bead Array (CBA) technology (BD Biosciences). Cells were seeded with the respective activators at 2×10^5 cells/well, culture supernatants were collected at different time points and analyzed on a FACSCanto (BD Biosciences) using a custom-made Flex Set with five different cytokine capture beads: LT-α, IL-10, IL-4, TNF-α and IFN-γ. Data were analyzed using the FCAP Array Software v1.0.1 running on BD FACSDiva (BD Biosciences).

2.3.4 **Protein isolation and phosphoimmunoblotting**

Cells were incubated at 37°C with pre-warmed PBS alone or with HMB-PP (1 nM) or OKT3 (1 μg/mL). Reactions were stopped by placing samples on ice and adding ice-cold PBS. Cell lysates were prepared and equal amounts of protein were analyzed by 10% SDS-PAGE electrophoresis, transferred onto nitrocellulose membranes, and immunoblotted with the following mAbs or antisera: Actin, phospho-Erk (Y204) (Santa Cruz Biotechnology), ZAP-70 and phospho-STAT5A/B (Y694/Y699) (Upstate Biotechnology), phospho-Akt (S473), phospho-GSK-3β (S9), phospho-JNK/SAPK (Y183/185), phospho-p38 MAPK (Y180/182) (Cell Signalling Technology), and phospho-LCK (Y505) (Transduction Laboratories). Immunodetection was performed

with horseradish peroxidase-conjugated secondary antibody and developed by chemiluminescence as described[303]. Whenever necessary membranes were striped using 15 mM TRIS pH 6.8 plus 2% SDS and β-Mercaptoethanol (100 mM) for 40 minutes at 57°C.

2.3.5 **RNA isolation and Affymetrix GeneChip analysis**

RNA labeling, hybridization to the Affymetrix GeneChip Human Genome U133 plus 2.0 Arrays and scanning was performed by the Affymetrix Core Facility, Instituto Gulbenkian de Ciência, Portugal as described below.

Total RNA was extracted using the RNeasy Mini Kit according to manufacture's protocol (Qiagen, Hilden, Germany). Concentration and purity was determined by spectrophotometry and integrity was confirmed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA). RNA was processed for use on Affymetrix (Santa Clara, CA, USA) GeneChip Human Genome U133 Plus 2.0 Arrays, according to the manufacturer's One-Cycle Target Labeling Assay. Arrays were scanned on an Affymetrix GeneChip scanner 3000 7G.

All the microarray data analysis was done using R and several packages available from CRAN (R Development Core Team, 2008) and Bioconductor. The raw data (CEL files) was normalized and summarized with the Robust MultiArray Average method from the *affy* package.

The differentially expressed genes were selected using linear models and empirical Bayes methods as implemented in *limma* package, verifying the *p*-values corresponding to moderated F-statistics, and selecting as differentially expressed genes those that had adjusted *p*-values lower than 0.005.

2.3.6 **Real-time quantitative PCR**

Total RNA was reverse-transcribed into cDNA using random hexamers and Superscript II first strand synthesis reagents (Invitrogen). qPCR was performed on ABI Prism 7700 Sequence Detection System using SYBR Green detection system (both from PE Applied Biosystems). Primers were designed using Primer3 v.0.4.0 online

program (http://primer3.sourceforge.net). Primer sequences are available upon request. For each transcript, quantification was done using the calibration curve method. β2-microglobulin was used as the internal control for normalization. All samples were run in triplicate and repeated three times. Analysis of the qPCR results was performed using the ABI SDS v1.1 sequence analysis software (Applied Biosystems).

2.3.7 Tumor cell cultures and *in vitro* killing assays

All tumor cell lines were cultured in complete 10% RPMI 1640 (as above), maintained at 1×10^5 up to 2×10^6 cells/mL by dilution and splitting 1:3 every 3–4 days.

For cytotoxicity assays, magnetically purified γδ PBL were pre-activated for 72 hours with 1–10 µg/mL αCD3 mAb (OKT3) or 1–10 nM HMB-PP either in the absence or presence of IL-2 (100 U/mL). Tumor cell lines were stained with CellTracer Far Red DDAO-SE (1 µM) (Molecular Probes, Invitrogen) and each 3×10^4 tumor cells were incubated with 3×10^5 γδ T-cells in RPMI devoid of activating compounds, for 3 hours at 37°C and 5% CO_2 on a round-bottom 96 well plate. Cells were then stained with Annexin V-FITC and analyzed by flow cytometry.

2.3.8 Confocal microscopy

Cells were stained at 4°C with mouse anti-human TCR Vγ9-PC5 (Beckman Coulter) primary antibody, and with anti-mouse Alexa Fluor 633 (Invitrogen, Molecular Probes) secondary antibody. Cells were then fixed with 4% Paraformaldeheyde for 15 minutes at 4°C. Nuclear DNA content was stained for with DAPI Fluoromount G (Southern Biotech). Immunofluorescence microscopy was performed with a LSM 510 META confocal microscope (Zeiss). Separate images were collected with a 63× objective for each fluorochrome and then overlaid to obtain a multicolor image.

2.3.9 Bioluminescent imaging of transplanted leukemia development in SCID mice

10^7 Molt-4 T-cell leukemia cells stably expressing firefly luciferase and GFP were injected i.v. in groups of 6 NOD/SCID mice per experiment, either in isolation or

together with 5×10^7 γδ PBL (>80% Vγ9$^+$), previously expanded and activated *in vitro* with 1 nM HMB-PP for 12 days. Treated mice received boosts of 5×10^7 γδ PBL i.v. on day 14 and 10,000 U IL-2 i.p. twice every week, whereas control mice received only IL-2. All mice were analyzed on a weekly basis by *in vivo* imaging (IVIS, Caliper Lifesciences) upon intra-peritoneal injection of luciferin. Photon signals were quantified with LivingImage software (Caliper Lifesciences). Mouse body weight was measured weekly, and animals suffering from wasting (loss of over 20% of initial body weight) were sacrificed.

Statistical analysis

Statistical significance of differences between subpopulations was assessed using Student's t-test and is indicated when significant as *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

2.4 RESULTS

2.4.1 Nanomolar amounts of HMB-PP replicate saturating TCR/CD3 ligation for activation of Vγ9$^+$ T-cells

In this study we used the anti-CD3ε monoclonal antibody (αCD3 mAb) OKT3 as a control for canonical T-cell activation through the TCR/CD3 complex, for direct comparison with HMB-PP. We began by testing the effect of several doses of each stimulating compound on human γδ T-cell activation, proliferation and survival. Concentrations of 1 nM HMB-PP and 1 µg/ml OKT3 produced identical profiles of expression of the activation marker CD69 in the Vγ9$^+$ T-cell population (Figure 1A), and displayed strikingly similar kinetics of activation without significant differences in cell viability (Figure 1B); they were therefore used in all subsequent experiments. Interestingly, whereas αCD3 mAb treatment reached a plateau of 60% CD69$^+$ cells at 1–10 µg/ml OKT3, 10 nM of HMB-PP were able to further increase the abundance of activated Vγ9$^+$ T-cells, to above 80% (Figure 1A).

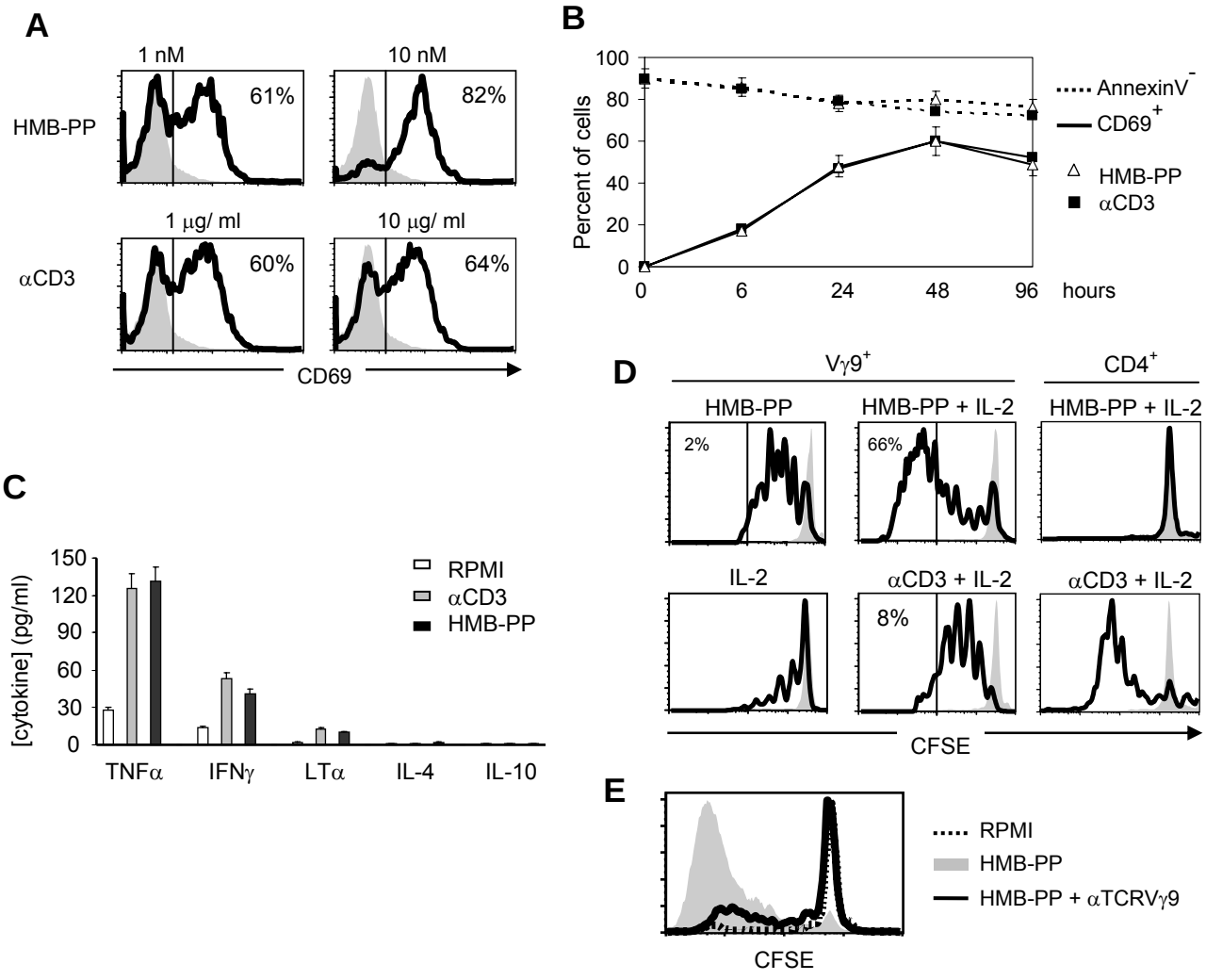


Figure 1. Nanomolar HMB-PP replicates saturating TCR/CD3 ligation for Vγ9$^+$ T-cell activation.

(**A**) Flow cytometry analysis for the expression of the activation marker CD69 in MACS-sorted (97–98% purity) γδ PBL, stimulated for 48 hours with the indicated amounts of HMB-PP or anti-CD3 mAb (OKT3). Shaded are non-stimulated Vγ9$^+$ T-cells. Percentages refer to cells above the threshold bar. (**B**) Time-course of the experiment described in (**A**) for 1 nM HMB-PP and 1 μg/ml OKT3; cells were also stained with Annexin V to assess their viability (Annexin V$^-$). (**C**) Cytokine bead array analysis of supernatants of MACS-sorted γδ PBL (of which 80–90% Vγ9$^+$) cultures after 24 hours of stimulation with HMB-PP or OKT3. RPMI refers to cells kept in media not supplemented with activating compounds. (**D**) CFSE dilution assays to monitor T-cell proliferation in total PBMC cultures supplemented with HMB-PP (1 nM) or OKT3 (1 μg/ml), with or without 100 U/mL rhIL-2. Cells (gated on Vγ9$^+$ or CD4$^+$) were analyzed by flow cytometry

after 4 days in culture; shaded are non-divided cells. Percentages indicate cells that have undergone more than 5 rounds of division. (**E**) CFSE dilution in gated Vγ9$^+$ T-cells within 6-day cultures of total PBMC activated with 1 nM HMB-PP in the presence or absence of blocking anti-TcRVγ9 antibody. Dashed is a control incubated in 10% RPMI without HMB-PP. Results shown in this figure are representative of 3 independent experiments.

Activated Vγ9Vδ2 T-cells are known to secrete large amounts of IFNγ and TNFα, very potent anti-tumor mediators *in vivo*. In accordance, treatment of sorted γδ PBL (80–95% Vγ9$^+$) with HMB-PP induced a typical Th1 cytokine profile, characterized by the preferential production of TNFα, IFNγ and LTα, in the absence of significant IL-4 or IL-10 (Figure 1C). Notably, the levels of Th1 cytokines produced after 1 nM HMB-PP treatment were similar to those induced by saturating amounts of αCD3 mAb (Figure 1C and data not shown), suggesting that low amounts of this phosphoantigen are able to fully exploit the TCR-mediated functional potential of Vγ9Vδ2 T-cells.

For the selective expansion of Vγ9$^+$ T-cells, HMB-PP has the advantage of not inducing αβ T-cell proliferation. Thus, HMB-PP treatment promoted the specific proliferation of Vγ9$^+$ T-cells within human PBL (Figure 1D). Importantly, this effect was completely abolished upon addition of a blocking antibody to the Vγ9$^+$ TCR (Figure 1E), demonstrating the TCR-dependence of HMB-PP activity.

While HMB-PP alone promoted up to 5 divisions of Vγ9$^+$ T-cells over 4 days, further proliferation required the co-administration of IL-2 (Figure 1D). A cooperative effect between phosphoantigens and IL-2 has been previously described[292,304], and in this study translated into a Vγ9$^+$ T-cell expansion of 30-fold within one week and 45-fold within two weeks of stimulation (Figure 2A). Moreover, addition of 100 units/mL IL-2 to HMB-PP cultures dramatically increased the total amounts of Th1 cytokines secreted by γδ T-cells by 20–80 fold (Figure 2B), which correlated with the induction of key transcription factor *t-bet* in cells stimulated with IL-2 or IL-2/HMB-PP combination (Figure 2C)

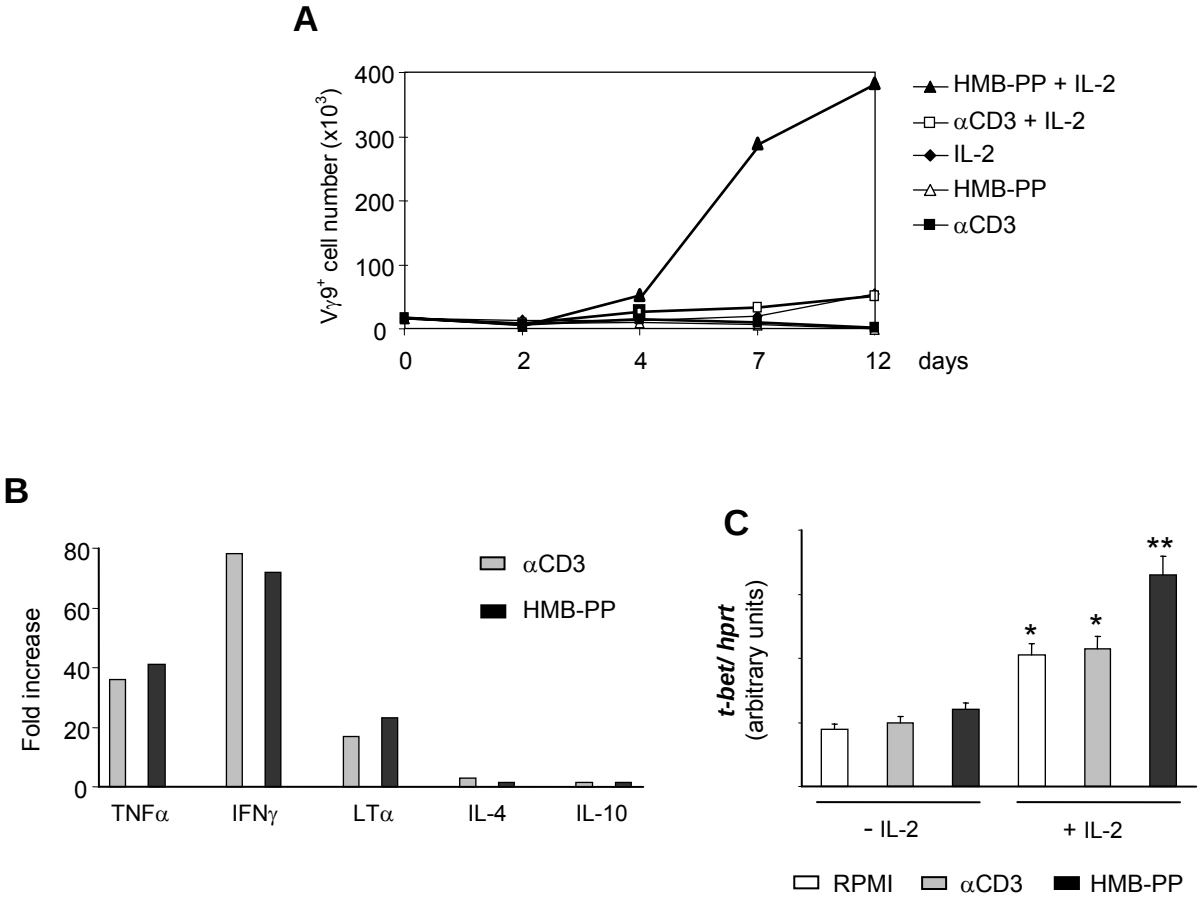


Figure 2. Exogenous IL-2 expands HMB-PP-activated Vγ9$^+$ T-cells and up-regulates their Th1 cytokine profile.

(**A**) Absolute numbers of Vγ9$^+$ cells in PBMC cultures stimulated with HMB-PP (1 nM) or OKT3 (1 ug/ml), supplemented or not with IL-2 (100 U/ml). Cells were analyzed by flow cytometry and light microscopy (Mossbauer chamber cell counts). (**B**) Cytokine bead array (CBA) analysis of supernatants of MACS-sorted γδ PBL (of which 80–90% Vγ9$^+$) after 24 hours of stimulation with HMB-PP or anti-CD3 mAb (OKT3). Represented is the ratio between the cytokine amounts produced in the presence (100 U/ml) and in the absence of IL-2. (**C**) Real-time PCR quantification of t-bet mRNA expression in activated Vγ9Vδ2 T-cells, normalized with β2-microglobulin. Cells were pre-incubated for 6 hours with the activating compounds (or kept in RPMI as control). Significant differences refer to cells cultured in RPMI in the absence of IL-2 (n = 3, *p<0.05 and **p<0.01).

2.4.2 HMB-PP rapidly triggers MEK/Erk and PI-3K/Akt signaling required for Vγ9$^+$ T-cell activation and anti-tumor function

Having characterized the cellular behavior of HMB-PP-stimulated Vγ9$^+$ T-cells, we next investigated the intracellular signaling mechanisms downstream of HMB-PP. Previous studies with less active phosphoantigens[192] reported a significant delay in the activation of kinase cascades when compared to direct TCR/CD3 complex ligation with OKT3 mAb[244,271]. Instead, for HMB-PP, we observed a very rapid (peaking around 7 min of stimulation), and absolutely identical to OKT3, kinetics of phosphorylation of the major signaling pathways implicated in TCR signal transduction: JNK, Erk and p38 MAPK; and PI-3K-associated Akt and GSK3β (Figure 3A, left panel). The same was valid in the presence of IL-2, in which kinase phosphorylation peaked earlier (immediately after 1 min of stimulation) but was still identical for HMB-PP or OKT3 combinations (Figure 3A, right panel). Of note, we verified that IPP could not replicate these signaling properties of HMB-PP, as illustrated by its failure to induce Akt phosphorylation within 60 minutes of stimulation (Figure 4). Furthermore, IPP treatment (even when used at 10^5 fold higher concentrations than HMB-PP) resulted in a modest production of TNFα and IFNγ within the first 6 hours of stimulation, when compared to HMB-PP (Figure 3B). These data reveal a thus far unique capacity of HMB-PP to trigger very rapid TCR-associated signaling, compatible with direct binding of the phosphoantigen to the TCR complex.

We next tested the requirement on intact PI-3K and MAPK pathways for γδ T-cell activation and anti-tumor function induced by HMB-PP. We pre-treated γδ T-cells with chemical inhibitors that specifically block those pathways and then analyzed the effects on cell activation, proliferation, TNFα secretion and tumor cell killing. Inhibition of PI-3K/Akt pathway using LY294002 resulted in approximately half of the cells losing their responsiveness to HMB-PP after 24–46 hours of stimulation (Figure 5A). Inhibition of the MEK/Erk pathway by UO126 produced even more dramatic effects, precluding HMB-PP-activation of approximately two thirds of Vγ9$^+$ T-cells. Moreover, inhibition of PI-3K/Akt and MEK/Erk signaling reduced TNFα production by HMB-PP-activated γδ T-

cells to around 20% and 10% of control levels, respectively, both in the absence and in the presence of IL-2 (Figure 5B).

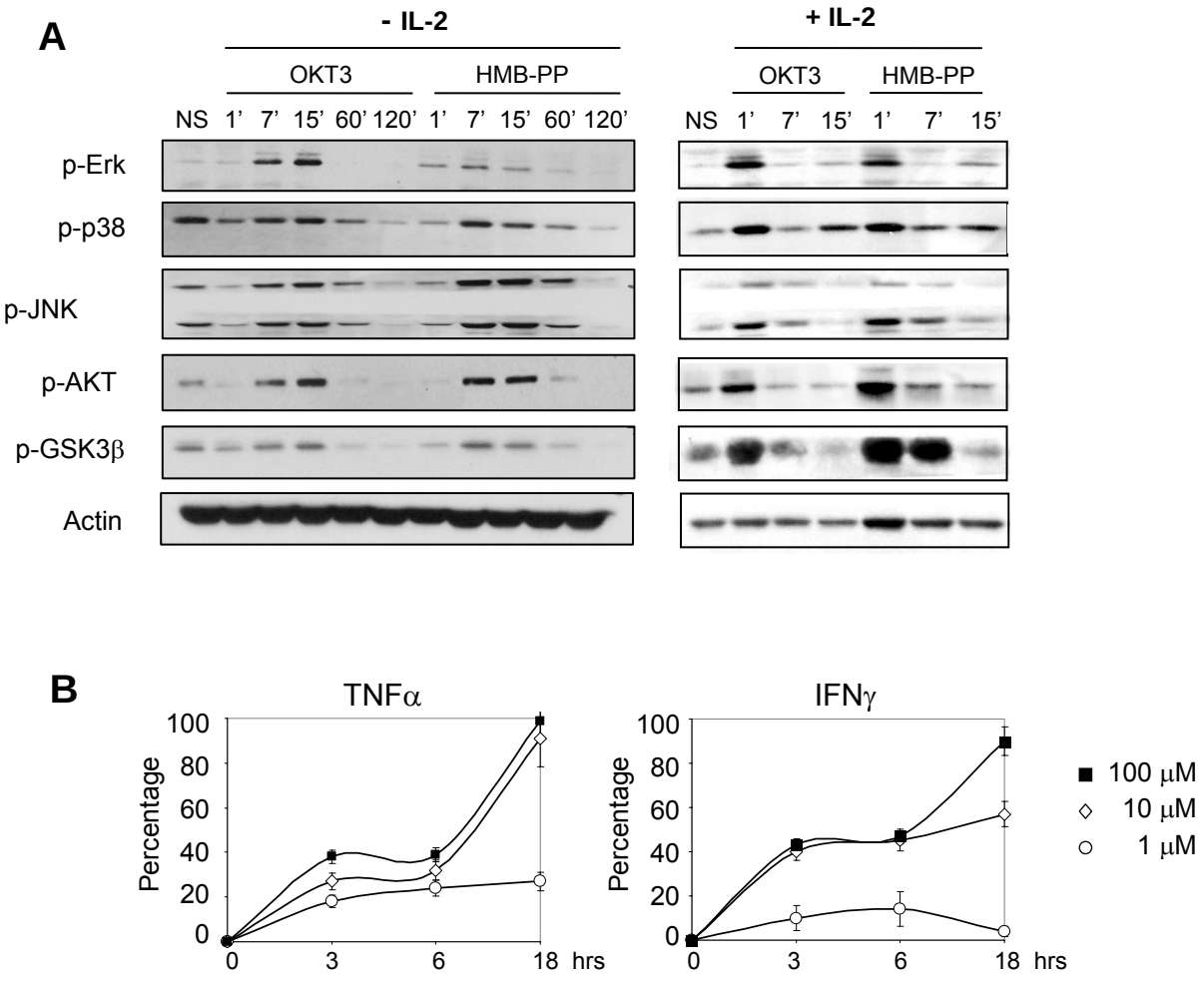


Figure 3. HMB-PP stimulation kinetically mimics Vγ9$^+$ TCR/CD3 signal transduction.

(**A**) Phosphoimmunoblotting for kinases implicated in TCR signaling. MACS-sorted γδ PBL (of which 80–95% Vγ9$^+$) were incubated with OKT3 (1 μg/ml) or HMB-PP (1 nM), in the absence (*left panel*) or presence (*right panel*) of 100 U/mL rhIL-2, for the times indicated, or kept in control media (NS, non-stimulated). Results shown in this figure are representative of 4 independent experiments. (**B**) TNFα and IFNγ levels were measured by CBA in the culture supernatants of MACS-sorted γδ PBL. Results were compared with the total amounts present in parallel cultures stimulated with 1 nM HMB-PP, and were expressed as percentages (IPP/HMB-PP).

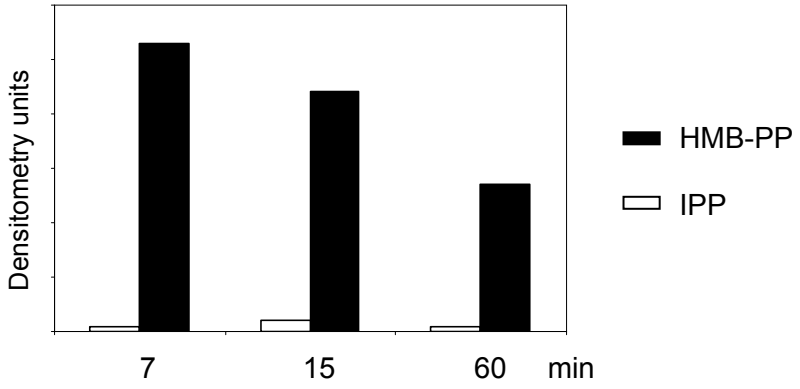


Figure 4. Akt phosphorylation in response to IPP versus HMB-PP stimulation of γδ PBL.

MACS-sorted γδ PBL were activated with 10 uM IPP or 1 nM HMB-PP for the indicated times. Cell lysates were analyzed by SDS-PAGE and immunoblotted for Phospho-Akt (P-Akt) or Beta-Actin on nitrocellulose membranes. Densitometry for P-Akt bands was normalized with Beta-Actin loading controls. Data correspond to the induction of Akt phosphorylation above basal levels, i.e., after subtraction of the unstimulated control levels.

These effects were remarkably mirrored in cultures supplied with αCD3 mAb, further demonstrating the similarity of these two activation regimens (Figures 5A and 5B).

In what regards γδ T-cell proliferation induced either by HMB-PP or by OKT3 (in the presence of IL-2), this was mostly dependent on intact PI-3K/Akt signaling, since UO126 had a more modest effect when compared with the severe block produced by LY294002 treatment, which reduced the proportion of γδ cells that divided twice or more over 4 days in culture, from over 80% to approximately 20% (Figure 5C).

Finally, the anti-tumor function of sorted γδ PBL (80–95% $V\gamma9^+$) was assessed through *in vitro* killing of the Jurkat leukemic target cell line. HMB-PP pre-treatment augmented γδ T-cell-mediated tumor cell death from around 20% (non-activated γδ) to 40% (1 nM HMB-PP) or 70% (10 nM HMB-PP) in a 6 hour assay (Figure 5D and data not shown). However, the addition of UO126 or/and LY294002 to the treatment reduced posterior leukemia targeting to basal (20–30%) levels; this was also the case for the more efficient (over 80% killing) combination of HMB-PP with IL-2 (Figure 5D). Collectively, these data demonstrate an absolute requirement of PI-3K/Akt- and

MEK/Erk-mediated signal transduction for HMB-PP-induced activation of anti-tumor Vγ9Vδ2 T-cells.

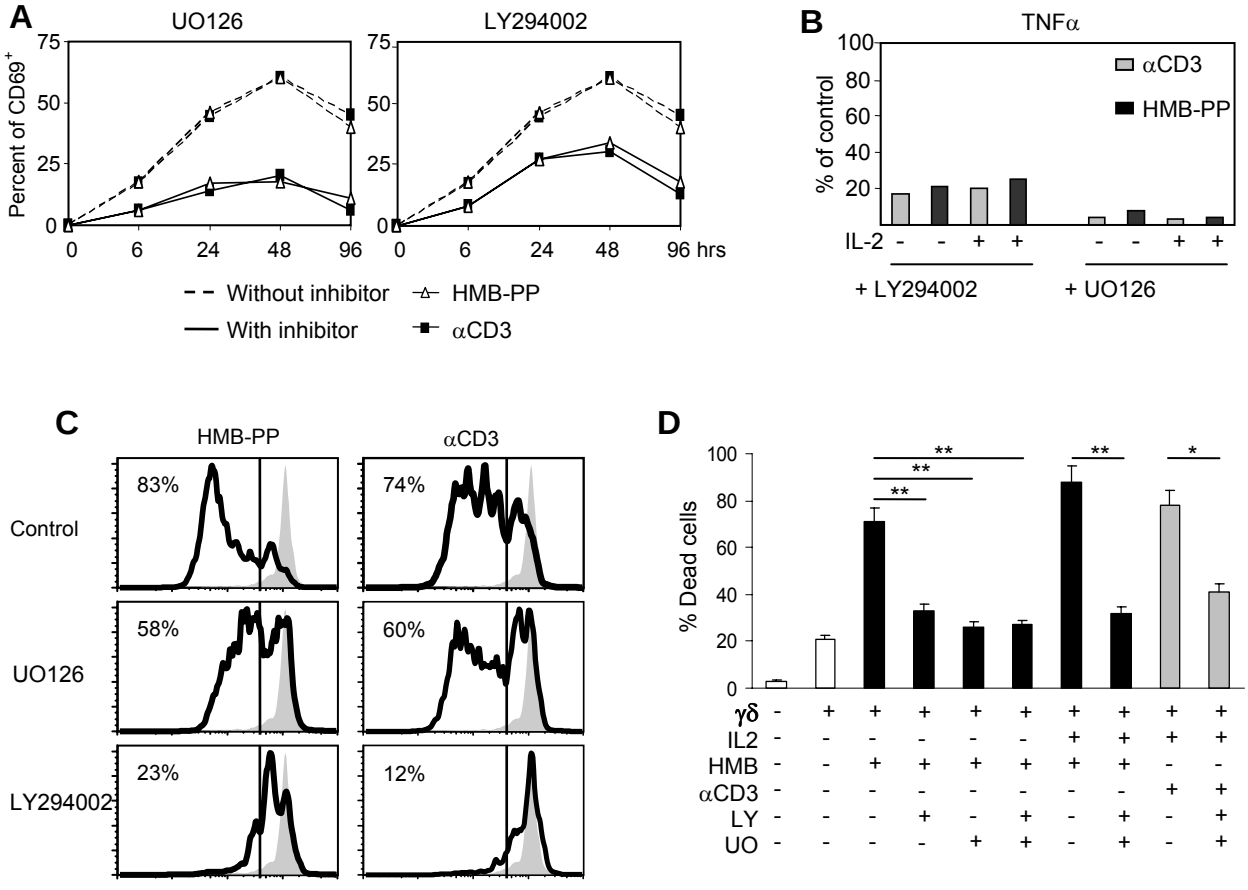


Figure 5. HMB-PP-mediated Vγ9$^+$ T-cell activation requires functional MEK/Erk and PI-3K/Akt signaling pathways.

Effects of MEK/Erk inhibitor UO126 and PI-3K/Akt inhibitor LY294002 on the activation and function of MACS-sorted γδ PBL (of which 85–95% Vγ9$^+$).

(**A**) Expression of activation marker CD69, assessed by flow cytometry. (**B**) secretion of TNFα after 24 hours of stimulation, measured by CBA. (**C**) Cell proliferation, assessed by CFSE dilution after 4 days in culture (percentages indicate cells that have undergone 2 or more rounds of division). (**D**) Jurkat leukemia cell killing, assessed by Annexin V staining and flow cytometry analysis after 6 hrs of co-incubation with pre-activated (for 3 days) γδ PBL. Results shown in this figure are representative of 3 independent experiments. Error bars represent SD and significant differences refer to controls without addition of chemical inhibitors (n = 3, *p<0.05 and **p<0.01).

2.4.3 HMB-PP signaling mimics the transcriptional events downstream of TCR ligation

Signaling cascades ultimately produce alterations in gene transcription, which can be effectively tracked by microarray analysis. We employed this technology to compare the transcriptomes of Vγ9Vδ2 T-cells activated with either HMB-PP or OKT3. Both stimuli produced dramatic transcriptional changes: when compared to non-stimulated cells, HMB-PP and OKT3 treatment resulted in 1359 and 1080 differences in gene expression of 4-fold or above, respectively (Figure 6A; full microarray data available on ArrayExpress via http://www.ebi.ac.uk/; accession E-MEXP-1601). These were consistent across 3 individual microarray experiments (Figure 7). Strikingly, a direct comparison of the two stimuli revealed that they affected essentially the same genes, as only 6 were differentially expressed (>4-fold) between them (Table 1). Therefore, the transcriptional program downstream of HMB-PP appears to be extremely similar to that induced by *bona fide* TCR signaling, as clearly illustrated by the Volcano plots of Figure 6A.

The gene expression program shared by HMB-PP treatment and direct TCR cross-linking involves, among many others targets (E-MEXP-1601), the very high (above 16-fold) up-regulation of pro-inflammatory genes IFNγ and LTα, chemokines CCL8, CCL2, CXCL9 and CXCL10, cell cycle mediator cyclin D2, activation co-receptor ICOS, cytolysis mediator Fas ligand (Fas-L), and components of cytokine receptors IL-2Rα (CD25) and IL-15Rα (Table 1), many of which are also induced by related phosphoantigens[305]. These results were validated by quantitative real-time PCR (qPCR), as shown on Figure 6B for a selection of genes.

Although we have concentrated here on genes upregulated upon stimulation, the profile of downregulated genes was also almost identical between the two treatments (E-MEXP-1601). Our results collectively suggest that HMB-PP essentially recapitulates the transcriptional program associated with *bona fide* TCR signaling. This phenomenon is further illustrated by a heatmap representation of gene expression levels across the samples, as depicted in Figure 7.

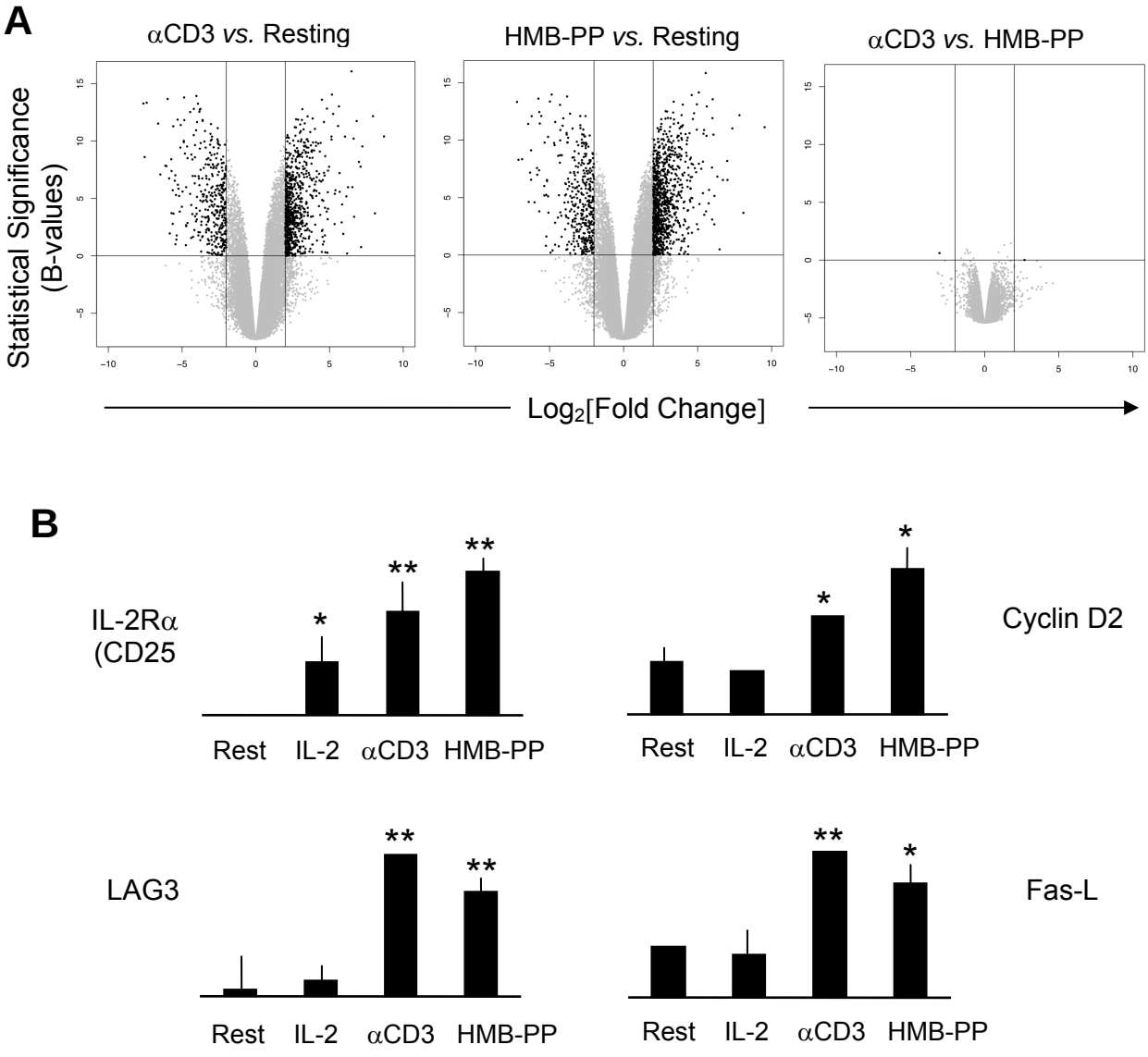


Figure 6. HMB-PP treatment reproduces the transcriptional alterations induced by TCR/CD3 ligation on γδ T-cells.

(**A**) Volcano plots of DNA microarray comparisons between αCD3 (OKT3) mAb-treated, HMB-PP-treated and non-stimulated ("resting") MACS-sorted γδ PBL (of which 85–95% $V\gamma9^+$). After 18 hours of incubation with the stimuli, RNA was extracted and submitted to Affymetrix GeneChip analysis. Represented are Fold-changes ("biological significance") versus statistical significance (B-values). Black dots represent genes over 4-fold differentially expressed (DE) between samples; all other probed genes are depicted in grey. Genes selected as differentially expressed had adjusted p-values lower than 0.005. Results are representative of 3 independent microarray experiments (see Figure 7). (**B**) Real-time PCR validation of microarray results for a

selection of genes similarly induced by OKT3 and HMB-PP (from Table **1**). Gene expression was quantified in independent samples of control and treated cells, also including an IL-2-treated sample. Error bars represent SD and significant differences refer to "resting" cells (n = 3, *p<0.05 and **p<0.01).

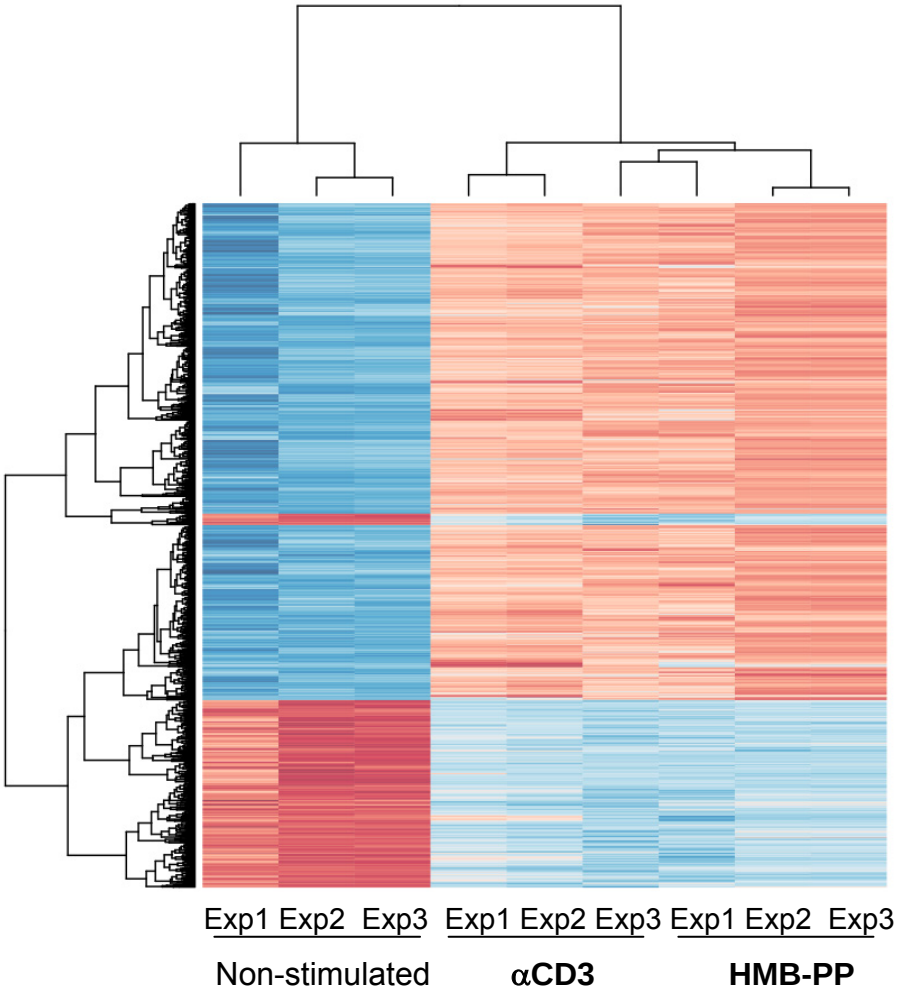


Figure 7. Heatmap of non-stimulated, HMB-PP-treated and anti-CD3 mAb (OKT3)-treated γδ T-cells.

The DNA microarray expression value for each gene is normalized across the samples; levels greater than the mean in a given sample are colored in red, and those below the mean are depicted in blue. Exp1-3 are triplicate independent microarray experiments. Note the striking similarity between HMB-PP-treated and anti-CD3-treated samples.

Similarly induced by HMB-PP and OKT3[(a)]

Link[b]	Gene	Description	Function	HMB[c]	OKT3[c]	Differ[d]
3458	*IFNγ*	Interferon-γ	Cytokine	9.53	8.70	0.83
6355	*CCL8*	Chemokine CC motif 8	Chemokine	8.09	8.08	0.01
114614	*MIRN155*	MicroRNA 155	MicroRNA	7.83	7.95	-0.12
4049	*LTα*	Lymphotoxin-α	Cytokine	7.35	6.64	0.71
6347	*CCL2*	Chemokine CC motif 2	Chemokine	6.49	6.19	0.30
3559	*IL2Rα*	IL-2R α chain	Cytokine-R	6.34	7.10	-0.76
4283	*CXCL9*	Chemokine CXC motif 9	Chemokine	6.14	6.14	0.00
3627	*CXCL10*	Chemokine CXC motif 10	Chemokine	5.75	4.87	0.88
894	*CCND2*	Cyclin D2	Cell cycle	5.62	5.25	0.37
3902	*LAG3*	Lymphocyte activation gene	Activation-R	4.46	4.08	0.38
29851	*ICOS*	Inducible T cell costimulator	Activation-R	4.4	5.40	-0.94
6504	*SLAMF1*	Signal transducer SLAM-1	Signaling	4.23	4.34	-0.11

Differentially induced by HMB-PP or OKT3

Link[b]	Gene	Description	Function	HMB[c]	OKT3[c]	Differ[d]
7412	*VCAM1*	Vascular cell adhesion-R	Adhesion	4.50	1.45	3.05
6373	*CXCL11*	Chemokine CXC motif 11	Chemokine	5.45	2.59	2.86
1493	*CTLA4*	Co-receptor CTLA-4	Activation-R	4.53	7.23	-2.70
112744	*IL17F*	IL-17 isoform F	Cytokine	1.29	4.81	-3.52
4094	*MAF*	Transcription factor Maf	Signaling	0.04	3.83	-3.79
6374	*CXCL5*	Chemokine CXC motif 5	Chemokine	0.04	7.14	-7.10

Table 1. Transcriptional changes induced by HMB-PP or OKT3 (anti-CD3 mAb) in Vγ9Vδ2 T cells.

Values are $\log_2$[fold change] compared to non-stimulated cells, based on triplicate microarray experiments. (-R, receptor)

[a] Listed is a selection of genes implicated in T cell activation. Full cDNA microarray data available on ArrayExpress (E-MEXP-1601).

[b] Locus link gene ID (for unequivocal gene identification)

[c] $\log_2$[fold change] relative to non-stimulated cells

[d] Difference in fold induction between HMB-PP-treated and OKT3-treated cells

2.4.4 HMB-PP does not induce Vγ9$^+$ TCR internalization and sustains the production of anti-tumor cytokines

Although our previous data demonstrated a striking parallel between HMB-PP- and OKT3-mediated γδ T-cell activation, previous reports on various phosphoantigens (other than HMB-PP) had revealed contradictory data on the modulation of surface Vγ9Vδ2 TCR levels[185,244]. This, added to recent data on the properties of HMB-PP interactions with TCR/CD3 complexes[218,302], prompted our investigation on whether HMB-PP stimulation induced TCR internalization in human γδ PBLs. In αβ T cells, activation by cognate antigen or anti-TCR/CD3 antibodies typically induces TCR internalization and consequently the down-modulation of its surface levels independently of the constitutive recycling of the complex[306,307]. Using two independent approaches, based on flow cytometry (Figure 8A) or confocal microscopy (Figure 8B), we consistently observed that HMB-PP-stimulated γδ T-cells maintained their high TCR surface expression, in stark contrast with the extensive down-regulation seen in OKT3-treated cells. This was the case both in the absence and in the presence of IL-2 (data not shown).

We next asked whether the lack of TCR internalization upon HMB-PP treatment could be associated with sustained activation of γδ T-cells. We tested the capacity of cells that had been treated for 2 days with either HMB-PP or OKT3, to respond to a second boost of stimulation (Figure 8C). Whereas HMB-PP-treated cells, which maintained high TCR levels on the cell surface after the initial 48 hour treatment (Figure 8A), produced high amounts (similar to primary activation) of anti-tumor Th1 cytokines in response to the secondary 24 hour stimulation with HMB-PP (Figure 8D), OKT3-treated cells failed to do so, presumably due to their inability to respond to the mAb once their TCR complexes have been internalized (Figure 8A–B). Although upon restimulation with HMB-PP, IFNγ became more abundant than TNFα (Figure 8D), contrary to the primary activation data (Figure 1C and Figure 2B), the cytokine profile of the two HMB-PP-based protocols were qualitatively very similar and consistently Th1-biased (Figure 8D and data not shown). These data show that HMB-PP is remarkably capable of sustaining Vγ9Vδ2 T-cell activation and the production of anti-tumor cytokines, which are critical parameters in immunotherapy protocols.

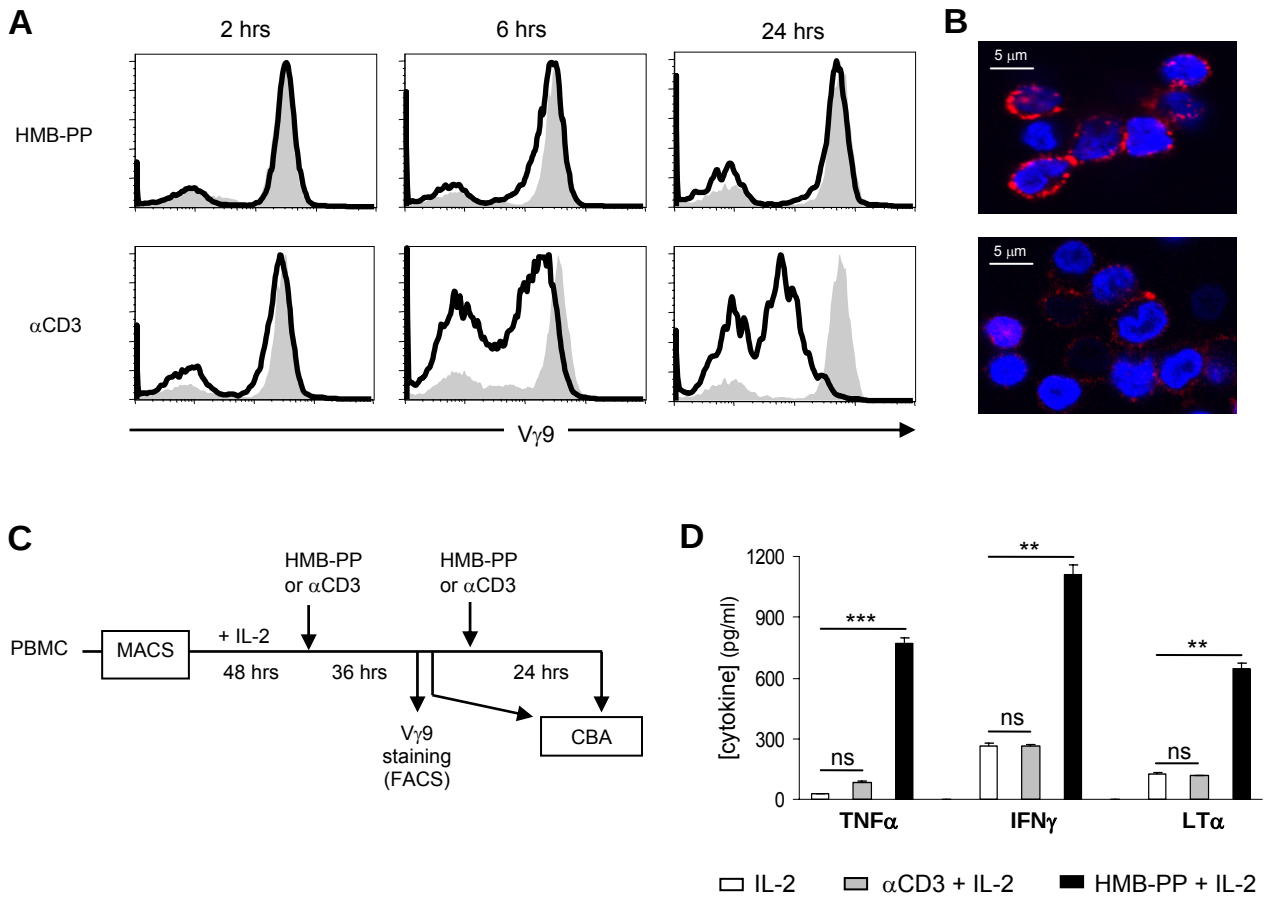


Figure 8. HMB-PP does not induce down-modulation of surface Vγ9$^+$ TCR and sustains cytokine production upon re-stimulation.

(**A**) MACS-sorted γδ PBL (of which 80–90% Vγ9$^+$) were incubated for the indicated times with HMB-PP or OKT3, and stained with anti-Vγ9 mAb for flow cytometry analysis. Bold lines represent treated cells, while shaded are non-stimulated Vγ9$^+$ cells (time = 0 hrs). (**B**) Confocal microscopy photos of γδ T-cells cultured for 24 hrs as in (A) and then stained for Vγ9$^+$ TCR. (**C–D**) Experimental design (**C**) and CBA analysis (**D**) of the re-stimulation response of MACS-sorted γδ PBL. After 36 hrs of stimulation, cells were re-plated for secondary activation during 24 hrs, when supernatants were collected and analyzed for Th1 cytokines by CBA. Error bars represent SD and differences refer to IL-2 controls (ns, non-significant; ***p*<0.01; ****p*<0.001). Results shown in this figure are representative of 2–5 independent experiments.

2.4.5 **HMB-PP plus IL-2 treatment promotes leukemia cell killing *in vitro* and *in vivo***

Having characterized the intracellular mechanisms of HMB-PP-mediated γδ T-cell activation, we next evaluated the anti-tumor potential of HMB-PP-based regimens. We selected leukemias as model tumors to employ in both *in vitro* and *in vivo* assays. The *in vitro* system previously used with Jurkat cells (Figure 5D) was applied to a larger panel of leukemia cell lines: Molt-4 (T-cell), RCH-ACV (pre-B cell) and HL-60 (myeloid) (Figures 9A–B). γδ PBL (80–95% $V\gamma9^+$) were treated with the different stimulating agents for 72 hours, and then transferred to plain media in co-culture with the leukemia cells. In just 3 hours, more than 80% of leukemia cells were killed by the γδ T-cells that had been stimulated with a combination of HMB-PP with IL-2 (compared to less than 20% by non-activated γδ T-cells), and such a regimen was at least as effective as saturating αCD3 plus IL-2 (Figures 9A–B). Of note, αCD3 mAb or HMB-PP used in isolation produced more modest increases in target-cell lysis (Figures 9A–B), highlighting the importance of exogenous IL-2 for the full activation of Vγ9Vδ2 T-cells[304,308] (Figure 2).

Taking into account the added relevance of pre-clinical *in vivo* systems for the evaluation of the anti-tumor potential of immunotherapy strategies, we adapted a model of transplantation of human tumors into lymphopenic SCID mice, previously used with human γδ T-cells by Kabelitz and colleagues[262], and added bioluminescent analysis of tumor development, which allows early detection of tumors and temporal evaluation throughout the course of treatment, in live animals and in real-time[309]. Four weeks after tumor injection, mice that had received HMB-PP plus IL-2-treated (activated and expanded over 12 days) γδ PBL showed significantly reduced tumor load (derived from Molt-4 leukemia cells) compared to control mice that did not receive γδ T-cells (Figures 9C–E). Furthermore, while most control had to be sacrificed at week 4 due to excessive body weight loss, γδ-treated animals resisted wasting for longer, up to week 6 (Figures 9C–D and data not shown). These results attest the capacity of HMB-PP-expanded and activated γδ T-cells to induce anti-tumor responses *in vivo*, and support the application of this phosphoantigen in conjugation with low amounts of IL-2 in clinical cancer settings.

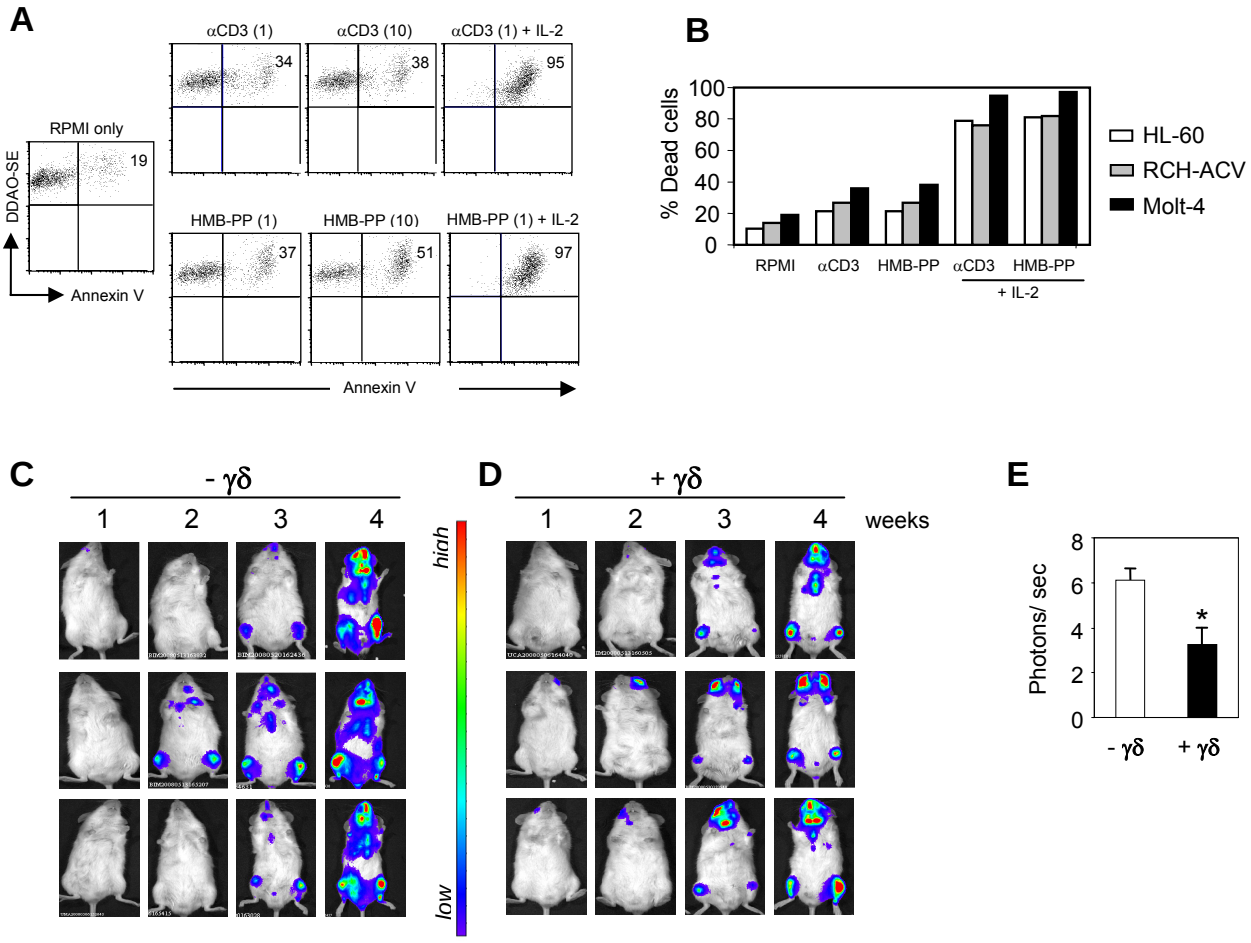


Figure 9. Leukemia cell killing by HMB-PP-activated γδ T-cells.

(A) *In vitro* lysis of Molt-4 leukemia cells. MACS-sorted γδ PBL (of which 85–95% Vγ9$^+$) were pre-activated for 72 hours with 1 or 10 µg/ml αCD3 mAb (OKT3), or 1 or 10 nM HMB-PP in the absence of IL-2, and also combined at the lower concentrations with IL-2 (100 U/ml). For the killing assay, DDAO-SE-labelled Molt-4 cells and pre-activated γδ PBL were co-incubated for 3 hours in media devoid of activating compounds. Samples were then stained with Annexin V to identify dying (Annexin V$^+$) tumor (DDAO-SE$^+$) cells by flow cytometry. **(B)** Data summary for killing assays (as in A) performed with three distinct leukemia cell lines. **(C–D)** Bioluminescent imaging of NOD/SCID mice inoculated with luciferase$^+$ Molt-4 leukemic cells, with **(D)** or without **(C)** co-injection of pre-activated γδ PBL, analyzed weekly as described in Materials and Methods. **(E)** LivingImage quantification of photon signals (tumor load) collected at day 28 of the experiment illustrated in **(C–D)**. Comparison of γδ-treated and control animals (n = 5, $p<0.05$). Data in this figure are representative of 3 **(A–B)** or 2 **(C–E)** independent experiments.

2.5 DISCUSSION

The stimulatory effect prenyl pyrophosphates have on Vγ9Vδ2 T-cells has been well documented and seems to require TCR expression, as indicated by antibody blocking and gene transfer experiments[166,310]. However, some of these experiments have been difficult to reproduce, and all attempts at showing cognate interactions between Vγ9Vδ2 TCRs and phosphoantigens in acellular systems (including surface plasmon resonance and X-ray crystallography of isolated complexes) have failed[301], probably due to the requirement of an unknown phosphoantigen-presenting molecule[271]. This has raised some skepticism on phosphoantigens as TCRγδ agonists, also stemming from the lack of precedent for such type of compounds interacting with any other variable region molecule, including all other γδ TCRs in humans or mice. However, recent data have highlighted particular properties of HMB-PP within the large family of phosphoantigens. Namely, HMB-PP induces the formation of high-density Vγ9Vδ2 TCR nanoclusters on the membrane of human γδ T-cells[218], and is bound on the surface of human APC by a tetramer reagent for the Vγ9Vδ2 TCR of rhesus macaques[302].

Following from these results on the extracellular dynamics of HMB-PP, our study aimed at clarifying the intracellular mechanisms of γδ T-cell activation mediated by HMB-PP. Our results show that very low amounts of HMB-PP are able to mimic the major effects of saturating ligation of the TCRγδ/CD3 complex, including the very rapid activation of MEK/Erk and PI-3K/Akt pathways to set up a transcriptional program, further enhanced by IL-2 signaling, that upregulates crucial target genes such as IFNγ or TNFα and endows cells with potent anti-tumor capacity. Interestingly, HMB-PP can produce all these intracellular events without down-modulating surface TCR levels, and this may be advantageous for sustaining the cell's activation status upon re-stimulation, as suggested by our cytokine secretion data. The crucial effect of HMB-PP on Vγ9Vδ2 T-cells may thus be the formation of high-density surface TCR nanoclusters[218] that may serve as platforms for intracellular signaling.

Our kinetic data on signal transduction further suggest that the interaction between HMB-PP and the Vγ9Vδ2 TCR is much more direct/stable than those of previously studied phosphoantigens, since downstream Erk phosphorylation, for example, peaks

simultaneously for HMB-PP and OKT3 treatments, in stark contrast with the delays of 115 min and 60 min, also relative to OKT3, observed respectively for the "pioneer" (naturally-occurring) IPP[244] and the more recent (synthetic) BrH-PP[271], currently in clinical trials as "Phosphostim". Of note, the concentration of HMB-PP we used was 50,000-fold and 3,000-fold *lower* than those used for IPP and BrH-PP, respectively. These data reveal a thus far unique capacity of HMB-PP to trigger very rapid TCR-associated signaling, compatible with direct binding to the Vγ9Vδ2 TCR, which remains to be formally shown and may require the assistance of an antigen-presenting molecule yet to identify[271,302]. This notwithstanding, we show for the first time, using a chemical inhibition strategy, that the major cellular effects of HMB-PP - γδ T cell activation, proliferation, Th1 cytokine secretion and anti-tumor cytotoxicity - are strictly dependent on Erk- and Akt-mediated signal transduction. HMB-PP stimulation therefore recruits the same signal transduction machinery employed by the γδ TCR, and is capable of doing so at minimal concentrations and within a strikingly short temporal scale that distinguish it from less potent phosphoantigens, whose stimulating effects on γδ T-cells most probably derive from their structural similarities with HMB-PP[192].

The use of a microbial compound for the activation of human anti-tumor lymphocytes fits the overall strategy of providing immune adjuvants (like viral nucleic acids for $CD8^+$ T-cells) for cancer therapy. Compared to other T-cell agonists, HMB-PP offers the advantage of specifically activating a T-cell population with overt effector function, devoid of known immune suppressive ("regulatory") subsets. Moreover, Vγ9Vδ2 T-cells are broadly reactive to tumors, potentially allowing them to be used to treat a variety of cancers.

The data presented in this report provide a framework for designing novel immunotherapy protocols using γδ T-cells, and encourage the use of HMB-PP in clinical settings. γδ T-cell-mediated tumor surveillance should evidently be seen as complementary to the adaptive component provided by MHC-restricted αβ T-cells upon priming by dendritic cells. Importantly, Vγ9Vδ2 T-cells can also induce monocyte and DC maturation[311-313], on one hand; and even act as CD80/86-expressing antigen-presenting cells that prime αβ T-cells, on the other[37]. Furthermore, γδ T-cells are prototypic representatives of unconventional lymphocytes with innate anti-tumor capacity, alike NK and NKT-cells, all of which recognize tumors independently of

classical MHC presentation[22]. We believe the success of cancer immunotherapy will critically depend on the integration of conventional and unconventional lymphocyte responses[314] to tackle the multiple immune evasion strategies developed by tumors.

3. IDENTIFICATION OF A PANEL OF TEN CELL SURFACE PROTEIN ANTIGENS ASSOCIATED WITH IMMUNOTARGETING OF LEUKEMIAS AND LYMPHOMAS BY PERIPHERAL BLOOD γδ T CELLS

3.1 ABSTRACT

3.1.1 Background / Objectives

Vγ9Vδ2 T lymphocytes are regarded as promising mediators of cancer immunotherapy due to their capacity to eliminate multiple experimental tumors, particularly within those of hematopoietic origin. However, Vγ9Vδ2 cell-based lymphoma clinical trials have suffered from the lack of biomarkers that can be used as prognostic of therapeutic success.

We have conducted a comprehensive study of gene expression in acute lymphoblastic leukemias and non-Hodgkin's lymphomas, aimed at identifying markers of susceptibility versus resistance to Vγ9Vδ2 T cell-mediated cytotoxicity.

3.1.2 Methodology / Principal Findings

We employed cDNA microarrays and quantitative real-time PCR to screen 20 leukemia and lymphoma cell lines, and 23 primary hematopoietic tumor samples. These data were analyzed using state-of-the-art bioinformatics, and gene expression patterns were correlated with susceptibility to Vγ9Vδ2 T cell mediated cytolysis *in vitro*.

We identified a panel of 10 genes encoding cell surface proteins that were statistically differentially expressed between "γδ-susceptible" and "γδ-resistant" hematopoietic tumors. Within this panel, 3 genes (*ULBP1*, *TFR2* and *IFITM1*) were associated with increased susceptibility to Vγ9Vδ2 T-cell cytotoxicity, whereas the other 7 (*CLEC2D*, *NRP2*, *SELL*, *PKD2*, *KCNK12*, *ITGA6* and *SLAMF1*) were enriched in resistant tumors. Furthermore, some of these candidates displayed a striking variance of expression among primary follicular lymphomas and T cell acute lymphoblastic leukemias.

Our results suggest that hematopoietic tumors display a highly variable repertoire of surface proteins that can impact on Vγ9Vδ2 cell-mediated immunotargeting. The prognostic value of the proposed markers can now be evaluated in upcoming Vγ9Vδ2 cell-based lymphoma/ leukemia clinical trials.

3.2 INTRODUCTION

γδ T lymphocytes display potent innate anti-tumor activity in both humans[258] and mice[11,315]. For example, mice genetically devoid of γδ T cells displayed increased susceptibility to skin tumor development induced experimentally by carcinogens[11,315], and to Transgenic Adenocarcinoma of the Mouse Prostate Model (TRAMP)[316]. More importantly, murine γδ T cells were shown to prevent (through perforin-mediated cytotoxicity) the development of spontaneous B cell lymphomas[51].

The major γδ T cell subset in human peripheral blood, Vγ9Vδ2 T lymphocytes, exert potent cytotoxicity towards tumor cell lines upon activation with small non-peptidic prenyl pyrophosphate intermediates of isoprenoid biosynthesis[192]. We and others have shown that, among such "phosphoantigens", 4-hydroxy-3-methyl-but-2-enylpyrophosphate (HMB-PP), a metabolite found in *Eubacteria* and *Protozoa*, is a very potent agonist of the Vγ9Vδ2 T cell receptor (TCR) that promotes cytotoxicity and the secretion of anti-tumor cytokines such as Interferon-γ (IFN-γ) and Tumor Necrosis Factor α (TNF-α)[192,317].

Phosphoantigen-activated Vγ9Vδ2 T cells can kill various solid tumor cell lines[258], and a particularly large number of hematopoietic cell-derived tumors[255,263,317], as well as freshly isolated tumor cells from patients with follicular B cell lymphoma or chronic lymphocytic leukemia (CLL)[283].

The well-established anti-tumor activity of Vγ9Vδ2 T cells has been recently explored in clinical trials for solid/epithelial[287,290,291] or liquid/hematopoietic tumors[286,289,318], which collectively showed promising yet limited success. The lack of response to therapy of some patients was attributed to deficient expansion of effector Vγ9Vδ2 T cells[289-291]. However, a large proportion of patients exhibiting significant and

sustained *in vivo* activation and proliferation of Vγ9Vδ2 T cells also failed to respond to treatment. Thus, in both prostate carcinoma[290] and non-Hodgkin's lymphoma[289], objective responses (partial remissions) were observed in just 33% of the patients that activated/ expanded their Vγ9Vδ2 T cells. These data emphasize the need for tumor biomarkers with prognostic value for γδ peripheral blood lymphocyte (γδ-PBL)-mediated immunotherapy.

Here we have conducted a comprehensive genome-wide expression study aimed at identifying lymphoma/ leukemia markers of susceptibility or resistance to γδ-PBL cytotoxicity. We set up an experimental system consisting of lymphoma/ leukemia cell lines with various degrees of susceptibility to γδ-PBL-mediated lysis, and performed comparative cDNA microarray analyses to characterize their gene expression profiles. These were validated through bioinformatics and quantitative RT-PCR (RT-qPCR), allowing us to define a panel of 10 candidate biomarkers whose expression displayed very marked variability among non-Hodgkin's lymphoma and acute lymphoblastic leukemia patients. Furthermore, one of these candidates, ULBP-1, was found to be a nonredundant determinant of leukemia/lymphoma susceptibility to γδ T cell cytotoxicity (Lança *et al.,* 2010)[319].

3.3 MATERIAL AND METHODS

3.3.1 *In vitro* cultures of human γδ-PBL and tumor cell lines

Peripheral blood was collected from healthy volunteers and PBMCs were isolated as described in[317]. γδPBL were expanded from isolated PBMCs for 12 days in RPMI 1640 complete media[317] supplemented with 100 U/mL of rhIL-2 (Roche Applied Science) and 1 nM HMB-PP (4-hydroxy-3-methyl-but-2-enylpyrophosphate) (Sup-RPMI). The percentage of $V\gamma9^+$ T cells in PBL increased from 3-14% at day 0 to 90-98% at day 12 (Figure 1). All tumor cell lines were cultured in complete 10% RPMI-1640 as previously described[317].

3.3.2 Leukemia and lymphoma primary samples

Pediatric B- or T-cell acute lymphoblastic leukemia cells, containing high (> 80%) leukemia involvement, were obtained from the peripheral blood and/or the bone marrow of patients at presentation, after informed consent and institutional review board approval (Instituto Português de Oncologia, Lisbon, Portugal). Fresh leukemia samples were enriched by density centrifugation over Ficoll-Paque and then washed twice in 10% RPMI-1640 medium supplemented with 2 mM L-glutamine (Sup-RPMI). Regarding lymphoma biopsies, lymph nodes were surgically removed, immediately frozen in liquid nitrogen and kept at -80ºC until further use (Department of Pathology, Hospital de Santa Maria – CHLN, Lisbon, Portugal). Upon diagnosis, we selected lymph nodes from lymphoma cases and reactive lymph nodes for our studies.

3.3.3 *In vitro* killing assays

For cytotoxicity assays, tumor cells (cell lines or primary samples) were stained with DDAO-SE (Molecular Probes, Invitrogen) and incubated at 1:10 ratio with γδ T cells in Sup-RPMI. Typically, $3x10^5$ HMB-PP-activated γδ-PBL (>90% Vγ9+) were co-incubated with $3x10^4$ tumor cells (pre-labeled with 1 μM DDAO-SE) with for 3-4 hours, then staining with Annexin V-FITC (BD Biosciences) and analyzed by flow cytometry.

3.3.4 RNA isolation, RT-qPCR and RNA interference

Total RNA from tumor cell lines was extracted using the RNeasy Mini Kit according to manufacture's instructions (Qiagen, Hilden, Germany). RNA from leukemia cells and samples was extracted with RNeasy Mini Kit according to manufacture's instructions. Concentration and purity was determined by spectrophotometry and integrity was confirmed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA). Total RNA was reverse-transcribed into cDNA as described in[317]. qPCR was performed on Rotor-Gene 6000 (Corbett) using SYBR Green detection system (PE Applied Biosystems). Glucoronidase beta (GUSB) and proteasome subunit beta type 6 (PSMB6) were used as endogenous controls in relative quantification using the standard curve method. Primers were designed using the

Roche Design Centre (Table 3). Lentiviral vectors expressing short hairpin RNA (shRNA) for the specific silencing of ULBP1 (CCT GGG AAG AAC AAA CTG AAA) were obtained from the RNAi Consortium and produced as previously described[319,320].

3.3.5 Target Synthesis and Hybridization to Affymetrix GeneChips

RNA from two independent cultures of each cell line (DAUDI, RAJI, RCH-ACV and 697) was processed for use on Affymetrix (Santa Clara, CA, USA) GeneChip HuGene 1.0 ST Arrays, according to the manufacturer's Whole Transcript Sense Target Labeling Assay. Briefly, 100 ng of total RNA containing spiked in Poly-A RNA controls (GeneChip Expression GeneChip Eukaryotic Poly-A RNA Control Kit; Affymetrix) was used in a reverse transcription reaction (GeneChip® WT cDNA Synthesis Kit; Affymetrix) to generate first-strand cDNA. After second-strand synthesis, double-stranded cDNA was used in an in vitro transcription (IVT) reaction to generate cRNA (GeneChip® WT cDNA Amplification Kit; Affymetrix). This cRNA was used for a second cycle of first-strand cDNA synthesis (GeneChip® WT cDNA Synthesis Kit; Affymetrix) which was fragmented and end-labeled (GeneChip® WT Terminal Labeling Kit; Affymetrix). The cDNA was used in a cocktail containing added hybridization controls and hybridized on arrays. Standard post hybridization wash and double-stain protocols (FS450_0007; GeneChip HWS kit, Affymetrix) were used on an Affymetrix GeneChip Fluidics Station 450. Arrays were scanned on an Affymetrix GeneChip scanner 3000 7G.

3.3.6 Microarray Data Analysis

All the microarray data analysis was done with R and several packages available from CRAN[321] and Bioconductor[322]. The raw data (CEL files) was normalized and summarized with the Robust MultiArray Average method from the affy package[323]. Unsupervised clustering analysis of the gene expression profiles for entire probe set data was assessed through hierarchical clustering (Euclidean distance and complete agglomeration method) and principal component analysis (prcomp function which calls a singular value decomposition method for non-symmetric matrices) as implemented in stats package[321]. Differentially expressed genes for each comparison were selected using linear models and empirical Bayes methods[324] as implemented in limma

package[325], verifying the p-values corresponding to moderated F-statistics, and selecting as differentially expressed genes those that had adjusted p-values adjusted using the Benjamini and Hochberg method[326] lower than 0.05. The enrichment of biological functions and pathways was analyzed using Ingenuity Pathway Analysis software (Ingenuity Systems, USA).

3.4 RESULTS

3.4.1 Highly variable susceptibility of acute leukemias and Non-Hodgkin's lymphomas to γδ-PBL cytotoxicity

In our laboratory we have studied a collection of 23 samples of acute lymphoblastic leukemias and non-Hodgkin's lymphomas, and a panel of 20 tumor cell lines of hematopoietic origin. The latter included acute lymphoblastic leukemia (ALL) (JURKAT, MOLT4, RCH-ACV, 697, CEM, TOM-1, RS4-11, B15, REH, Bv173) and acute myelogenous leukemia (AML) (HL-60, HEL, THP-1) cell lines; and non-Hodgkin Burkitt's (DAUDI, RAJI, RAMOS), follicular (DOHH2) and lymphoblastic (Oz) lymphoma cell lines (Table 1). Although the capacity of peripheral blood γδ T cells to target multiple tumor cell lines of hematopoietic origin is well documented[255,263,317], we observed that a substantial fraction of cell lines (Figure 2 A-B) and patient samples (Figure 2A and not shown) were strikingly resistant to γδ-PBL (obtained from healthy donors) pre-activated (as illustrated by high CD69 levels) with HMB-PP, the most potent natural Vγ9Vδ2 T cell activator known to date[192,317] (Figure 1). For example, the B-ALL cell lines Bv173, REH and 697 (Figure 2 A-B), and six primary samples obtained from B-ALL patients (Figure 2A and not shown), remained mostly alive (Annexin V^-) in co-cultures with fully-activated (100% $CD69^+$; not shown) γδ-PBL. Similar data was obtained with primary T-ALL samples and the cell line CEM (Figure 2A). This resistance to γδ-PBL cytotoxicity contrasted sharply with the extensive killing observed for the B-ALL line RCH-ACV and the T-ALL line MOLT-4 (Figure 2A), among various other hematopoietic tumors (Figure 2B).

Table 1. Description of the hematopoietic tumor cell lines used in this study.

	Cell line	Cell type	Diagnosis	Karyotype/ Genotype	Immunological markers	PubMed ID
Acute lymphoblasctic leukemia – ALL	**Bv173**	Pre-B cell	Pre-B cell leukemia	Hyperdiploid, t(9;22)(q34;q11)-associated *BCR-ABL* fusion transcripts *B2-A2*	CD3 -, CD10 +, CD13 +, CD19 +, CD20 +, CD34 -, CD37 -, CD79a -, cyCD79a +, CD80 -, CD138 -, HLA-DR +, sm/cylgG -, sm/cylgM -, sm/cykappa -, sm/cylambda –	6572735
	RS4-11	Pre-B cell and monocyte precr. (dual origin)	Pre-B cell leukemia	Hyperdiploid, t(4;11)(q21;q23) carries MLL-AF4 fusion gene	CD3 -, CD10-, CD4 +, CD13 +, CD14 (+), CD15 +, CD19 -, CD33 (+), CD34 -, CD68 +, HLA-DR +	3917311
	RCH-ACV	Pre-B cell	Pre-B cell leukemia	Hyperdiploid, Trisomy 8 and t(1;19)(q23;p13.3) – E2A-PBX1 fusion	CD3 -, CD10 (+), CD13 -, CD19 +, CD20 -, CD34 (+), CD37 -, CD79a -, cyCD79a +, CD80 -, CD138 -, HLA-DR +, sm/cylgG -, smIgM -, cylgM +, sm/cykappa -, sm/cylambda -	3455845
	B15	Pre-B cell	Pre-B cell leukemia	Pseudodiploidy, diverse duplications, deletions and translocations	CD3 -, CD10 +, CD13 +, CD19 +, CD20 -, CD34 +, CD37 -, CD38 +, CD79a +, cyCD79a +, CD80 -, CD138 -, HLA-DR +, sm/cylgG -, smIgM -, cylgM +, sm/cykappa -	3162827
	TOM-1	Pre-B cell	Pre-B cell leukemia	Hyperdiploid, carries t(9;22)(q34;q11) effecting BCR-ABL rearrangement	CD3 -, CD10 +, CD13 +, CD19 +, CD20 +, CD34 +, CD37 +, CD38 +, CD79a -, cyCD79a +, CD80 -, CD138 -, HLA-DR +, sm/cylgG -, sm/cylgM -, sm/cykappa -, sm/cylambda -	3103721
	697	Pre-B cell	Pre-B cell leukemia	Diploid, t(1;19)(q23;p23) ; E2A-PBX1 fusion	CD3 -, CD10 +, CD13 -, CD19 +, CD20 (+), CD34 -, CD37 -, CD38 +, CD79a -, cyCD79a +, CD80 -, CD138 -, HLA-DR +, cylgM +, cylgG -	6982733

	REH	Pre-B cell	Pre-B cell leukemia	Pseudodiploid, carries t(4;12)(q32;p13) and del (12) producing respective TEL-AML1 fusion and deletion of residual TEL	CD3 -, CD5 -, CD10 +, CD13 -, CD19 +, CD20 +, CD21 -, CD34 -, CD37 -, CD38 +, CD71 +, CD79a -, cyCD79a +, CD80 -, CD86 -, CD138 +, HLA-DR +, sm/cyIgG -, sm/cyIgM -, sm/cykappa -,	197411
	NALM-6	PreB cell	Pre-B cell leukemia	Near-diploid, carries t(5;12)(q33.2;p13.2)	CD3 -, CD10 +, CD13 (+), CD19 +, CD34 (+), CD37 -, cyCD79a +, CD80 -, CD138 +, HLA-DR +, sm/cyIgG -, cyIgM +, smIgM -, sm/cykappa -, sm/cylambda	83966
	CEM	T-cell	T-cell leukaemia	Diploid, diverse deletions and translocation	CD2 -, CD3 +, CD4 +, CD5 +, CD6 +, CD7 +, CD8 -, CD13 -, CD14 -, CD15 +, CD19 -, CD33 -, CD34 -, CD68 -, CD90 -, HLA-DR -, TCRalpha/beta +, TCRgamma/delta -	14278051
	JURKAT	T-cell	T-cell leukemia	Pseudodiploid, Diverse chromosomal aberrations, including deletions and translocation t(5;10)(q11;p15)	CD2 +, CD3 +, CD4 (+), CD5 +, CD6 +, CD7 +, CD8 -, CD13 -, CD19 -, CD34 +, TCRalpha/beta +, TCRgamma/delta -	68013
	MOLT-4	T-cell	T-cell leukemia	Hypertetraploid, diverse chromosomal aberrations, including t(7;7)(p15;q11)	CD2 +, CD3 (+), CD4 +, CD5 +, CD6 +, CD7 +, CD8 +, CD13 -, CD19 -, CD34 +, TCRalpha/beta -, TCRgamma/delta -	4567231
Acute myelogenous leukemia - AML	**HL-60**	promyeloblast	Promyelocytic leukemia	Pseudodiploid, diverse chromosomal aberrations, including deletions and translocations	CD3 -, CD4 +, CD13 +, CD14 -, CD15 +, CD19 -, CD33 +, CD34 -, HLA-DR -	288488
	HEL	Erythroid precursors	Erythroleukemia	Hypotriploid, diverse chromosomal aberrations, including deletions, duplications and translocations	CD3 -, CD13 +, CD14 -, CD15 +, CD19 -, CD33 +, CD41 +, CD42 +, CD71 +, CD235a +	6177045
	THP-1	Monocyte	Monocytic leukemia	Near-tetraploid, carries t(9;11) associated with AML-M5	CD3 -, CD4 +, CD13 +, CD14 (+), CD15 +, CD19 -, CD33 (+), CD34 -, CD68 +, HLA-DR +	6970727

Chronic myelogenous leukemia - CML	**K562**	Pre-Erythrocyte/ granulocyte/ monocyte	Myeloid leukemia	Diploid, t(9;22) - associated BCR-ABL fusion transcripts B3-A2	CD3 -, CD13 (+), CD15 +, CD19 -, CD33 +, CD 71 +, CD 235a +;	95026
Burkitt's lymphoma	**DAUDI**	Mature B cell	Burkitt's Lymphoma	Diploid, t(8;14)(q24;q32)	CD3 -, CD10 +, CD13 -, CD19 +, CD20 +, CD34 -, CD37 +, CD38 +, CD79a +, cyCD79a +, CD80 +, CD138 -, HLA-DR +, sm/cyIgG -, sm/cyIgM +, sm/cykappa +, sm/cylambda -	4178827
	RAMOS	Mature B cell	Burkitt's Lymphoma	Hypodiploid, diverse deletions, duplications and translocations, including t(8;14)(q24;q32.2) effecting IGH/MYC rearrangement	CD3 -, CD10 +, CD13 -, CD19 +, CD20 +, CD34 -, CD37 +, CD38 +, CD79a +, cyCD79a +, CD80 +, CD138 -, HLA-DR +, sm/cyIgG -, sm/cyIgM +, sm/cykappa -, sm/cylambda +	181343
	RAJI	Mature B cell	Burkitt's lymphoma	Diploid, t(8,14)(q24;q32) translocation	CD3 -, CD10 +, CD13 -, CD19 +, CD20 +, CD34 -, CD37 +, CD79a +, CD80 +, CD138 -, HLA-DR +, sm/cyIgG -, cyIgM +, sm/cykappa -, sm/cylambda -	14086209
Follicular lymphoma	**DOHH-2**	Mature B cell	Follicular B cell Lymphoma	Hyperdiploid; diverse chromosomal translocations incl. t(8;14)(q24;q32) and t(14;18)(q32;q21)	CD3 -, CD10 +, CD13 -, CD19 +, CD20 +, CD34 -, CD37 +, CD38 +, CD79a +, CD80 +, CD138 -, HLA-DR +, sm/cyIgG +, sm/cyIgM -, sm/cykappa -, sm/cylambda +	1849602
Lymphoblastic lymphoma	**OZ**	Pre-B cell	Pre-B non-hodgkin lymphoma	t(14;18)(q32;q21) translocation which involves the Bcl-2 gene	CD3 -, CD10 +, CD13 -, CD19 +, CD20 -, CD38 +, CD79a +, HLA-DR +, sm/cyIgG -, cyIgM -, sm/cykappa -, sm/cylambda -	9600110

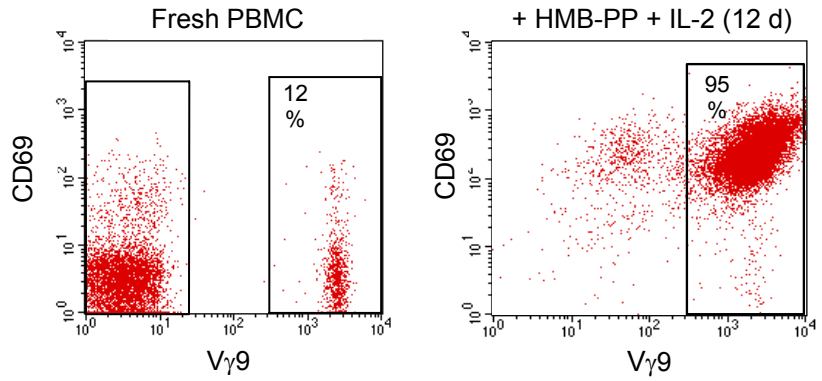


Figure 1. Selective Vγ9$^+$ T cell expansion and activation.

Percentage of Vγ9$^+$ T cells in fresh PBMCs (left plot) and upon stimulation with HMB-PP (1 nM) and IL-2 (100 U/ml) for 12 days (right plot). CD69 reports the activation status of the cells. These results are representative of all healthy donors used in this study (range of Vγ9$^+$ enrichment: 90-98%).

For systematic analysis of our killing assay data, we considered tumor samples with over 70% lysis as susceptible to γδ-PBL-mediated lysis ("γδ-susceptible"), and those under 30% lysed as "γδ-resistant". Importantly, susceptibility was independent of the γδ-PBL donor, as the pattern of susceptible/ resistant lines was equivalent for three independent healthy donors (Figure 3A). Moreover, the differences in susceptibility to γδ T cells were maintained when tumor cell lines were incubated with shortly (12 hrs)-activated γδ T cells (Figure 3B), further supporting the segregation between susceptible and resistant cell lines. As primary samples are difficult to obtain in order to reproduce and expand experiments aimed at dissecting the molecular mechanisms of tumor lines for the initial candidate searches, and later extended our findings to patient susceptibility to γδ-PBL cytotoxicity, we focused on our well-established panel of cell lines for the initial candidate searches and latter extended our findings to patient samples.

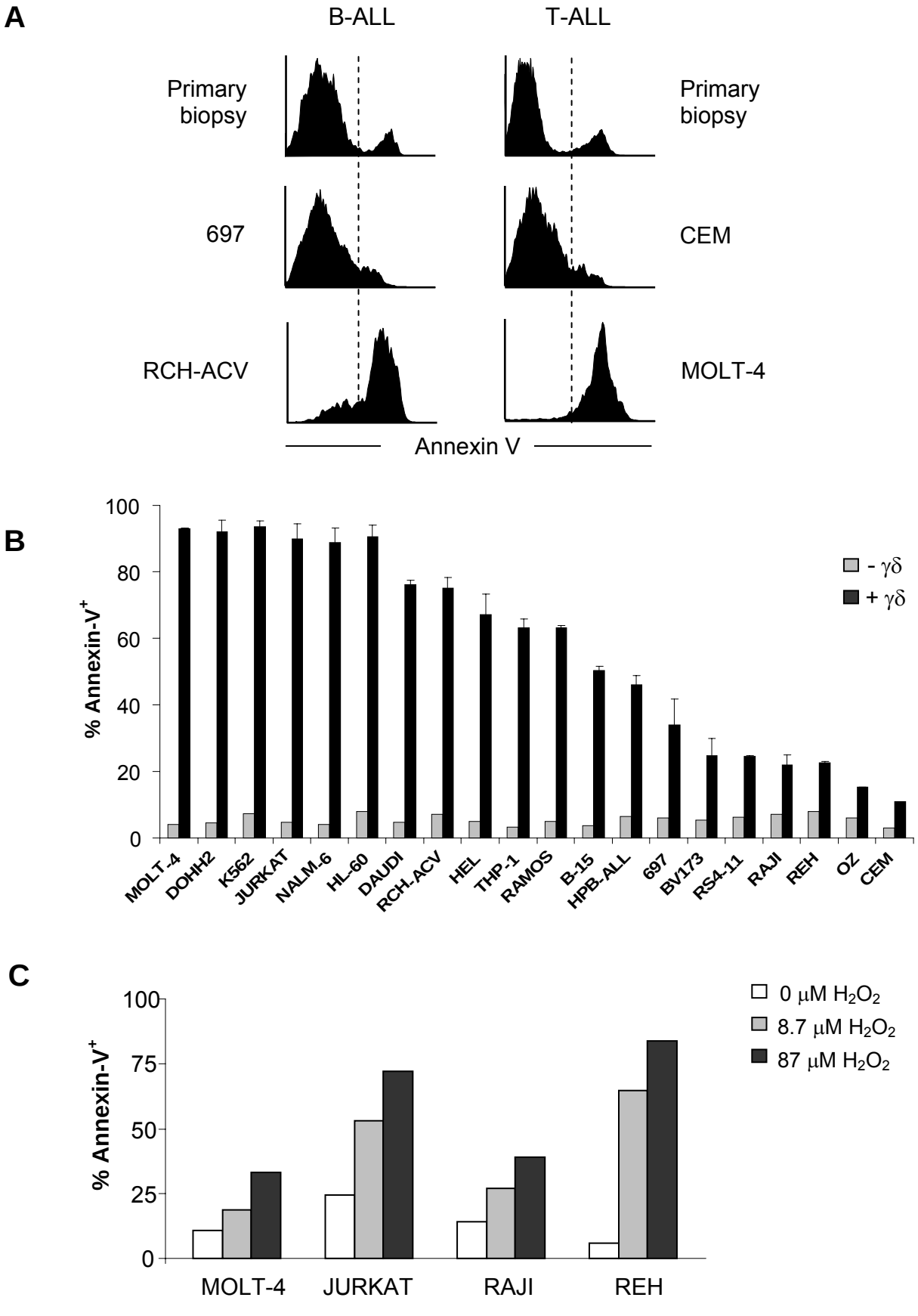
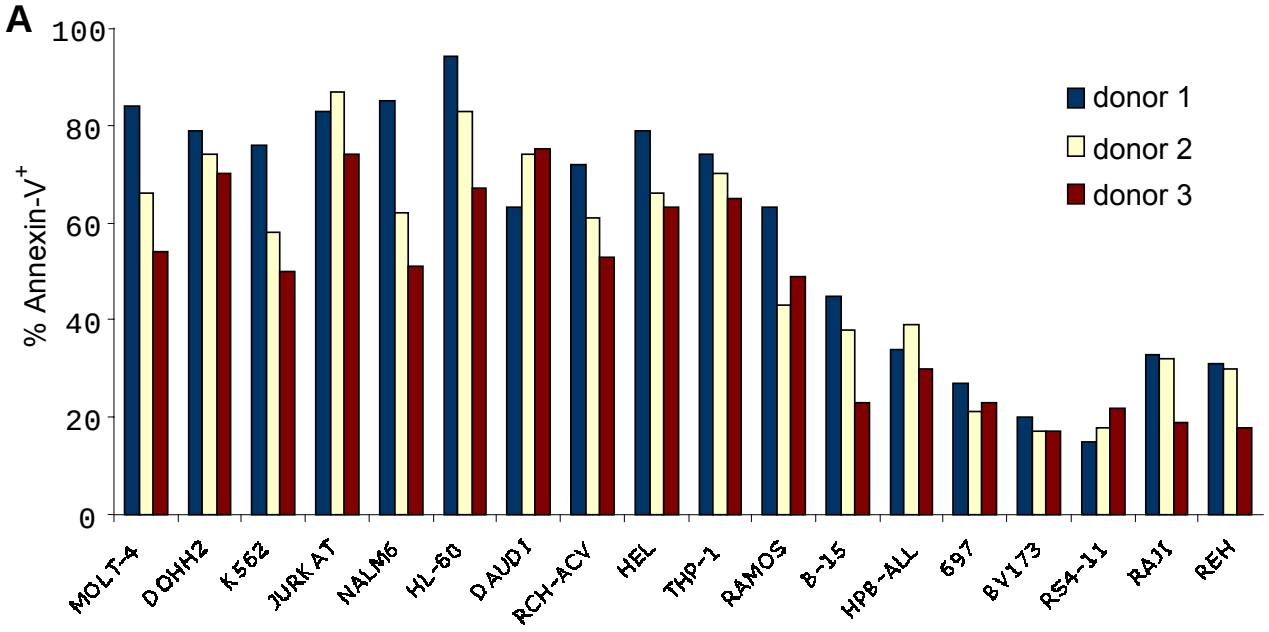

A
B-ALL
T-ALL
Primary biopsy
Primary biopsy
697
CEM
RCH-ACV
MOLT-4
Annexin V
B
% Annexin-V+
100
80
60
40
20
0
- γδ
+ γδ
MOLT-4
DOHH2
K562
JURKAT
NALM-6
HL-60
DAUDI
RCH-ACV
HEL
THP-1
RAMOS
B-15
HPB-ALL
697
BV173
RS4-11
RAJI
REH
OZ
CEM
C
% Annexin-V+
100
75
50
25
0
0 μM H2O2
8.7 μM H2O2
87 μM H2O2
MOLT-4
JURKAT
RAJI
REH

Figure 2. Differential susceptibility of leukemia and lymphoma cells to γδ-PBL cytotoxicity.

(A) Annexin V staining for apoptotic tumor cells after 4 hours of co-incubation with HMB-PP-activated γδ-PBL. Tumors were B-ALL (left panels) or T-ALL (right panels) cells, either primary samples or the indicated cell lines. **(B)** Summary of killing assays (as in A) with 20 leukemia or lymphoma cell lines (described in Table 1). Error bars correspond to triplicate assays. **(C)** Effect of increasing concentrations of H_2O_2 on leukemia/ lymphoma cell apoptosis (% Annexin V^+).

We first considered that tumor resistance to γδ-PBL cytotoxicity could stem from intrinsic anti-apoptotic mechanisms developed by some leukemia/ lymphoma cell lines. However, when we tested the effect of a pro-apoptotic stimulus - H_2O_2 –, we observed no association between resistance to apoptosis and to γδ-PBL cytotoxicity. Namely, the cell lines Jurkat (γδ-susceptible) and REH (γδ-resistant) were more sensitive to non-saturating concentrations of H_2O_2 than the cell lines MOLT-4 (γδ-susceptible) and RAJI (γδ-resistant) (Figure 2C). This suggests that susceptibility to γδ-PBL cytotoxicity is not related to their response to other death stimuli, and probably involves a specific protein expression program (involved in tumor/ γδ-PBL interactions) that we set out to characterize.



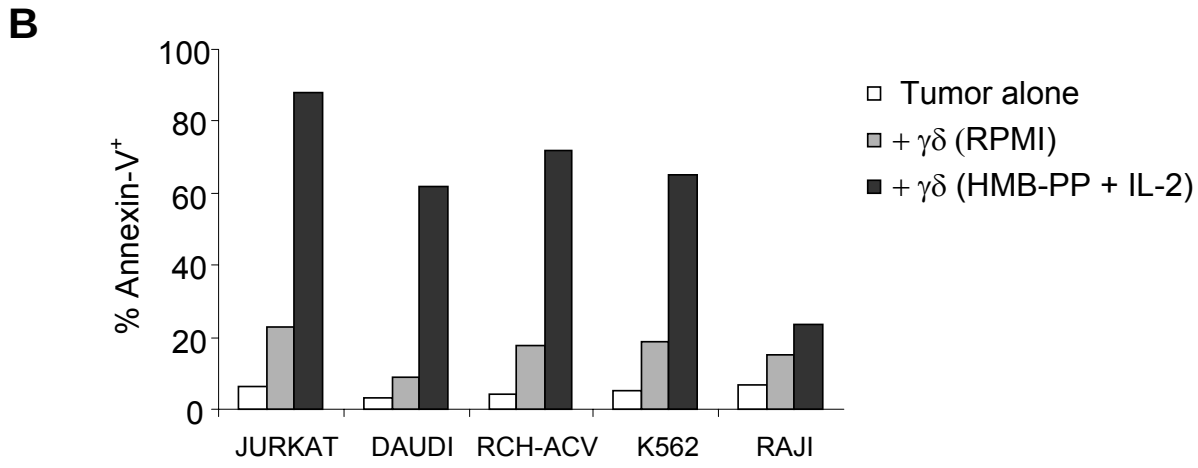


Figure 3. γδ-PBL-mediated lysis of leukemia/ lymphoma cells.

Killing assay results for leukemia/ lymphoma cell lines using 12 day-cultured (as in Figure 1) γδ-PBL obtained from three independent healthy donors (**A**); and γδ T cells pre-isolated by MACS and cultured for 12 hours on plain medium (RPMI) or supplemented with HMB-PP and IL-2 (**B**). Plotted are percentages of Annexin-V^+ (dead) cells.

3.4.2 Genome-wide comparisons between γδ-susceptible and γδ-resistant hematopoietic tumors

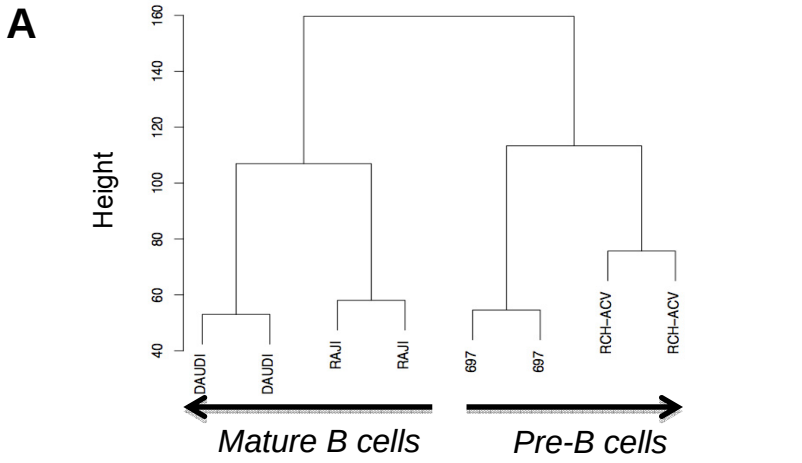
The observed differences in susceptibility γδ-PBL cytotoxicity among hematopoietic tumors emphasize the importance of defining gene signatures that may predict the effectiveness of γδ T cell-based immunotherapies in the clinic. We performed a genome-wide analysis aimed at comparing the mRNA expression profiles γδ-susceptible and γδ-resistant tumors. We employed cDNA microarrays to examine two pairs of hematopoietic tumor cell lines sharing the same cytogenetic alterations and cellular phenotypes (Table 1): the Burkitt's lymphomas DAUDI (susceptible) and RAJI (resistant); and the B-ALL lines RCH-ACV (susceptible) and 697 (resistant).

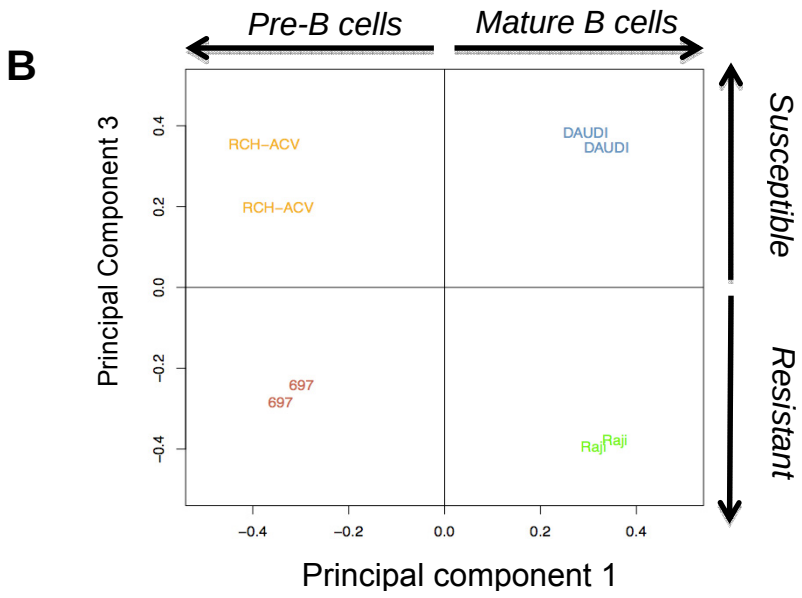
First, samples were grouped according to the similarity of gene expression patterns using unsupervised clustering analysis (no group specification *a priori*). Based on the entire probe set data, two main groups could be defined, which corresponded to the original cell type (Figure 4A): pre-B (697 and RCH-ACV) and mature B cells (DAUDI

and RAJI). We next applied principal component analysis (PCA), which identifies new variables, the principal components, which are linear combinations of the original variables (genes expression levels) and represent the largest variation found between samples[327] Although the original cell type was the major source of variation between all samples (53,3% of total variation), PCA showed that component 3 was responsible for the segregation (16,4% of total variation) according to the susceptibility to γδ-PBL cytotoxicity (Figure 4B): susceptible (DAUDI and RCH-ACV) *versus* resistant (RAJI and 697).

To identify gene expression variations associated with susceptibility to γδ-PBL cytotoxicity, and to suppress the variations due to the transformed cell type (pre-B or mature B cells), we first compared tumors with identical origin, i.e., DAUDI *versus* RAJI, and RCH-ACV *versus* 697 (Supplementary Tables 2-3). We then used Bayesian linear models[324] and selected the common genes between both analyses: 340 genes (155 up and 185 down-regulated in γδ-susceptible tumors) presented similar gene expression variations and were considered for further analysis (Supplementary Table 4). Bioinformatics analysis revealed an enrichment for functions related to cell-to-cell signaling and interaction, hematological system development and function, immune cell trafficking (p-value < 0.05, Supplementary Table 5). Some of the top pathways affected were interferon signaling, crosstalk between dendritic cells and natural killer cells and molecular mechanisms of cancer (p-value < 0.05, Supplementary Table 6).

The gene expression variations observed also suggested that, consistent with our previous experimental data (Figure 2C), the segregation between susceptible and resistant tumors is not associated with expression of anti- or pro-apoptotic genes (Figure 4C and Supplementary Table 7). Thus, up-/ down-regulation of pro-/ anti-apoptotic genes did not correlate with susceptibility to γδ-PBL cytotoxicity. Moreover, apoptotic related functions and pathways were not enriched in the panel of 340 genes (Supplementary Table 6). Based on these results, we favored the hypothesis that susceptibility or resistance to γδ-PBL cytotoxicity is conferred by signals presented at the tumor/ γδ-PBL interface, i.e., on the surface of leukemia/ lymphoma cells.


A
Height
160
140
120
100
80
60
40
DAUDI
DAUDI
RAJI
RAJI
697
697
RCH-ACV
RCH-ACV
Mature B cells
Pre-B cells


B
Pre-B cells
Mature B cells
Susceptible
Resistant
Principal Component 3
0.4
0.2
0.0
−0.2
−0.4
RCH-ACV
RCH-ACV
DAUDI
DAUDI
697
697
Raji
Raji
−0.4
−0.2
0.0
0.2
0.4
Principal component 1

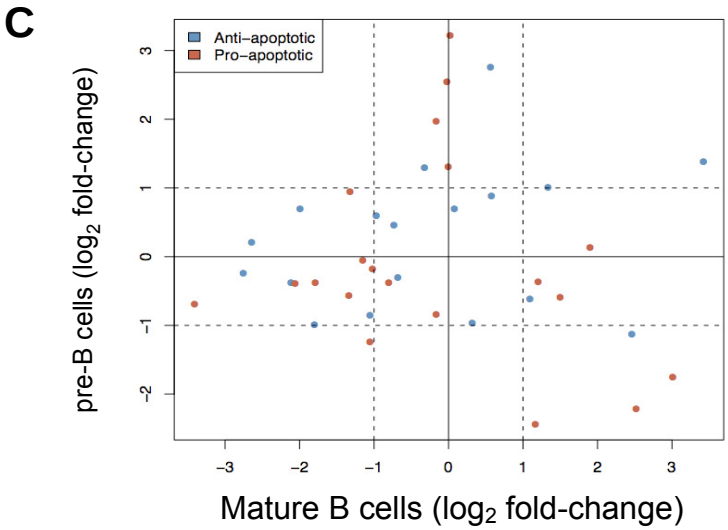

C
Anti−apoptotic
Pro−apoptotic
pre-B cells (log2 fold-change)
3
2
1
0
−1
−2
−3
−2
−1
0
1
2
3
Mature B cells (log2 fold-change)

Figure 4. Comparison of gene expression in tumor cell lines susceptible or resistant to γδ-PBL cytotoxicity.

Bioinformatics analyses of cDNA microarray comparisons between the Burkitt's lymphomas DAUDI and RAJI; and the B-ALL lines RCH-ACV and 697. **(A)** Unsupervised hierarchical clustering analysis. Samples with similar gene expression patterns are grouped together and connected with branches, producing a clustering tree (or dendrogram) on which the branch length inversely reflects the degree of similarity between samples. **(B)** Principal Component Analysis. The samples are plotted according to the first and third principal components (corresponding to the largest variation found between samples). **(C)** Variations in expression levels of anti- or pro-apoptotic genes in susceptible *versus* resistant tumor cell lines. Dashed lines indicate 2 fold-changes (in logarithmic scale) in the expression ratio susceptible/ resistant.

3.4.3 A set of cell surface proteins segregates between γδ-susceptible and γδ-resistant leukemia/ lymphoma cell lines

T cells recognize their targets through cell surface antigens. We therefore focused our analysis of the panel of 340 genes on those encoding plasma membrane proteins (with extracellular domains), using a fold change threshold of 2 (log FC >1). These consisted of 8 genes up-regulated and 19 genes down-regulated in γδ-susceptible tumors, when compared to resistant tumors (Supplementary Table 8). The mRNA expression levels of the 27 candidates were assessed by RT-qPCR (in independent samples) as to validate the microarray results. Upon statistical analysis of the data, 22 out of the 27 genes were confirmed as differentially expressed in the two pairs of cell lines used for microarray comparisons: of these, 6 genes were up-regulated and 16 genes were down-regulated in γδ-susceptible tumors (Figure 5A). In order to have more stringent selection criteria, we extended our expression studies to a broader panel of cell lines, including 6 susceptible and 4 resistant cell lines (Figure 6). This revealed 10 genes with significant expression variation between susceptible and resistant tumors (p-value < 0.05 from Mann-Whitney test) (Figure 5B). Thus, our final panel of candidate markers of susceptibility to γδ-PBL cytotoxicity consisted of 3 genes enriched in γδ-

susceptible tumors (ULBP1, TFR2 and IFITM1), and 7 genes enriched in γδ-resistant leukemias/ lymphomas (CLEC2D, NRP2, SELL, PKD2, KCNK12, ITGA6 and SLAMF1) (Table 2).

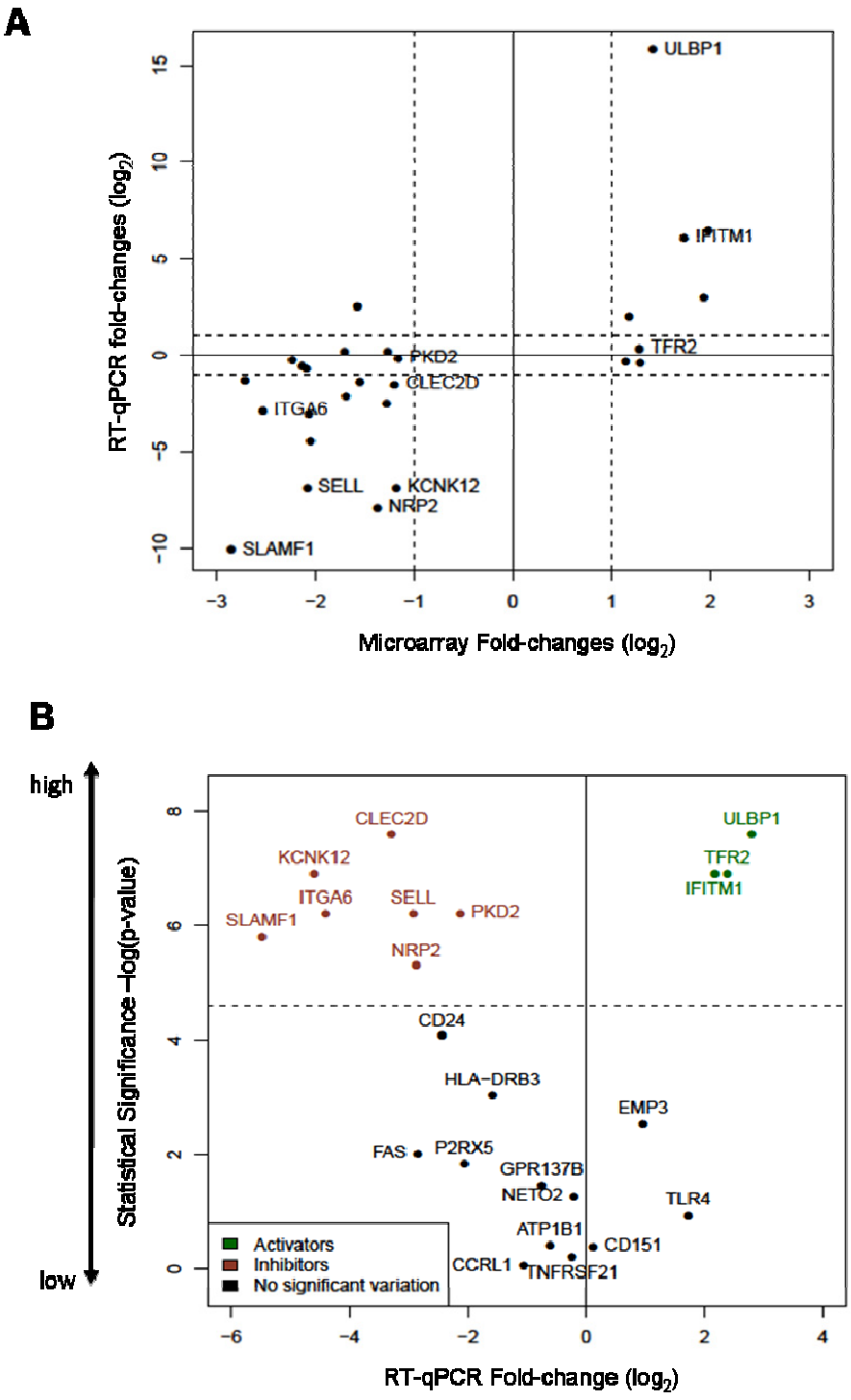


Figure 5. Variations in expression of genes encoding cell surface proteins that segregate between γδ-susceptible and γδ-resistant leukemia/ lymphoma cell lines.

(A) RT-qPCR validation of microarray results for the comparisons of Figure 4. The mRNA expression levels were normalized to GUSB and PSMB6 for each cell line. Plotted are the averages of relative expression levels in DAUDI *versus* RAJI (DAUDI/ RAJI) and RCH-ACV *versus* 697 (RCH-ACV/ 697). Dashed lines indicate 2 fold-change values (in logarithmic scale). **(B)** Statistical analysis of RT-qPCR results (detailed in Figure 6) in 6 susceptible and 4 resistant cell lines. Statistical significance was assessed by Mann-Whitney test (-log *p* value). Dashed line represents the statistical threshold $p = 0.01$.

Symbol	Description	Biological function	*p* value
Enriched in γδ-susceptible tumors			
ULBP1	UL16 binding protein	Ligand for NKG2D on NK and T cells; induces cytotoxicity, cytokine secretion	0
IFITM1 (CD225)	Interferon-induced transmembrane protein 1	Involved in cell proliferation and malignancy	0
TFR2	Transferrin receptor 2	Cellular uptake of transferrin-bound Iron	0.004
Enriched in γδ-resistant tumors			
CLEC2D	C-type lectin 2, D	Ligand for the NK inhibitory receptor CD161	0.002
SELL	Selectin L	Adhesion of T cells to endothelial cells	0.001
SLAMF1	Signaling lymphocytic activation molecule 1	Bidirectional T cell to B cell stimulation	0
KCNK12	Potassium channel K, 12	Potassium channel	0
ITGA6	Integrin alpha 6	Integrin; receptor for laminin	0.014
PKD2	Polycystic kidney disease 2	Calcium channel	0.017
NRP2	Neuropilin 2	Co-receptor for VEGF; implicated in tumor growth and vascularization	0.018

Table 2. Panel of cell surface proteins associated with the susceptibility or resistance of lymphomas/ leukemias to γδ T cell cytotoxicity.

The statistical difference between the average gene expression in the 6 susceptible *versus* the 4 resistant tumors of Figure 4 was assessed by Mann-Whitney test ($p<0.05$).

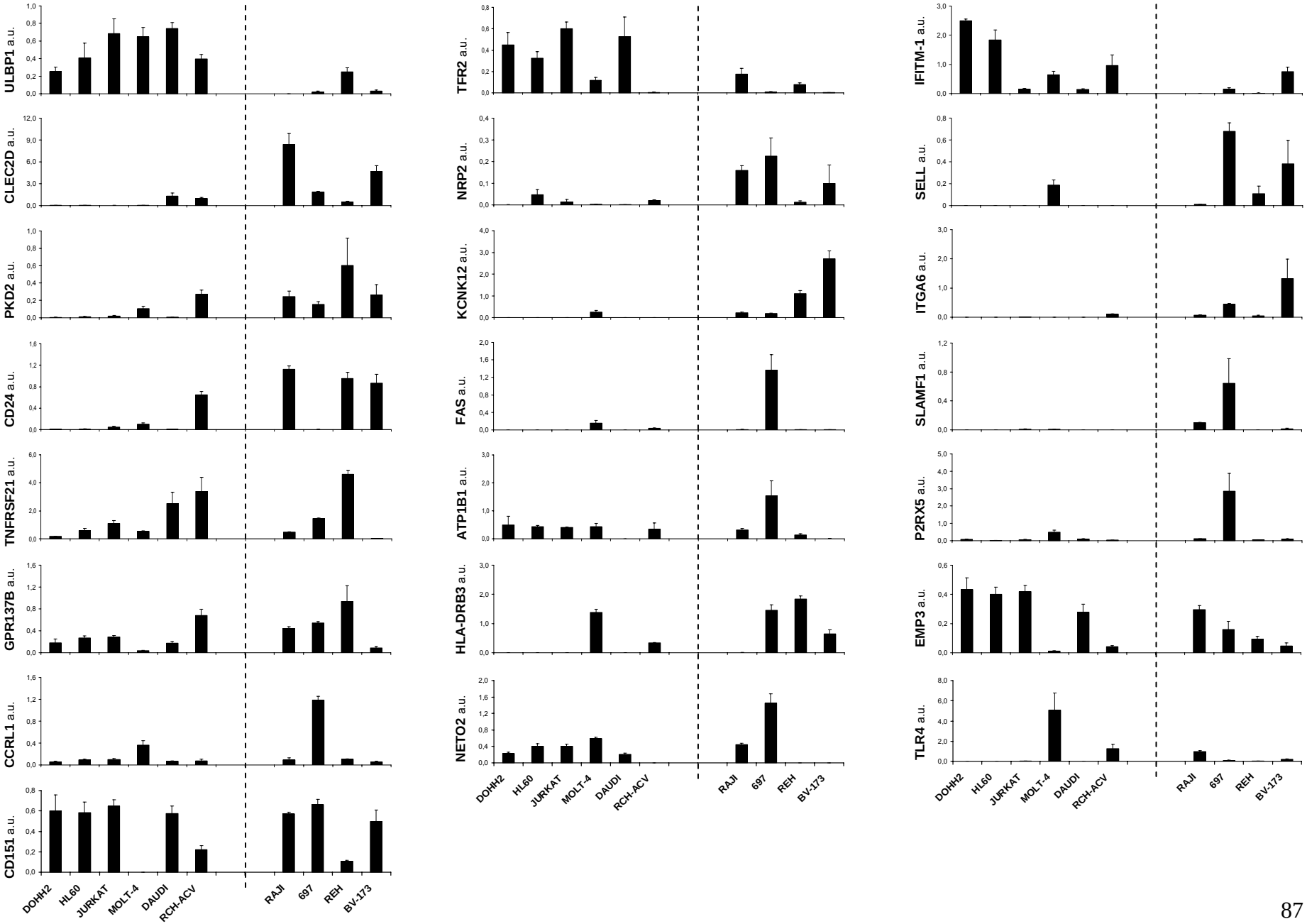

ULBP1 a.u.
CLEC2D a.u.
PKD2 a.u.
CD24 a.u.
TNFRSF21 a.u.
GPR137B a.u.
CCRL1 a.u.
CD151 a.u.
TFR2 a.u.
NRP2 a.u.
KCNK12 a.u.
FAS a.u.
ATP1B1 a.u.
HLA-DRB3 a.u.
NETO2 a.u.
IFITM-1 a.u.
SELL a.u.
ITGA6 a.u.
SLAMF1 a.u.
P2RX5 a.u.
EMP3 a.u.
TLR4 a.u.
DOHH2
HL60
JURKAT
MOLT-4
DAUDI
RCH-ACV
RAJI
697
REH
BV-173

Figure 6. Real-time quantitative PCR data for the 22 candidate genes.
Real-time quantitative PCR data for the 22 candidate genes encoding cell surface proteins identified as differentially expressed by cDNA microarray analysis (as described in Figure 5), in a panel of 6 γδ-susceptible and 4 γδ-resistant cell lines (separated by dashed line). mRNA expression levels were normalized to housekeeping genes GUSB and PSMB6. Error bars correspond to three independent experiments.

3.4.4 Heterogeneity of expression of candidate markers in primary leukemia and lymphoma samples

We next determined the expression levels of each candidate marker in primary samples obtained from T-cell acute lymphoblastic leukemia (T-ALL) and non-Hodgkin's lymphoma (NHL) patients. Within the latter we sampled patients with common indolent (follicular) or aggressive (diffuse large B cell - DLBCL) lymphomas. Gene expression levels in samples were compared with healthy PBMCs (for ALL) and reactive follicles (for NHL), taken as references (0 on log scale) in Figure 7 A-B. Hence, a positive or negative (log scale) variation indicates higher or lower expression in tumors than in the control samples, respectively. Overall, the tumors exhibited very variable gene expression profiles. For example, among susceptibility-associated genes, ULBP1 was overexpressed in a large number of primary samples, while TFR2 was only enriched in three FL samples (FL 1, FL 2 and FL 8), and IFITM1 was strongly depleted in various tumors (Figures 7A and B). On the other hand, all resistance-associated genes were overexpressed in FL sample 3, by contrast with the majority of primary samples analyzed. Moreover, some markers, such as ITGA6 or SELL, essentially did not vary among the various patients (Figures 7A-B). Collectively, these data revealed a striking heterogeneity in the expression of particular candidate genes in primary tumors. When compared to our results with tumor cell lines (Figure 6), these clinical data possibly reflect distinct selective pressures on the expression of the genes that compose the candidate panel, the consequence of which should now be evaluated in clinical trials.

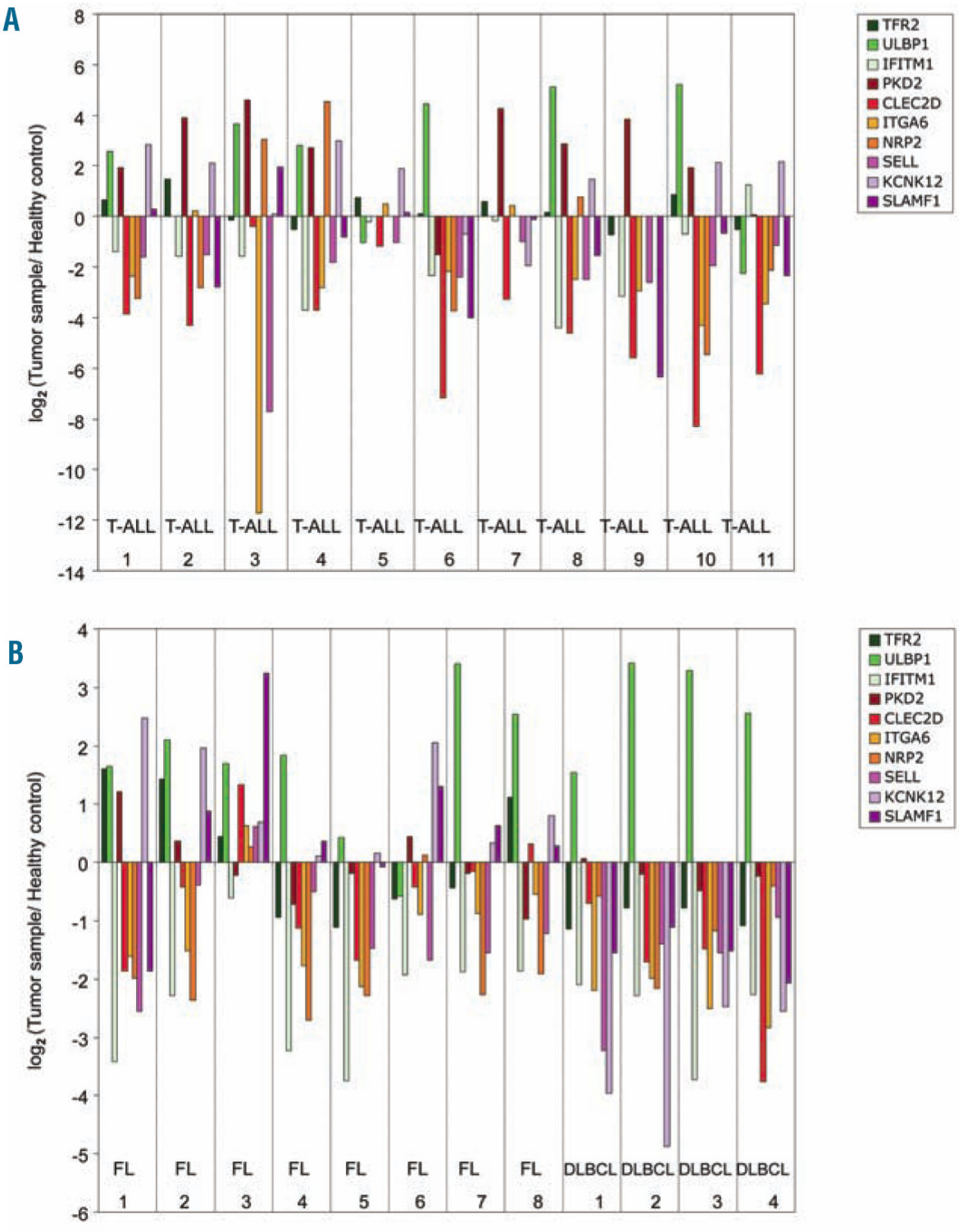


Figure 7. Quantification of mRNA expression levels of γδ-susceptibility markers in acute lymphoblastic leukemia and non-Hodgkin's lymphoma patients.

(A) RT-qPCR analysis of mRNA expression in 11 T cell acute lymphoblastic leukemia (T-ALL) samples, normalized to housekeeping genes (GUSB and PSMB6) and to reference PBMCs from healthy individuals. Values were converted to logarithmic scale. **(B)** RT-qPCR analysis of mRNA expression in 8 follicular lymphoma (FL) and 4 diffuse large B cell

lymphoma (DLBCL) samples, normalized to housekeeping genes (GUSB and PSMB6) and to a reference sample - reactive follicles – obtained through the same procedure. Values were converted to logarithmic scale.

3.4.5 The MHC class Ib protein ULBP-1 is a nonredundant determinant of leukemia/lymphoma susceptibility to γδ T cell cytotoxicity

ULBP-1, a molecule that is known to stimulate NK cell cytotoxicity (Table 2), also presented the highest degree of overexpression in leukemias and lymphomas (Figures 6 and 7). This prompted us to assess the physiologic role of this NKG2DL in leukemia targeting by γδ-PBL through loss-of-function studies using RNA interference. *ULBP1* mRNA and protein expression levels were efficiently and specifically decreased on shRNA transfection (Figure 8A and data not shown).

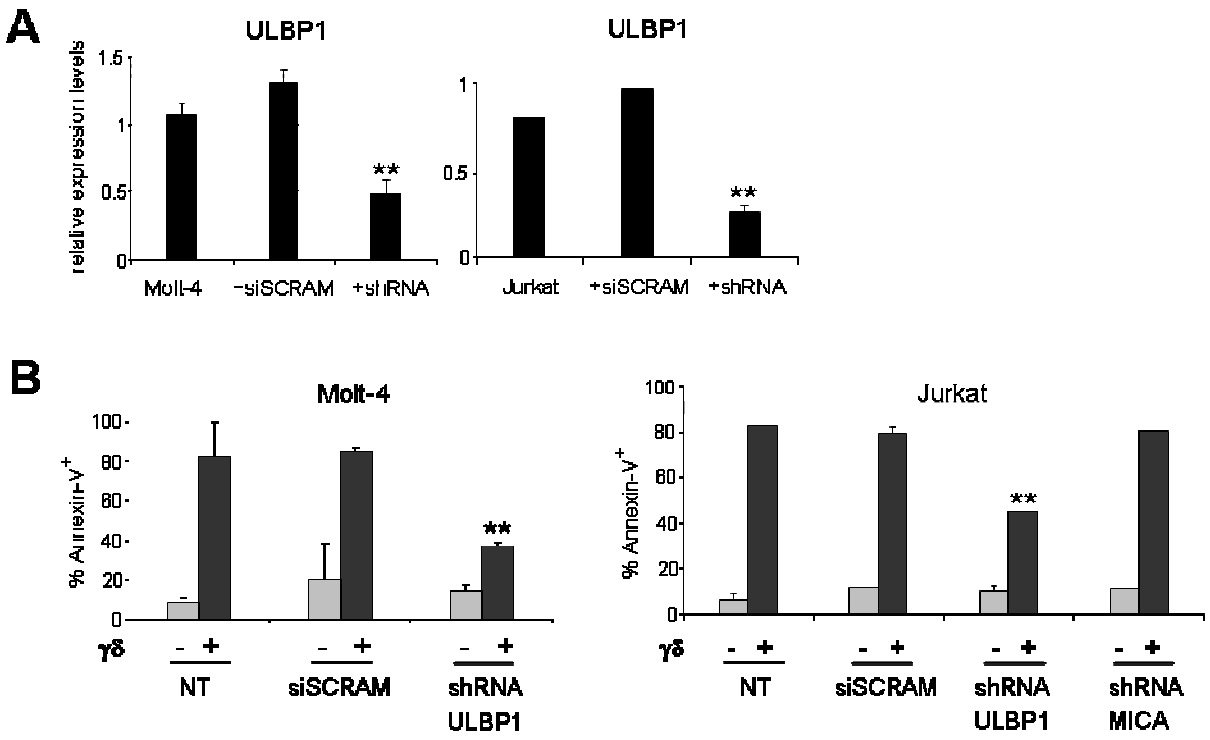


Figure 8. ULBP1 is required for Vγ9Vδ2 T-cell recognition of leukemia/ lymphoma cells.

(**A**) Lentiviral shRNA-mediated knockdown of ULBP1 and MICA in Molt-4 or Jurkat leukemia cells was confirmed by quantitative RT-PCR using GUSB and PSMB6 as endogenous

references. Cells were infected with 10 μL of high-titer virus (10^7 CFU/mL) in media containing polybrene, submitted to selection 48 hours later, and collected for analysis 96 hours after infection. siSCRAM is an shRNA of scrambled (unspecific) sequence, used as an infection control. Error bars represent SD (n = 3). $^{**}P < .01$. (**B**) Molt-4 or Jurkat leukemia cells, subjected to ULBP1 or MICAsh RNA knockdown (as in panel A), were used in *in vitro* killing assays either in the presence (+) or absence (–) of γδ-PBLs (as in Figure 2B). Nontransduced (NT) and siSCRAM-transduced cells were used as controls. This work was performed in collaboration with Telma Lança in the host laboratory[319].

Loss of ULBP1 expression caused a significant reduction (35%-50%) of γδ-PBL–mediated lysis of leukemia lines (Figure 8B), essentially "converting" these susceptible tumors into γδ-resistant lines. These data collectively suggest that ULBP1 plays a crucial and nonredundant role in γδ-PBL recognition of leukemias.

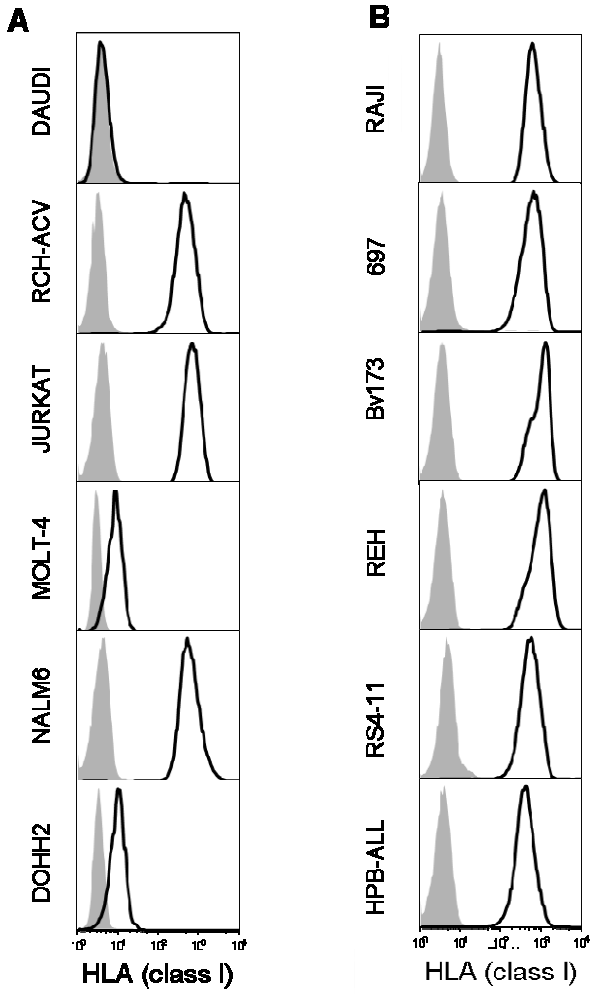


Figure 9. HLA class I expression on leukemia/ lymphoma cells.

Surface HLA class I expression in γδ-susceptible cell lines (**A**) or γδ-resistant cell lines (**B**), as analyzed by flow cytometry with anti-HLA-ABC mAb (W6/32). Shaded is isotype control staining.

Table 3. List of primers used in this study for RT-qPCR analysis

Gene	Gene ID	Primer forward (5´-3´)	Primer reverse (5´-3´)
ATP1B1	NM_001001787.1	CCGCCAGGATTAACACAGAT	TCCTCGTTCTTTCGGTTCAC
CCR7	NM_001838.2	TTCCAGGTATGCCTGTGTCA	ATAGGTCAACACGACCAGCC
CCRL1	NM_016557.2	CTAAAGTCCCCAGCCAATCA	TGATGTTCCTAGGTAGCGGG
CD151	NM_004357.4	AACACGGAGCTCAAGGAGAA	AGCGGATCCACTCACTGTCT
CD24	NM_013230.2	ACCCACGCAGATTTATTCCA	GAGACCACGAAGAGACTGGC
CD58	NM_001779.2	ATGAAATGGAATCGCCAAAT	TTGCTCCATAGGACAATCCC
CD70	NM_001252.3	TGGTACACATCCAGGTGACG	GAGGCGGGAGAGCAGATT
CLEC2D	NM_001004419.2	TCCAAGACCTGTCATGGTTTC	ACTGGCACCTTTGTCATTCA
CLSTN3	NM_014718.3	ATGGCGTAACTCCCAACAAG	TCATCACAGACCTGCTCCAG
EMP3	NM_001425.2	TGTCACTCCTCTTGCTGGTG	ACATTACTGCAGGCCCATGT
FAS	NM_000043.3	CAAGGGATTGGAATTGAGGA	TACTCCTTCCCTTCTTGGCA
GPR137B	NM_003272.3	TACTCAAATACCGGTTGCCC	GATGGAGAGAGAGACGGCAC
HLA-DBP1	NM_002121	CCTGAATGGACAGGAGGAAA	TACTCCGGGCAGAATCAGAC
HLA-DRB3	NM_022555.3	GCTGAAGTGCAGATGACAATTT	GAACTGAGGAAGCCACAAGG
IFITM1	NM_003641.3	ACTGAAACGACAGGGGAAAG	GTGTGGTTTTCTGCGTGGA
ITGA6	NM_000210.2	GGGAGTACCTTGGTGGATCA	AGCATGGATCTCAGCCTTGT
KCNK12	NM_022055.1	TCGCTCTACTTCTGCTTCGTC	GTTGAGCACCTGCTTGATGA
NETO2	NM_018092.3	GAACCAGCAATGGAGGTCAT	TCCAAGTGATCAAACCGACA
NRP2	NM_003872.2	GCAGTGGACATCCCAGAAAT	CGATGATGGTGATGAGGATG
P2RX5	NM_002561.2	GGGCACCTGTGAGATCTTTG	TGACGTCCATCACATTGCTT
PKD2	NM_000297.2	TGGAAATTCGCATTCACAAA	GATGCTCAAAGTTGGGGAAA
PTCH1	NM_001083603.1	TAACGCTGCAACAACTCAGG	GAAGGCTGTGACATTGCTGA
SELL	NM_000655.3	CACCCAGAGGGACTTATGGA	CAATTTCCGCCTTGTTTTGT
SLAMF1	NM_003037.2	CAGGCCCTCCACGTTATCTA	TTCTGGAGTGGAGACCTGCT
TFR2	NM_003227.3	GAATGATTCAGGGTCAGGGA	ACCGTCACATTAGGGTCAGC
TLR4	NM_138554.3	TGAGCAGTCGTGCTGGTATC	CAGGGCTTTTCTGAGTCGTC
TNFRSFR21	NM_014452.3	GTGGGCTGATGGAAGACAC	CAGGAGAGCGGAATTCTCAA
ULBP1	NM_025218.2	ACTGGGAACAAATGCTGGA	TGCCAGCTAGAATGAAGCAG
GUSB	NM_000181.2	TGCAGCGGTCTGTACTTCTG	CCTTGACAGAGATCTGGTAATTCA
PSMB6	NM_002798.1	GGCGGCTACCTTACTAGCTG	AAACTGCACGGCCATGATA

3.5 DISCUSSION

The success of immunotherapy to tackle tumors, in particular those that prevail after chemo- or radiotherapy, critically depends on two factors: the specific activation of effector anti-tumor lymphocytes; and the molecular recognition of tumor cells by activated lymphocytes. Concerning γδ T cells, research over the last 15 years has identified very potent and specific phosphoantigens, most notably HMB-PP[192,317], that seem to fulfill the first requirement. However, despite suggestions of phosphoantigens themselves[192,208,272], or a F1-ATPase-related structure complexed with delipidated apolipoprotein A-I[207], or the non-classical MHC protein ULBP4[55] being responsible for tumor cell recognition by Vγ9Vδ2 PBL, the issue is still highly controversial. This naturally impacts on our ability to design effective therapeutic protocols based on γδ-PBL immunotargeting of tumors. Thus, only 33% of patients with prostate carcinoma[290] or non-Hodgkin's lymphoma[289] showed objective responses despite large activation and expansion of their Vγ9Vδ2 T cells *in vivo*. These considerations stress the importance of identifying tumor molecular signatures that may predict the response to activated γδ-PBL.

In this study we set out to identify cell surface proteins involved in interactions between leukemia/ lymphoma cells and γδ-PBL. Taking tumor cytolysis (*in vitro*) as functional readout, we screened a panel of 20 leukemia and lymphoma cell lines that faithfully reproduced the susceptibility/ resistance of primary tumors (Figure 2A). The use of cell lines permitted experimental reproducibility and hence statistical robustness for the gene expression undertaken. Upon the identification of candidate markers, we analyzed their expression in 23 samples derived from T-ALL and NHL (FL and DLBCL) patients.

The choice of cDNA microarrays as screening tools was based on a multiplicity of previous studies that demonstrated how powerful and reliable they are in defining cancer molecular signatures[328]. Our analyses led to the identification of a large panel of genes differentially expressed between "γδ-susceptible" and "γδ-resistant" tumors. Importantly, we verified that there was no correlation between intrinsic anti-apoptotic properties and resistance to γδ-PBL cytotoxicity, both in terms of gene expression and response to a death stimulus. Thus, susceptibility or resistance to γδ-mediated lysis is more likely to be related with tumor recognition and immune evasion strategies, the molecular basis of which remains to be elucidated. Of note, MHC

class Ia expression did not consistently segregate between γδ-susceptible and γδ-resistant tumor cell lines (Figure 9). For example, among susceptible lines, DAUDI and MOLT-4 expressed very low or undetectable levels, whereas JURKAT and RCH-ACV displayed high levels of surface MHC class I (Figure 9). These data exclude a mechanism of "missing self" as the basis for γδ T cell recognition of hematopoietic tumors.

Building upon stringent biological and statistical selection criteria, we narrowed our microarray data down to 10 genes encoding cell surface proteins (with extracellular domains), whose expression segregated with susceptibility *versus* resistance to γδ-PBL cytotoxicity. We believe the provision of this gene profile to the biomedical community is of great value. Thus, we propose the expression of each candidate gene to be evaluated during upcoming γδ T cell-based clinical trials. The genes with highest predictive value will constitute novel leukemia/ lymphoma biomarkers, for which standardized quantification essays should be developed. This will endow clinicians with a key tool for the indication and monitoring of γδ T cell-based immunotherapies.

Furthermore, within the panel of 10 candidate markers, some are likely to play non-redundant roles in leukemia/ lymphoma cell recognition by γδ-PBL. Thus, proteins that are enriched in γδ-susceptible tumors may provide activation signals, whereas markers of resistance may convey inhibitory signals to γδ-PBL. Provocatively, 7 of the candidates are known to intervene in immune responses: 4 of them (ULBP1, IFITM1, CLEC2D and SLAMF1) provide stimulatory (or inhibitory) signals through receptors expressed on lymphocytes, while 3 (NRP2, SELL and ITGA6) control lymphocyte adhesion. IFITM1 was shown to modulate NK cell responses and its expression correlated with improved survival of gastric cancer patients[329]. By contrast, the expression of CLEC2D, a ligand for the inhibitory receptor CD161, inhibits NK cell responses and was associated with increased malignancy grade of glioblastoma[77]. NRP2 is another protein that can favor cancer progression by acting as a coreceptor for Vascular Endothelial Growth Factor (VEGF) and stimulating tumor growth[330]. We will proceed with individual knock-down (RNA interference) experiments in a functional (tumor killing) bioassay to dissect the role of each of the candidates in γδ-PBL targeting of leukemias and lymphomas. Given that some of these molecules can also provide costimulatory or inhibitory

signals to NK cells, we also plan to address their role in NK cell targeting of hematopoietic malignancies.

ULBP1 is a ligand for the NKG2D receptor expressed on all cytotoxic lymphocyte lineages, including 100% of Vγ9Vδ2 T cells, which has been clearly implicated in anti-tumor responses[22,98,331,332]. We showed that the expression levels of ULBP1 determine leukemia/ lymphoma susceptibility to γδ T cell–mediated cytolysis, and therefore, we propose ULBP1 to be tested as a biomarker in upcoming γδ T cell–based cancer clinical trials. Importantly, we observed a frequent down-regulation of ULBP1 expression in primary samples from leukemia patients (Figure 7A), which may underlie an immune evasion mechanism developed during tumor progression.

In sum, this study establishes a panel of 10 putative markers of leukemia/ lymphoma susceptibility to γδ-PBL cytotoxicity. The expression data collected from primary samples showed a striking heterogeneity for particular candidate genes, most notably ULBP1, whereas other genes, such as IFITM1, ITGA6 or SELL, essentially did not vary among patients. It is therefore predictable that different components of the proposed panel will have very distinct behaviors when associated to therapeutic outcome in clinical trials. It will also be interesting to infer to what extent immunoselection may have conditioned the expression of these markers in tumors evolving in a dynamic interaction with γδ T lymphocytes. This will significantly add to our understanding of anti-tumor immunity and to our capacity to modulate it for cancer immunotherapy.

4. DIFFERENTIATION OF HUMAN PERIPHERAL BLOOD Vδ1$^+$ T CELLS EXPRESSING THE NATURAL CYTOTOXICITY RECEPTOR NKP30 FOR RECOGNITION OF LYMPHOID LEUKEMIA CELLS

4.1 ABSTRACT

4.1.1 Background / Objectives

The success of cancer immunotherapy depends on productive tumor cell recognition by killer lymphocytes. γδ T cells are a population of innate-like lymphocytes endowed with strong, MHC-unrestricted cytotoxicity against tumor cells. This notwithstanding, we recently showed that a large proportion of human hematologic tumors is resistant to γδ peripheral blood lymphocytes (PBLs) activated with specific agonists to the highly prevalent Vγ9Vδ2 TCR. Although this probably constitutes an important limitation to current γδ T cell–mediated immunotherapy strategies, we describe here the differentiation of a novel subset of Vδ2$^-$ Vδ1$^+$ PBLs expressing natural cytotoxicity receptors (NCRs) that directly mediate killing of leukemia cell lines and chronic lymphocytic leukemia patient neoplastic cells.

4.1.2 Methodology / Principal Findings

We show that Vδ1$^+$ T cells can be selectively induced to express NKp30, NKp44 and NKp46, through a process that requires functional phosphatidylinositol 3-kinase (PI-3K)/AKT signaling on stimulation with γ_c cytokines and TCR agonists. The stable expression of NCRs is associated with high levels of granzyme B and enhanced cytotoxicity against lymphoid leukemia cells. Specific gain-of-function and loss-of-function experiments demonstrated that NKp30 makes the most important contribution to TCR-independent leukemia cell recognition. Thus, NKp30$^+$ Vδ1$^+$ T cells constitute a novel, inducible and specialized killer lymphocyte population with high potential for immunotherapy of human cancer.

4.2 INTRODUCTION

Tumors develop in hosts endowed with a highly complex immune system that includes various lymphocyte subsets capable of recognizing and destroying transformed cells. It is now widely accepted that, although lymphocytes may constantly patrol tumor formation, cancer cells develop molecular strategies to evade immune surveillance, which are competitively selected under the pressure of the host immune system[14]. This dynamic process, termed "cancer immunoediting," is thought to constitute a major obstacle to cancer immunotherapy[14].

Among multiple immune evasion mechanisms, we have recently shown that leukemia and lymphoma primary cells often down-regulate the non-classical MHC protein, ULBP1, which is critical for recognition of hematologic tumors by γδ T cells expressing the counter-receptor NKG2D[319]. γδ T cells are innate-like lymphocytes that account for 1%-10% of peripheral blood lymphocytes (PBL) of healthy people and are capable of targeting a significant fraction of hematologic tumor cell lines tested in the laboratory[333]. However, we have demonstrated that many lymphoid leukemia cells are resistant to fully activated Vγ9Vδ2 T cells[319,333], the dominant subset of γδ PBLs. Furthermore, clinical trials involving the in vivo administration of activators of Vγ9Vδ2 T cells have shown limited success, with objective responses restricted to 10%-33% of patients with either hematologic or solid tumors[289,290,299]. Even more modest has been the outcome of trials involving the adoptive transfer of activated and expanded $V\delta2^+$ cells, because no objective responses have been reported[299]. In fact, the simple ex vivo expansion of autologous $V\delta2^+$ T cells, whose surveillance the tumor managed to escape in vivo, may be condemned to little therapeutic effect on reinjection into the patient. Therefore, we believe it is critical to invest in strategies that endow γδ T cells with additional recognition machinery to detect tumors that have resisted the natural components present in vivo.

Besides Vγ9Vδ2 T cells, $V\delta1^+$ T cells are also endowed with potent antitumor cytolytic function, particularly as tissue-associated or tumor-infiltrating lymphocytes[54,251,334,335]. Moreover, $V\delta1^+$ T cells can constitute up to 30% of all γδ PBLs and may thus represent an important alternative population for adoptive cell therapy. However, this possibility remains poorly explored.

In this study we identified and characterized a novel $V\delta1^+$ PBL subset capable of targeting hematologic tumors highly resistant to fully activated Vγ9Vδ2 PBLs. We

show that this Vδ1$^+$ population owes its specialized killer function to induced expression of natural cytotoxicity receptors (NCRs), which have been mostly regarded as NK-specific markers. Instead, we show that, although neither Vδ1$^+$ nor Vδ2$^+$ cells express NCRs constitutively, these can be selectively up-regulated in Vδ1$^+$ cells by AKT-dependent signals provided synergistically by γ_c cytokines (IL-2 or IL-15) and TCR stimulation. We further show that NKp30 and NKp44 are both functional in NCR$^+$ Vδ1$^+$ PBLs, and synergistically contribute to enhanced targeting of lymphocytic leukemia cells, with NKp30 playing the major role in this process. Thus, NKp30$^+$ Vδ1$^+$ PBL constitute a novel promising population for adoptive cell immunotherapy of hematologic malignancies.

4.3 Methods

Ethics statement. Research involving clinical samples was conducted according to the principles expressed in the Helsinki Declaration. All procedures were approved by the review board of Instituto Português de Oncologia de Lisboa (Portugal).

4.3.1 Isolation of human peripheral blood γδ T cells

Peripheral blood was collected from anonymous healthy volunteers, diluted in a 1:1 ratio (volume-to-volume) with PBS (Invitrogen Gibco), and centrifuged in Ficol-Paque (Histopaque-1077; Sigma-Aldrich) in a volume ratio of 1:3 (1 part ficol to 3 parts diluted blood) for 30 minutes at 1 500 rpm and 25°C. The interfase containing mononuclear cells was collected and washed (in PBS), and γδ T cells were isolated (to above 95% purity) by magnetic cell sorting via positive selection (with a FITC-labeled anti-TCRγδ antibody) or via negative selection (with a cocktail of Biotin-labeled antibodies; Miltenyi Biotec). When noted, Vδ1$^+$ cells were further purified by magnetic cell sorting via positive selection with a FITC-labeled anti-Vδ1 TCR antibody (Fisher Scientific) and anti-FITC microbeads (Miltenyi Biotec).

4.3.2 Cell culture

Isolated γδ PBLs were cultured at 10^6 cells/mL at 37°C, 5% CO^2 in round-bottom 96 well plates with RPMI 1640 and 2 mM l-glutamine (Invitrogen Gibco) supplemented with 10% FBS (Invitrogen Gibco), 1 mM sodium pyruvate (Invitrogen Gibco), and 50 mg/mL of penicillin and streptomycin (Invitrogen Gibco). The cells were expanded in the presence of 100 U/mL of rhIL-2 (Roche Applied Science), with or without 10 nM of HMB-PP (4-hydroxy-3-methyl-but-2-enyl pyrophosphate; Echelon Biosciences) and 1μg/mL of phytohemagglutinin (PHA; Sigma-Aldrich). Cells were washed and the culture medium was replaced every 5-6 days. To study the induction of NKp30 expression, γδ PBLs were cultured in the presence or absence of 100 U/mL of rhIL-2 (Roche Applied Science), 1μg/mL of soluble anti-CD3 antibody (eBioscience; clone OKT3), and 20ng/mL of rhIL-15 (Biolegend). For TCR blockade, freshly isolated γδ PBL were CFSE-labeled and then incubated for 7 days with anti-TCRγδ (Beckman Coulter; clone IMMU510) diluted 1:20 in complete medium supplemented with 1μg/mL PHA and 100 U/mL rhIL-2. To study the effects of chemical inhibitors of signal transduction, the MEK inhibitor UO126 and the PI-3K inhibitor LY294002 (both from Calbiochem) were added at 10 mM for a 2-hour incubation period and then maintained in culture with 100 U/mL rhIL-2 and 1μg/mL PHA for 7 days.

4.3.3 Flow cytometry cell sorting

For sorting of γδ PBL based on the expression of NKp30 and $V\delta1^+$ TCR, cells from PHA and IL-2–activated cultures were stained with anti-NKp30 (Biolegend; clone P30-15), anti-Vδ1 (Thermo Fisher Scientific; clone TS8.2), and sorted on a FACSAria cell sorter (BD Biosciences).

4.3.4 Leukemia patient samples

B-cell chronic lymphocytic leukemia cells were obtained from the peripheral blood of patients at presentation, after informed consent and institutional review board approval (Instituto Português de Oncologia de Lisboa, Portugal). Samples were enriched by density centrifugation over Ficol-Paque and then washed twice in 10% RPMI 1640.

4.3.5 *In vitro* tumor killing assays

All tumor cell lines (details provided in previous chapter) were cultured in complete 10% RPMI 1640, maintained at 10^5 up to 10^6 cells/mL by dilution and splitting in a 1:3 ratio every 3-4 days. For cytotoxicity assays, magnetically purified γδ PBL were preactivated for 7-19 days in the presence of IL-2 (100 U/mL) and either 1µg/mL PHA or 10 nM HMB-PP. For receptor blocking, γδ PBLs were incubated for 2 hours with the blocking antibodies anti-NKp30 (clone F252), anti-NKp44 (clone KS38), anti-NKp46 (clone KL247), anti-TCRγδ (Beckman Coulter, clones IMMU510 or B1.1), or anti-Vδ1 TCR (Fisher Scientific, clones TCS1 or TS8.2). The blocking antibodies were maintained in the culture medium during the killing assays. Tumor cell lines or leukemia primary samples were stained with CellTrace Far Red DDAO-SE (1 µM; Molecular Probes, Invitrogen) and each batch of 3 × 10^4 tumor cells was incubated with 1.5 × 10^5 to 3 × 10^5 γδ T cells in RPMI for 3 hours at 37°C and 5% CO^2 on a round-bottom plate with 96 wells. Cells were then stained with Annexin V–FITC (BD Biosciences) and analyzed by flow cytometry. For the redirected killing assays, PHA and IL-2–activated γδ PBL were incubated for 4 hours with the NCR agonists anti-NKp30 (clone AZ20), anti-Nkp44 (clone Z231) or anti-NKp46 (clone Bab281) during a standard ^{51}Cr release assay.

4.3.6 Flow cytometry analysis

Cells were labeled with the following fluorescent monoclonal antibodies: anti-CD3–PerCP-Cy5.5 (eBioscience; clone OKT3); anti-TCRγδ–FITC (eBioscience; clone B1.1); anti-CD69–PE (BD Pharmingen; clone FN50); anti-NKG2D–PE/Cy7 (Biolegend; clone 1D11); anti-2B4–APC (Biolegend, clone C1.7); anti–DNAM-1–Alexa-Fluor647 (Biolegend; clone DX11); anti-NKp30–APC (Biolegend; clone P30-15); anti-Vδ2 TCR-PE (Biolegend; clone B6); anti-NKp44–APC (Biolegend; clone P44-8); anti-NKp46–AlexaFluor647 (Biolegend; clone 9E2); anti-Vδ1 TCR-FITC (Thermo Fisher Scientific; clone TS8.2); anti-NKp30–PE (Biolegend; clone P30-15); anti–Mouse IgG1κ-APC Isotype Ctrl (Biolegend; clone MOPC-21); anti–Mouse IgG1κ-PE Isotype Ctrl (Biolegend, clone MOPC-21); anti-CD27–APC/Cy7 (Biolegend, clone O323); and anti-CD56–APC (Biolegend, clone HCD56). Cell proliferation was measured by following a standard CFSE staining protocol (CellTrace CFSE Cell Proliferation Kit, Invitrogen; final concentration 0.5 mM), while

apoptosis was assessed by Annexin V–FITC (BD Pharmingen) staining. Cells were analyzed on a FACSCanto flow cytometer (BD Biosciences).

4.3.7 RNA isolation and cDNA production

Total RNA was extracted using the RNeasy Mini Kit according to the manufacturer's protocol (QIAGEN). Concentration and purity was determined by spectrophotometry and integrity was confirmed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay (Agilent Technologies). Total RNA was reverse-transcribed into cDNA using random hexamers and Superscript II first strand synthesis reagents (Invitrogen).

4.3.8 Real-time quantitative PCR

Real-time quantitative PCR (qPCR) was performed on ABI Prism 7500 FAST Sequence Detection System using SYBR Green detection system (both from Applied Biosystems). Primers were designed using Primer3 v.0.4.0 online program (http://primer3.sourceforge.net). For each transcript, quantification was done using the calibration curve method. β_2-microglobulin (*B2M*), Glucoronidase β (*GUSB*) and proteasome subunit β type 6 (*PSMB6*) were used as housekeeping controls for normalization of gene expression. The following primers were used: *B2M*, forward CTAT CCAG CGTA CTCC AAAG ATTC, reverse CTTG CTGA AAGA CAAG TCTG AATG; *PSMB6*, forward GGCG GCTA CCTT ACTA GCTG, reverse AAAC TGCA CGGC CATG ATA; *GUSB*, forward TGCA GCGG TGTA CTTC TG, reverse CCTT GACA GAGA TCTG GTAA TCA; *B7H6*, forward TCAC CAAG AGGC ATTC CGAC CT, reverse ACCA CCTC ACAT CGGT ACTC TC; *NKP44*, forward CCGT CAGA TTCT ATCT GGTG GT, reverse CACA CAGC TCTG GGTC TGG; *NKP46*, forward AAGA CCCC ACCT TTCC TGA, reverse TGCT GGCT CGCT CTCT AGT; *GZMB*, forward GGGG GACC CAGA GATT AAAA, reverse CCAT TGTT TCGT CCAT AGGA G. All samples were run in triplicate and repeated 3 times. Analysis of the qPCR results was performed using the ABI SDS v1.1 sequence analysis software (Applied Biosystems).

Statistical analysis

Differences between subpopulations were assessed using the Student *t* test and are indicated when significant as **P* < .05; ***P* < .01; and ****P* < .001 in the figures.

4.4 RESULTS

4.4.1 Enhanced cytotoxicity of γδ PBL cultures activated with pan-T-cell mitogen

We compared the antitumor killing capacity of γδ PBL cultures (always maintained in the presence of IL-2) activated either with PHA, a plant lectin that acts as a potent T-cell mitogen[336], or the specific Vγ9Vδ2 TCR agonist HMB-PP[218,317]. Although both regimens were similarly efficient at activating γδ PBLs, as evaluated by CD69 up-regulation and cell proliferation (Figure 1A), we noted that samples activated with PHA were consistently better killers of hematopoietic tumor cell lines than samples (of the same donor origin) stimulated with HMB-PP (Figure 1B-C). This was valid across all donors tested (Figure 1B, Table 1, and data not shown) and was associated with higher expression of *GZMB* (Figure 1D), a key component of the lymphocyte cytolytic machinery. Of note, freshly isolated γδ PBLs, which lack *GZMB* expression (Figure 1D), displayed very poor antileukemia cytotoxicity (< 10% killing; not shown), as previously reported[317].

The superior cytotoxic function of PHA-stimulated γδ PBL cultures was a surprising finding, because we and others have shown that HMB-PP is a very potent activator of the highly dominant Vγ9Vδ2 PBL subset[317]. We were particularly interested that, compared with HMB-PP–activated γδ PBL, PHA-stimulated cultures displayed improved cytotoxicity against various resistant leukemia cell lines, such as Bv-173, REH or HPB-ALL (Figure 1B-C), which we had shown to lack expression of the critical NKG2D ligand ULBP1[319,333]. Of note, PHA-stimulated γδ T cells did not target normal (healthy) PBMC (Figure 2). These data demonstrate that the pan–T-cell mitogen PHA is capable of increasing the cytolytic potential of medium-term (1-3 weeks) γδ PBL cultures against leukemia cells, which could be of great value for adoptive cell immunotherapy.

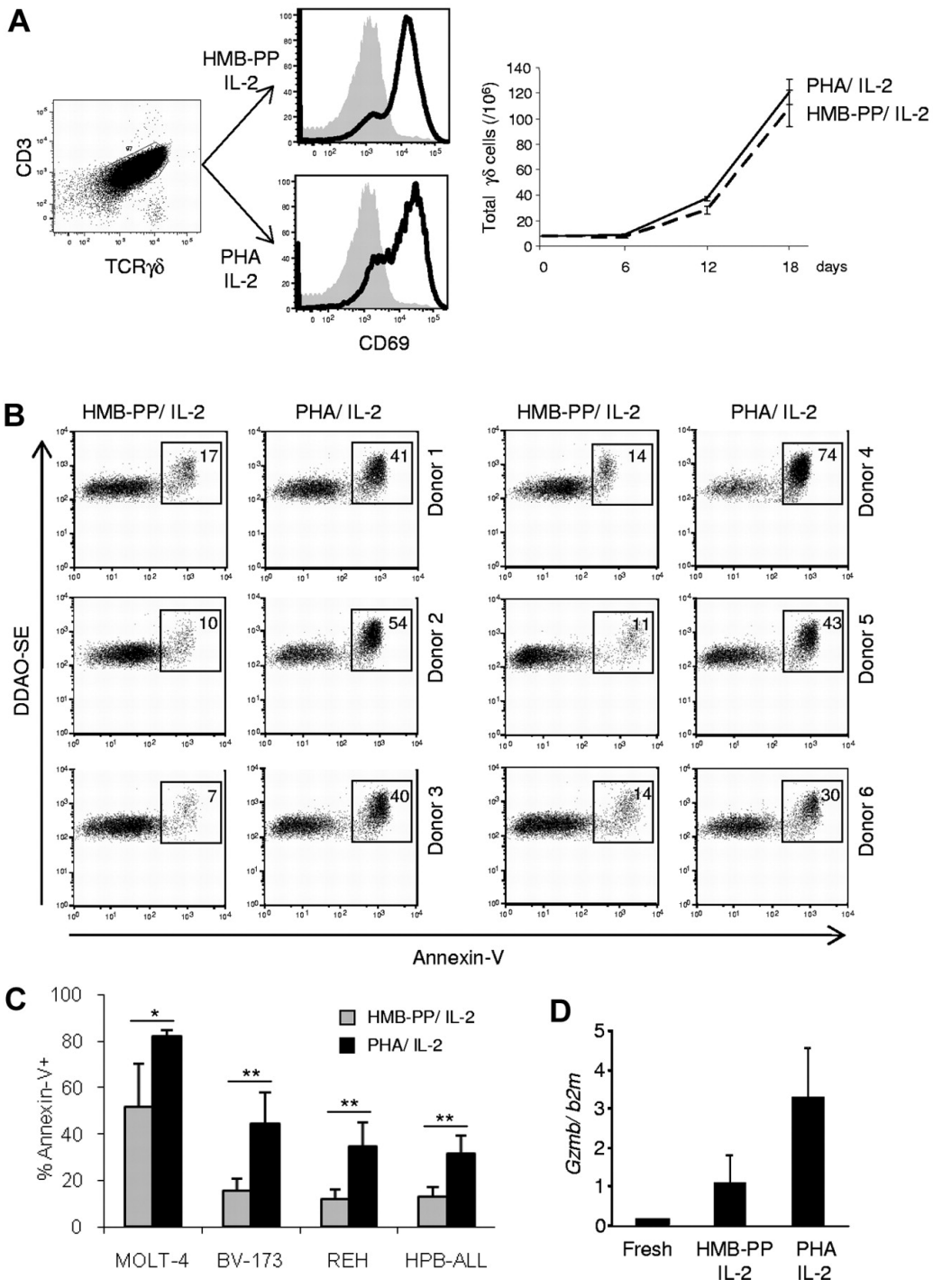


Figure 1. Enhanced antileukemia cytotoxicity of γδ PBL cultures activated with pan–T-cell mitogen.

(**A**) γδ peripheral blood lymphocytes (γδ PBLs) were MACS-sorted from the peripheral blood of healthy volunteers (left panel), and stimulated with either HMB-PP and IL-2 or PHA and IL-2 for 4 to 19 days. Activation was evaluated by flow cytometry for CD69 up-regulation (middle panels; levels in freshly isolated control cells are shaded), and total cell numbers are shown on the right panel. (**B-C**) Preactivated (for 14 days, as in panel A) γδ PBLs were coincubated with DDAOse-labeled leukemia cells for 3 hours. Tumor cell lysis was

evaluated by Annexin-V staining using flow cytometry. (**B**) Representative results of 6 different donors for the Bv173 leukemia cell line. Percentages refer to Annexin-V$^+$ tumor cells. Basal tumor cell apoptosis (in the absence of γδ PBL) was < 5%. (**C**) Summary of the results of 6 different donors with 4 leukemia target cell lines. Error bars represent SD (n = 6, **P* < .05; ***P* < .01). (**D**) Real-time PCR quantification of *GzmB* mRNA levels in freshly isolated, HMB-PP and IL-2–activated and PHA and IL-2–activated γδ PBL. Data in this figure are representative of 2 to 3 independent experiments with similar results.

Donor	1	2	3	4	5	6
% Vδ1$^+$	5	20	20	15	7	45
% Vδ2$^+$	89	75	70	81	88	48

Table 1. Percentages of Vδ1$^+$ versus Vδ2$^+$ cells within γδ PBLs of six healthy individuals used as donors in Figure 1B.

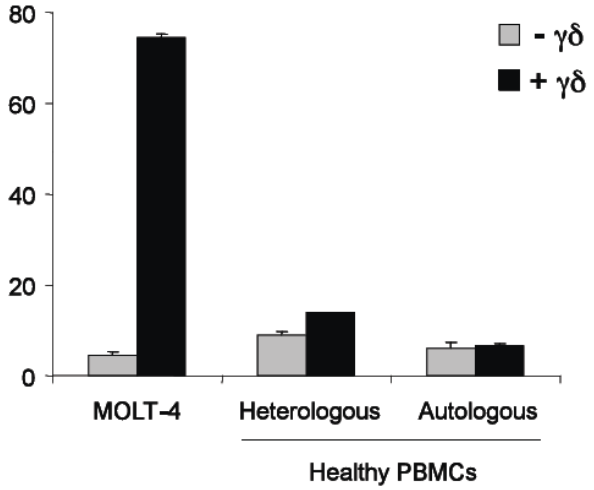


Figure 2. PHA/ IL-2 activated NCR$^+$ Vδ1$^+$ PBLs do not target healthy PBMCs.

MACS-purified Vδ1$^+$ PBLs were activated with PHA / IL-2 and then used in killing assays (performed as described in Figure 1) against the leukemia cell line MOLT-4, or PBMCs (autologous or heterologous) obtained from healthy donors. Error bars represent SD (n=3).

4.4.2 Induction of NCR expression on γδ PBLs activated with pan-T-cell mitogen

We next investigated the mechanism(s) underlying the enhanced cytotoxicity of PHA-activated γδ PBL cultures. We considered that this could be explained by differential expression of receptors such as NKG2D[22,148,319], DNAM-1[224,230], or 2B4[337], all previously shown to participate in tumor cell recognition by killer lymphocytes. However, none of these candidates was differentially expressed between PHA-activated and HMB-PP–activated γδ PBL cultures (Figure 3A). By contrast, and unexpectedly, the natural cytotoxicity receptor NKp30, an important trigger of NK cell cytotoxicity[95], was specifically found on PHA-stimulated γδ PBLs (Figure 3B; Figure 4A-B). Furthermore, the other NCR family members, NKp44 and NKp46, were also selectively expressed in these samples (Figure 3C-D; see next paragraph).

The proportion of $NKp30^+$ cells increased steadily with culture time (Figure 3E), suggesting an association of NKp30 induction with cell proliferation. Although unlikely because of the very low background in fresh samples (Figure 4B), it was possible that a minute subset constitutively expressing NKp30 could be preferentially expanded in PHA-stimulated γδ PBL cultures. However, experiments with highly (> 99%) FACS-purified $NKp30^-$cells demonstrated that $NKp30^-$ cells were able to acquire NKp30 expression as efficiently as unsorted cells on PHA and IL-2 stimulation (Figure 5). Moreover, under such conditions, $NKp30^-$ and $NKp30^+$ cells proliferated to similar extent (not shown), further arguing against preferential expansion of $NKp30^+$ cells under such conditions. These results suggest that NKp30 expression is induced de novo on γδ PBL activation by PHA and IL-2 treatment, which is coupled to cell proliferation.

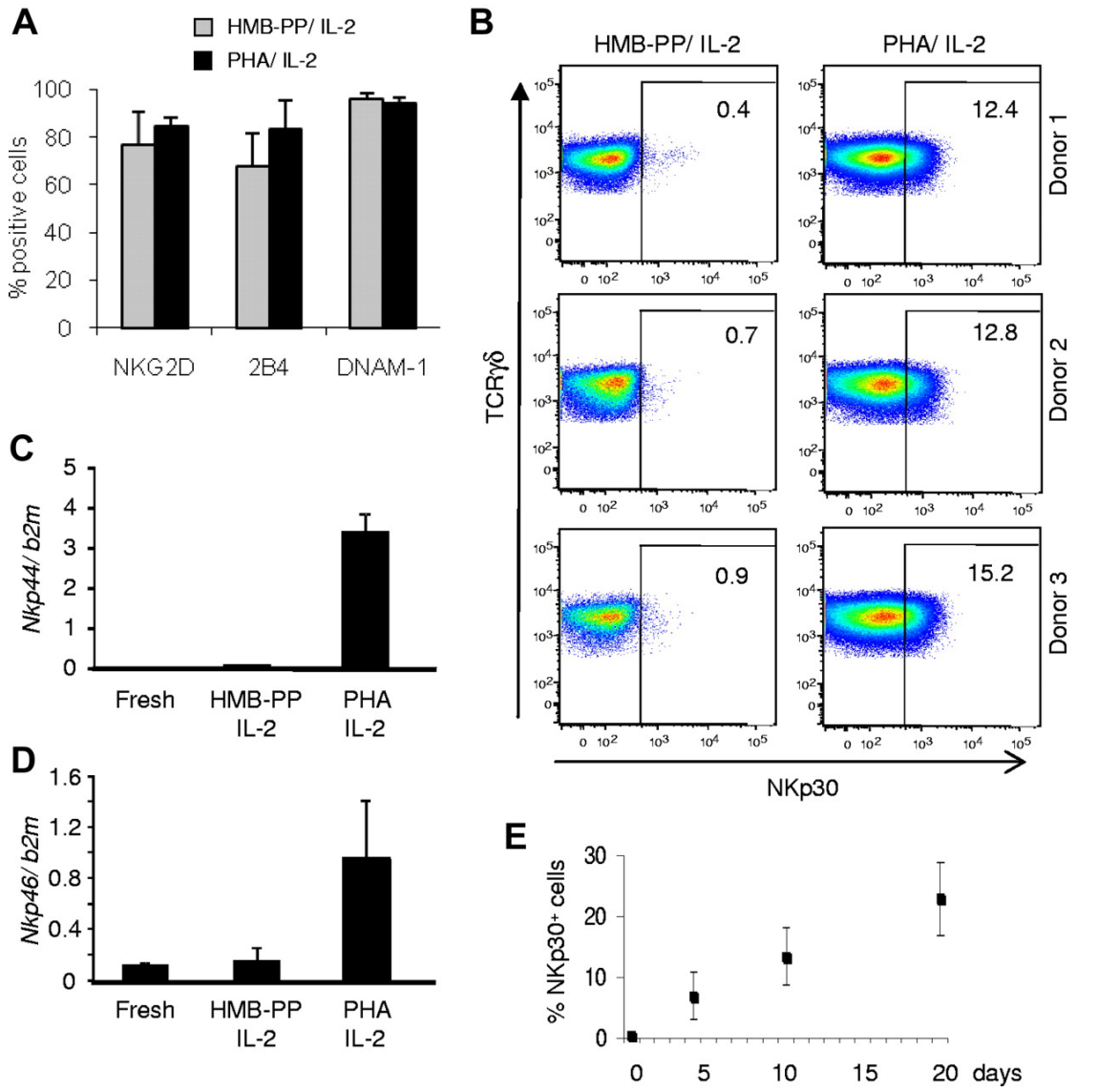


Figure 3. Induction of natural cytotoxicity receptor expression in γδ PBLs activated with pan–T-cell mitogen.

γδ PBLs were cultured as described in Figure 1 for 4-19 days and analyzed by flow cytometry for surface expression of various NK receptors. (**A**) Results for NKG2D, 2B4 and DNAM-1 in 10-day cultures activated either with HMB-PP and IL-2 (gray) or PHA and IL-2 (black), derived from 6 independent healthy donors. Error bars represent SD (n = 6; *P* > .05). (**B**) Expression of NKp30 in the same cultures of (A). FACS plots correspond to cultures derived from 3 individual donors. Percentages refer to NKp30$^+$ γδ PBLs. Isotype control staining is presented in Figure 4A. (**C-D**) Real-time PCR quantification of *Nkp44* (C) and *Nkp46* (D) mRNA levels in freshly isolated, HMB-PP and IL-2–activated and PHA and IL-2–activated γδ PBL. (**E**) Evolution of the percentage of NKp30$^+$ cells in the cultures described in (A), analyzed up to day 19. Error bars represent SD (n = 5). Data in this figure are representative of 2 to 4 independent experiments with similar results.

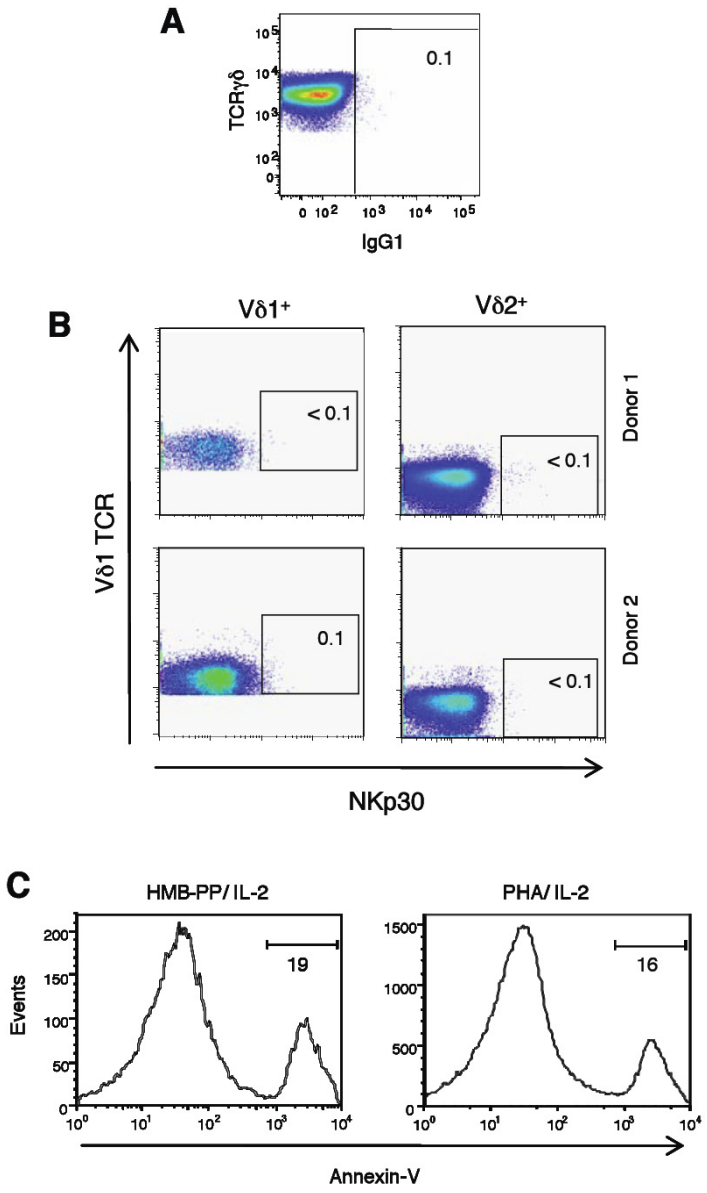


Figure 4. Control NKp30 expression and cell apoptosis in resting or activated γδ PBLs.

(**A**) IgG1κ isotype control staining for NKp30 in pre-gated TCRγδ$^+$ cells from PHA/ IL-2-activated cultures (as described in Figures 1 and 3). Data are representative of 6 independent experiments. (**B**) NKp30 and Vδ1 TCR expression in γδ PBLs freshly-isolated from two healthy donors. Data are representative of fifteen different healthy donors. (**C**) Vδ2$^+$ PBLs are not preferentially susceptible to PHA-induced apoptosis. γδ PBLs were cultured with HMB-PP/ IL-2 or PHA/ IL-2 as described in Figure 1; after 7 days, cells were stained with Annexin-V and analyzed by flow cytometry by gating on Vδ2$^+$ cells.

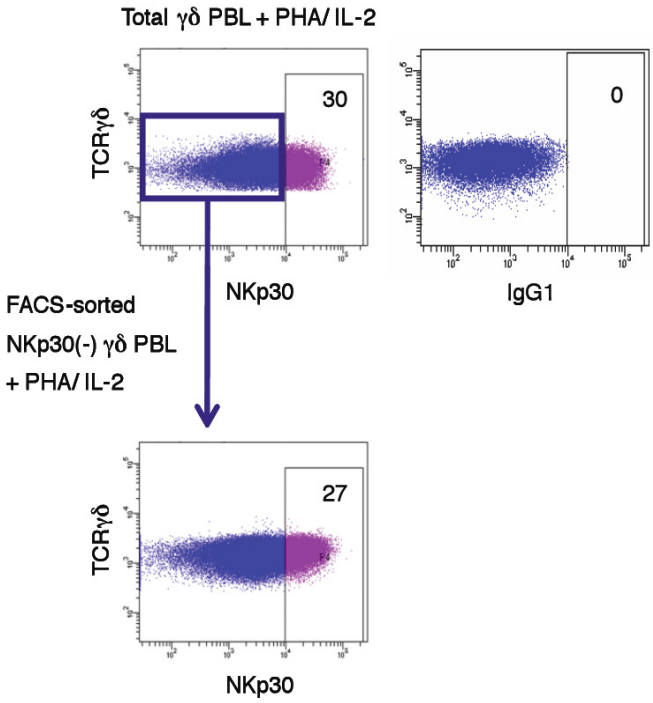


Figure 5. NKp30 expression is induced de novo on γδ PBLs.

Induction of NKp30 expression in total γδ PBL (upper panel) or in FACS-sorted (>99% pure) NKp30$^{(-)}$ γδ PBL from PHA/ IL-2-activated γδ PBL cultures (middle panel). The lower panel shows isotype control staining. Cells were stimulated with PHA/ IL-2 for 14 days and then analyzed on a FACSAria cytometer.

4.4.3 NCRs are selectively expressed by proliferating Vδ1^{+} T-cells

Considering that HMB-PP had been shown to be an optimal agonist of Vγ9Vδ2 cells[218,317], we hypothesized that our findings derived from PHA-mediated activation of a distinct γδ PBL subset. Consistent with this, we observed that, by contrast with HMB-PP, treatment with PHA preferentially expanded Vδ2^{-} cells among γδ PBL (Figure 6A). We verified that this was not because of differences in Vδ2^{+} cell apoptosis in the 2 experimental conditions (Figure 4C). The most likely Vδ2^{-} population to expand so markedly (Figure 6A) were Vδ1^{+} cells, because other subsets are very rare in the peripheral blood of healthy adults[338]. When Vδ1 versus Vδ2 TCR usage was assessed, a dramatic Vδ1^{+} cell enrichment was found in PHA-activated cultures (> 80% of all γδ T cells after 19 days; Figure 6B; Figure 7).

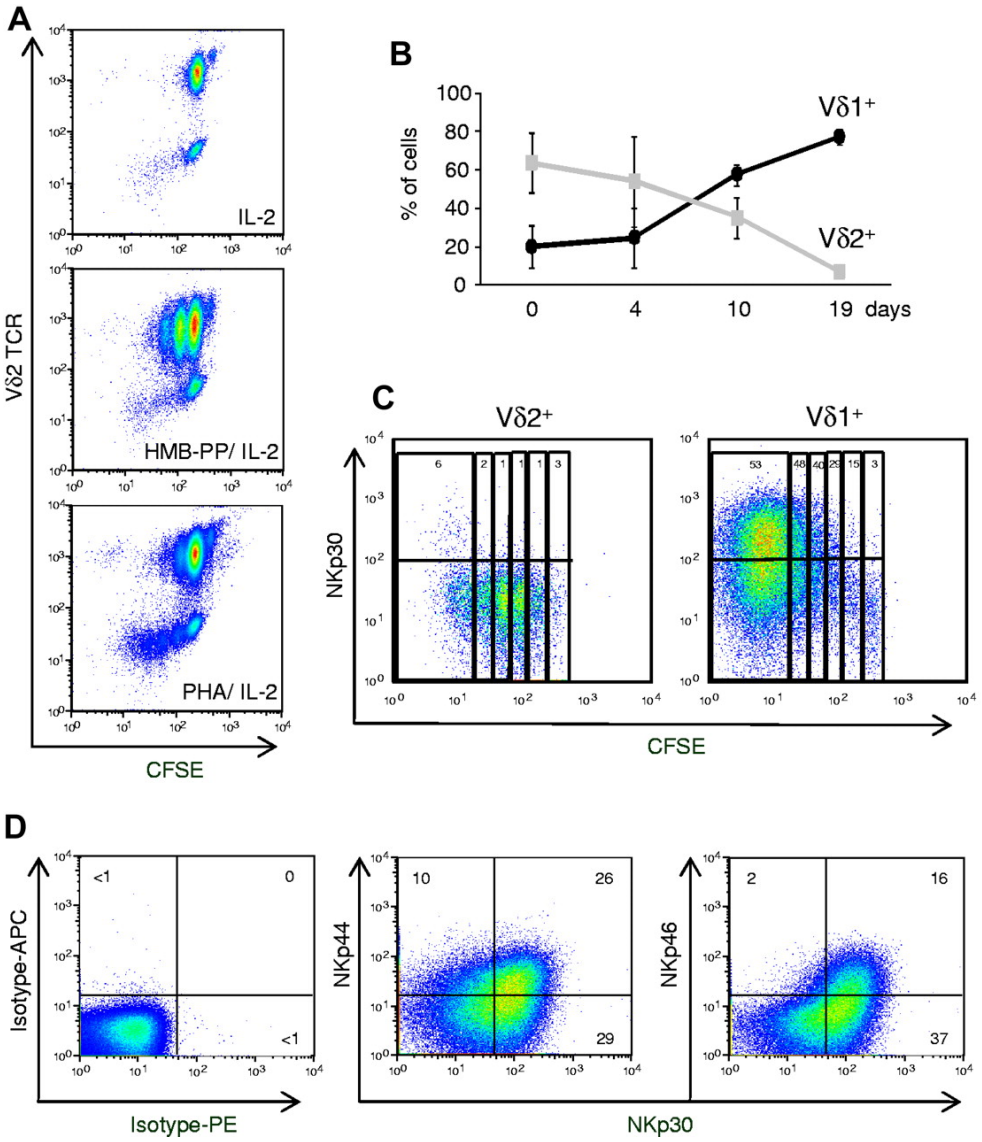


Figure 6. Natural cytotoxicity receptors are selectively expressed on proliferating Vδ1$^+$ T cells.

(**A**) γδ PBLs were labeled with CFSE and cultured as described in Figure 1, or in the absence of T cell mitogens (ie, IL-2 alone). Flow cytometry analysis of CFSE dilution and Vδ2 TCR expression after 7 days in culture. (**B**) Percentage of Vδ1$^+$ or Vδ2$^+$ cells among total γδ PBLs cultured up to 19 days with PHA and IL-2. Error bars represent SD (n = 3). (**C**) NKp30 expression in PHA and IL-2–activated γδ PBL subsets. Vδ1$^+$ or Vδ2$^+$ cells were FACS-sorted from peripheral blood, labeled with CFSE and cultured with PHA and IL-2 for 7 days. Percentages refer to NKp30$^+$ cells within each cell division (according to CFSE levels and indicated by vertical rectangles). (**D**) Expression of NKp30, NKp44 and NKp46 in Vδ1$^+$ T cells after 19 days of PHA and IL-2 stimulation. Isotype mAb control stainings are also shown. Data in this figure are representative of 2-3 independent experiments with similar results.

Conversely, and as described[317], HMB-PP–activated cultures were progressively dominated by Vδ2$^+$ cells (Figure 6A; Figure 7).

The induction of NKp30 expression was examined in parallel cultures of isolated Vδ1$^+$ or Vδ2$^+$ cells, which were stimulated with PHA and IL-2. Although neither freshly isolated Vδ1$^+$ nor Vδ2$^+$ cells expressed NKp30 (Figure 4B), this NCR was strongly induced (on PHA and IL-2 treatment) in Vδ1$^+$ but not Vδ2$^+$ cells (Figure 6C). Moreover, by following CFSE dilution, we demonstrated a striking accumulation of NKp30$^+$ cells with progressive division of Vδ1$^+$ cells (Figure 6C). These data suggest that activation of Vδ1$^+$ cells in PHA and IL-2 cultures induces NKp30 expression concomitantly with cell proliferation.

Whereas high percentages (> 50%) of NKp30$^+$ cells were usually detected after 2 to 3 weeks in culture, NKp44 (~ 30%) and NKp46 (< 20%) were expressed in lower proportions of Vδ1$^+$ cells (Figure 6D). Furthermore, most of NKp44$^+$ or NKp46$^+$ Vδ1$^+$ cells also expressed NKp30 (Figure 6D). We therefore considered NKp30 as the most informative marker of the inducible NCR$^+$ Vδ1$^+$ subset, and we set out to further characterize its differentiation.

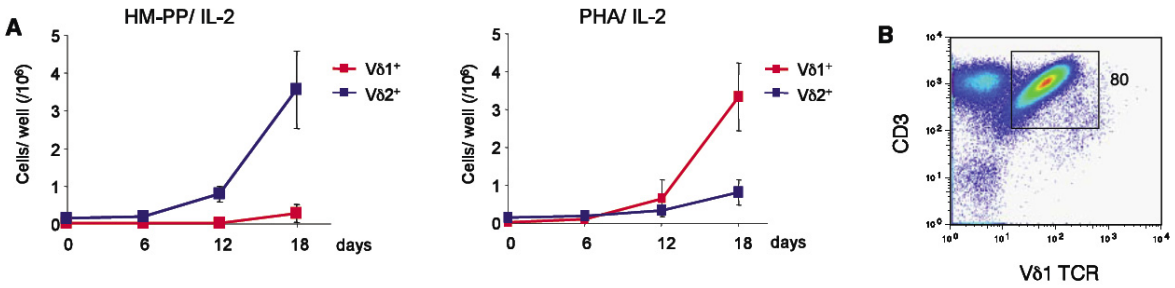


Figure 7. Vδ1$^+$ T-cell enrichment in γδ PBL cultures activated with PHA/ IL-2.

(**A**) MACS-sorted γδ PBLs from healthy donors were cultured with HMB-PP/ IL-2 or PHA/ IL-2 (as described in Figure 1), and analyzed for Vδ1 versus Vδ2 TCR expression. (**B**) Enrichment of CD3$^+$ Vδ1$^+$ TCR$^+$ cells in γδ PBL cultures after 19 days of activation with PHA/ IL-2.

4.4.4 NKp30 induction requires AKT-dependent γc cytokine and TCR signals

We next dissected the specific signals required for the differentiation of NCR^+ $V\delta1^+$ T cells. First, the 2 components of the activation protocol, IL-2 and PHA, were dissociated. IL-2, or its related γ_c cytokine, IL-15, alone were sufficient to induce some NKp30 expression, but the effect was modest compared with PHA and IL-2 (or PHA and IL-15) combinations (Figure 8A and not shown). On the other hand, PHA alone was not able to keep the cultures viable (data not shown), consistent with the critical role of γ_c cytokines in the survival of γδ T cells, particularly on activation and proliferation[304,317].

Although PHA has been a widely used T cell mitogen, it is also a nonphysiologic compound capable of cross-linking a series of surface receptors, including the TCR[339]. We hypothesized that the molecular mediator of PHA stimulation could be the $V\delta1^+$ TCR complex. We therefore compared the ability of PHA and the OKT3 mAb, which specifically cross-links CD3ε chains of the TCR complex, to induce NKp30 expression (when combined with IL-2 or IL-15) in $V\delta1^+$ T cells. OKT3 was fully capable of mimicking PHA in these assays (Figure 8A-B), thus inducing NKp30 in proliferating $V\delta1^+$ T cells (Figure 9). Moreover, TCRγδ blockade in PHA and IL-2 cultures prevented NKp30 induction (Figure 8C). These data suggest that PHA treatment provides TCR signals to induce NCR expression on $V\delta1^+$ PBL. Moreover, the differences between cytokine alone or combination treatments with OKT3 (or PHA) highlight a marked synergy between γ_c cytokine and TCR signals in this process (Figure 8A-B).

To further explore the molecular mechanisms of NCR induction, we used chemical inhibitors of key signal transduction pathways downstream of γ_c cytokine receptors and/or TCR signaling. Although blocking JAK signaling triggered extensive cell death before any NCR induction (not shown), coincubation with the PI-3K/ AKT inhibitor LY294002 specifically prevented NKp30 induction in proliferating $V\delta1^+$ T cells (Figure 8D). AKT is involved in transducing both γ_c cytokine and TCR signals[340], including TCRγδ signals[317]. By contrast, the MAPK/Erk inhibitor UO126 had no detectable effect on NKp30 induction in proliferating $V\delta1^+$ T cells (Figure 8D).

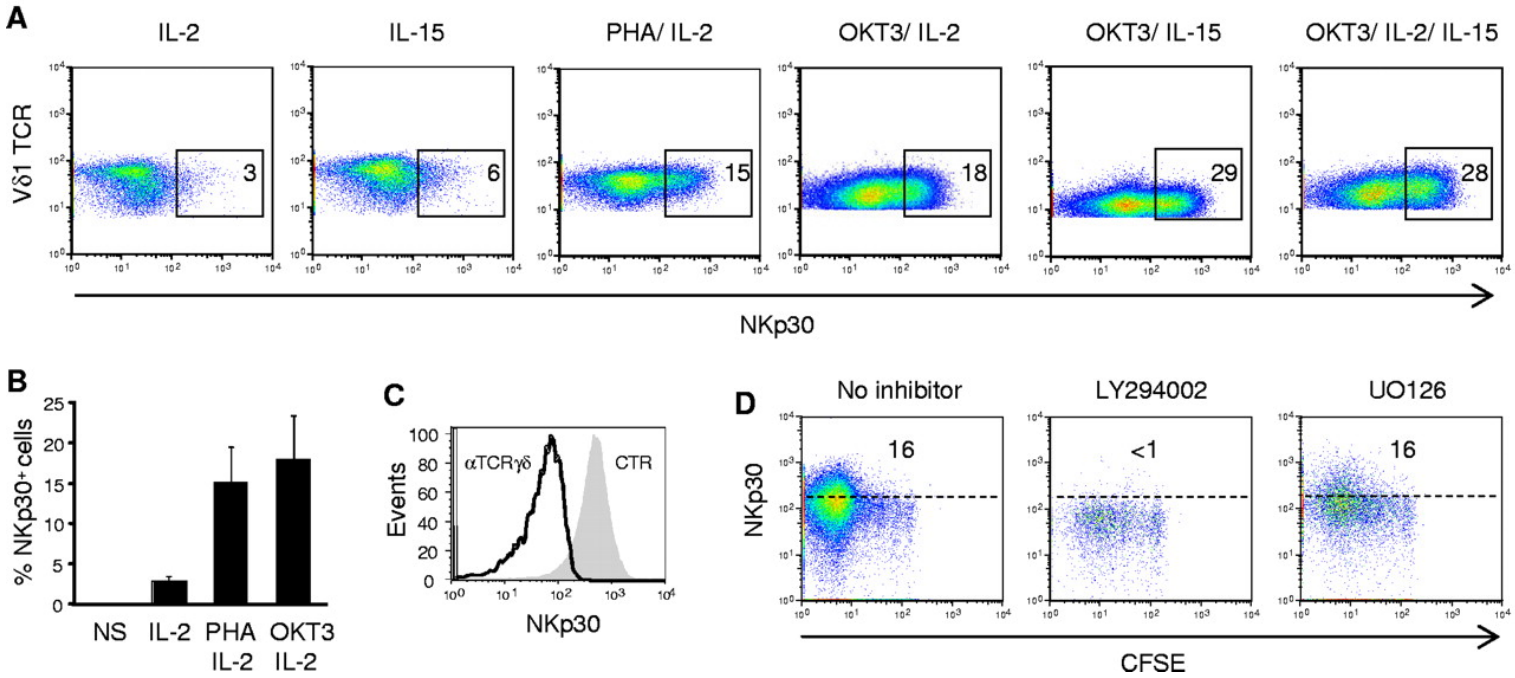


Figure 8. AKT-dependent γ_c cytokine and TCR signals induce NKp30 expression in Vδ1$^+$ T cells.

(**A-B**) Flow cytometry analysis of NKp30 expression on pregated Vδ1$^+$ T cells from γδ PBL cultures after 7 days in the presence of IL-2 or IL-15, alone or in combination with PHA or OKT3 (anti-CD3ϵ mAb). (**C**) Effect of blocking anti-TCRγδ mAb on NKp30 induction in PHA and IL-2–activated γδ PBLs. The shaded gray area is pregated NKp30$^+$ cells in 7-day control cultures. (**D**) Effect of chemical inhibitors LY294002 and UO126 on NKp30 induction in PHA and IL-2–activated γδ PBLs, prelabeled with CFSE. Data in this figure are representative of 2 to 3 independent experiments with similar results.

Importantly, the selective effect of LY294002 dissociated NCR induction from cell proliferation, thus demonstrating that Vδ1$^+$ T-cell proliferation is necessary (Figure 6C; Figure 9) but not sufficient (Figure 8D) to induce NKp30 expression. Collectively, these data demonstrate that AKT-dependent γ_c cytokine and TCR signals synergize to induce NKp30 expression in Vδ1$^+$ T cells.

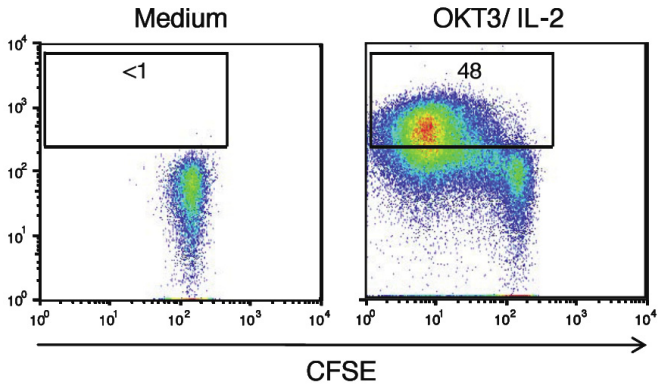


Figure 9. TCR plus IL-2 signals induce NKp30 expression in proliferating Vδ1$^+$ T-cells. NKp30 expression in Vδ1$^+$ T-cells, FACS-sorted from peripheral blood, labeled with CFSE and cultured with or without OKT3 mAb and IL-2 for 7 days.

4.4.5 Functional NKp30 and NKp44 trigger tumor cell killing by Vδ1$^+$ PBLs

Although the previous data established clear associations between NKp30 expression and increased cytotoxicity of γδ (Vδ1$^+$) PBL cultures, the functional role of NCRs in this system remained to be formally demonstrated. We therefore undertook gain-of function and loss-of-function experiments to evaluate the effect of NCR modulation on Vδ1$^+$ enriched (> 80%; Figure 6B) PBL cultures, which expressed NCRs at levels similar to those in Figure 6D (not shown). First, using a reverse Ab-dependent cytotoxicity assay, we showed that cross-linking of NKp30 or NKp44, but not NKp46, produced significant increases in lysis of the P815 tumor cell targets (Figure 10A). These data demonstrate that induced NKp30 and NKp44 are functional and mediate tumor cell killing.

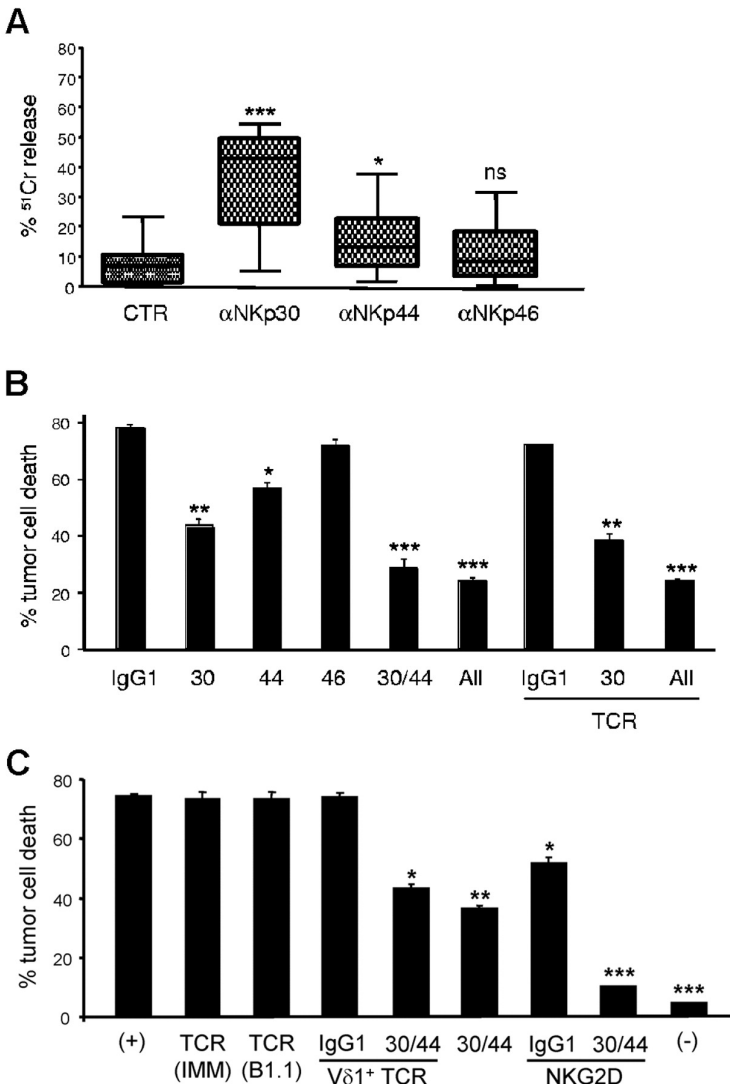


Figure 10. NKp30 and NKp44 mediate tumor cell killing by NCR$^+$ γδ PBLs.

(**A**) Functional evaluation of NKp30, NKp44 and NKp46 using specific monoclonal antibodies in a 4-hour ^{51}Cr release redirected killing assay (at 2:1 effector:target ratio) of the FcγR$^+$ P815 target cell line by γδ PBLs activated and expanded with PHA and IL-2. Data are presented as mean and SD of 8 independent experiments performed in triplicate (*P < .05, ***P < .001; ns = not statistically significant). (**B**) γδ PBLs activated and expanded for 18 days in PHA and IL-2 were incubated (at 5:1 effector:target ratio) for 3 hours with the leukemia cell line MOLT-4 (as in Figure 1). Effect of blocking antibodies to NKp30, NKp44, NKp46 and TCRγδ (IMMU510) on tumor cell killing. (**C**) Vδ1$^+$ PBLs were MACS-sorted after 20 days in PHA and IL-2 cultures for the assay described in (B), but using blocking antibodies to pan-TCRγδ (IMMU510 and B1.1), Vδ1$^+$ TCR (TCS1), NKG2D or NKp30 and NKp44, or the depicted combinations. (+) refers to control cultures without inhibitory antibodies; (-) refers to tumor cell cultures without Vδ1$^+$ PBLs. Error bars represent SD (n = 3, *P < .05; **P < .01; ***P < .001).

To assess if they played nonredundant roles in targeting leukemia cells, we performed receptor blockade experiments using NCR-specific mAbs (kindly provided by Dr A. Moretta, University of Genova, Italy). We observed significant reductions in tumor cell lysis on NKp30 and NKp44 blockade (Figure 10B). As expected from the results in Figure 10A, NKp46 blockade did not affect tumor cell killing. Interestingly, a synergistic effect between NKp30 and NKp44 was also clearly observed. Of note, TCR$\gamma\delta$ blockade in any setting (alone or in combination with anti-NCR mAbs) was a neutral event during the killing assay (Figure 10B).

To further establish the TCR-independence of NCR$^+$ Vδ1$^+$ PBL cytotoxicity, we isolated PHA-activated Vδ1$^+$ cells to very high purity (Figure 11) and used 3 different well-described anti-TCR blocking antibodies, including one (TCS1) specific for the Vδ1$^+$ TCR. Again, we observed no effect on leukemia cell killing (Figure 10C). By contrast, inhibition of NKG2D had a significant (Figure 10C) and dose-dependent (Figure 11C) impact on tumor lysis.

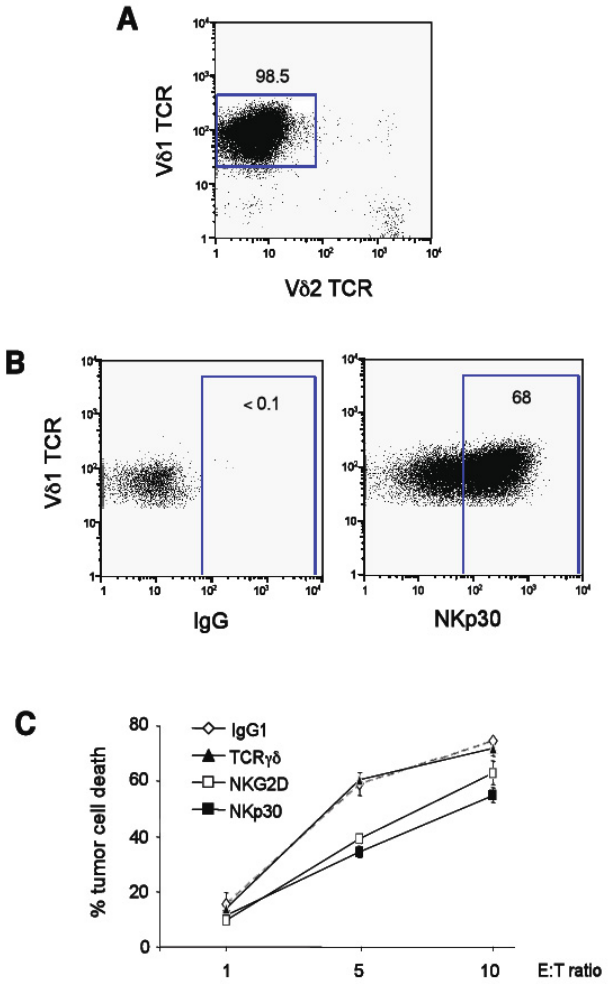


Figure 11. Dose-dependent inhibitions of NKp30 and NKG2D impact on leukemia cell recognition by NCR$^+$ Vδ1$^+$ PBLs.

MACS-sorted (negatively-selected) $\gamma\delta$ PBLs were cultured for 20 days in PHA/ IL-2 and then positively-selected by MACS with Vδ1 TCR beads, followed by 3 days of activation in PHA/ IL-2. (**A-B**) Phenotype of the NCR$^+$ Vδ1$^+$ cells used in the assays. (**C**) Percentage of dead MOLT-4 leukemia cells after 3 hrs of co-incubation with NCR$^+$ Vδ1$^+$ PBLs at various effector: target ratios, in the presence of the indicated mAbs. Error bars represent SD (n=3).

In fact, when we combined NCR and NKG2D inhibition, NCR$^+$ Vδ1$^+$ PBLs could not kill above background levels (Figure 10C). These data suggest that leukemia cell targeting by NCR$^+$ Vδ1$^+$ PBLs is a TCR-independent event mostly mediated by the synergistic function of NKp30, NKp44 and NKG2D.

4.4.6 NKp30$^+$ Vδ1$^+$ PBL are specialized killers that target resistant primary lymphocytic leukemias

To fully characterize the antitumor potential of NCR$^+$ Vδ1$^+$ PBL, we used FACS to sort NKp30$^+$ cells to a high degree of purity (> 99%; Figure 12A) and performed a series of functional assays. As expected (Figure 6D), sorted NKp30$^+$ cells also expressed NKp44 and NKp46 (Figure 12B), and the 3 NCRs were largely stable on the surface of the purified cells when cultured for 2 weeks with IL-2 alone (Figure 12C). These data demonstrate the feasible expansion of a stable NCR$^+$ Vδ1$^+$ T cell subset.

When the cytotoxic function of NKp30$^+$ cells was assessed, an increased targeting of the resistant leukemia cell line Bv173 (among others; not shown) was observed (in comparison with NKp30$^-$ counterparts; Figure 12D). This correlated with higher expression of granzyme B (Figure 12E). Moreover, NKp30 expression also associated with higher degree of CD56 expression (Figure 13), which has been previously linked to cytotoxicity of human lymphocytes, including Vδ2$^+$ T cells[341].

Finally, we performed functional killing assays with primary samples obtained from B-cell chronic lymphocytic leukemia patients. We previously showed that such specimens are considerably resistant to γδ PBL activated and expanded with the specific Vγ9Vδ2 TCR agonist HMB-PP[333], a finding confirmed in this study (Figure 12F-G). Importantly, HMB-PP and IL-2–activated γδ PBLs do not express NCRs (Figure 3B). We therefore compared their antitumor cytolytic activity with that of NKp30$^+$ cells isolated from γδ PBL cultures activated with PHA and IL-2. We observed that NKp30$^+$ γδ PBLs, obtained from 6 different donors, were consistently more efficient at eliminating primary B-CLL cells (Figure 12F-G). These data collectively suggest that highly cytotoxic NKp30$^+$ Vδ1$^+$ PBL are promising new candidates for adoptive cell immunotherapy of hematologic malignancies.

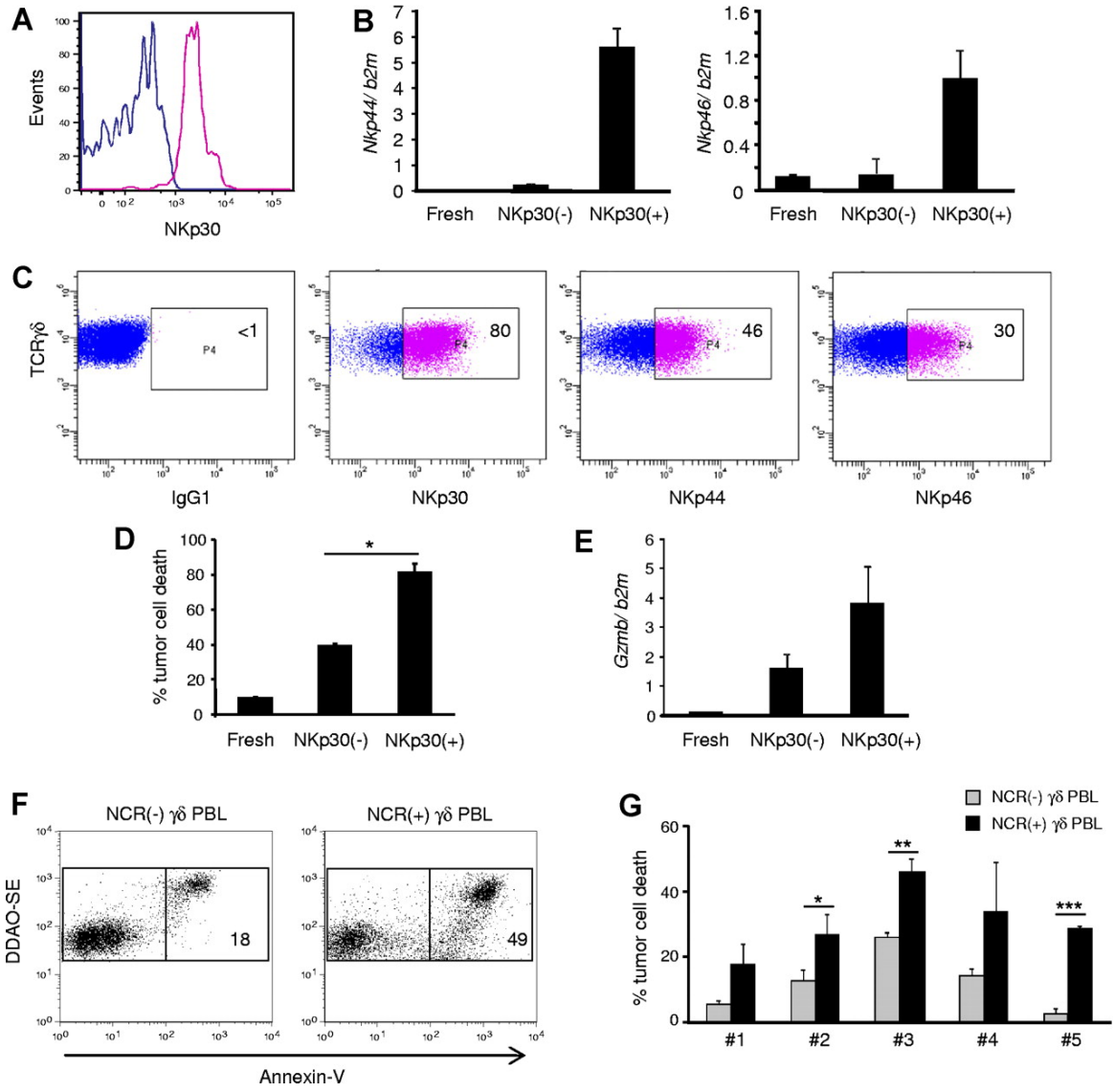


Figure 12. NKp30$^+$ γδ PBLs are a stable subset endowed with enhanced cytotoxicity against chronic lymphocytic leukemia cells.

NKp30$^+$ and NKp30$^{(-)}$ γδ PBLs were FACS-sorted from 14-day PHA and IL-2–activated cultures. (**A**) Reanalysis of NKp30 expression in the purified populations. (**B**) Real-time PCR quantification of *Nkp44* (left) and *Nkp46* (right) mRNA levels in NKp30$^-$ or NKp30$^+$ γδ T cells, compared with freshly isolated γδ PBLs. Error bars represent SD (n = 3). (**C**) Sorted NKp30$^+$ γδ PBLs were cultured in the presence of IL-2. Analysis of NKp30, NKp44 and NKp46 expression after 14 days. (**D**) NKp30$^-$ or NKp30$^+$ γδ T cells, or freshly isolated γδ PBLs, were used in killing assays with the leukemia cell line Bv173 (as in Figure 1). Tumor cell death was evaluated by Annexin-V staining (n = 3, **P* < .05). (E) Real-time PCR quantification of *GzmB* mRNA levels in freshly isolated, NKp30$^{(-)}$ or NKp30$^+$ γδ T cells. Error bars represent SD (n = 3). (F-G) Representative plots (**F**) and data summary (**G**) for 5 primary B-cell chronic lymphocytic leukemia samples that were used in killing assays (as in Figure 1) with γδ PBLs

obtained from 6 distinct donors and activated with either HMB-PP and IL-2 or PHA and IL-2. NCR(-) γδ PBL from HMB-PP and IL-2–activated cultures (gray bars) were compared with NCR(+) γδ PBL from PHA and IL-2–activated cultures (black bars). Error bars represent SD (n = 6, **P* < .05; ***P* < .01; ****P* < .001).

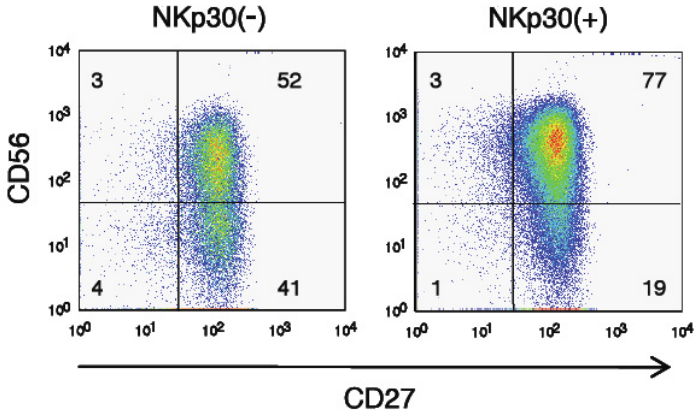


Figure 13. Increased expression of CD56 in NCR^{+} Vδ1^{+} PBLs.

Flow cytometry data for CD56 and CD27 expression in Vδ1^{+} PBLs activated with PHA/ IL-2 for 19 days and gated on either NKp30$^{(-)}$ or NKp30^{+} cells.

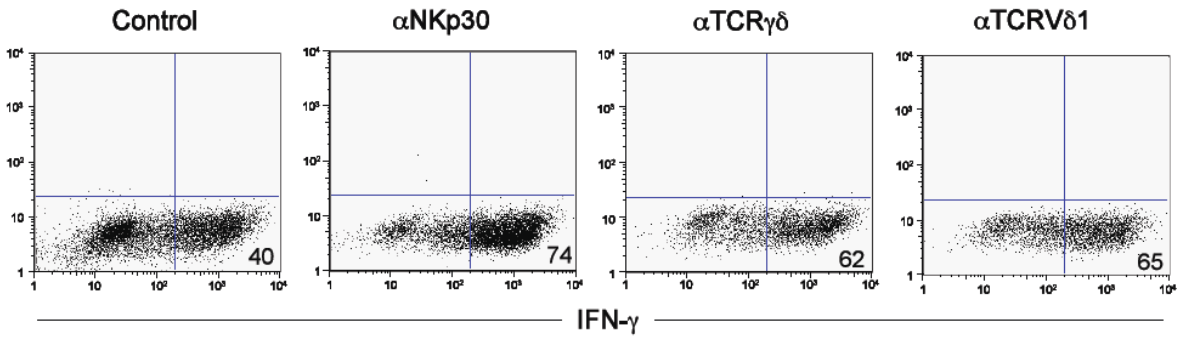


Figure 14. NKp30 triggering promotes IFN-γ production by NCR^{+} Vδ1^{+} PBLs.

MACS-purified Vδ1^{+} PBLs were activated with PHA/ IL-2 for 5 days and then stimulated with the indicated plate-bound mAbs in the presence of IL-2. Control refers to IgG1 mAb. Cells were stained intracellularly for IFN-γ and analyzed by flow cytometry.

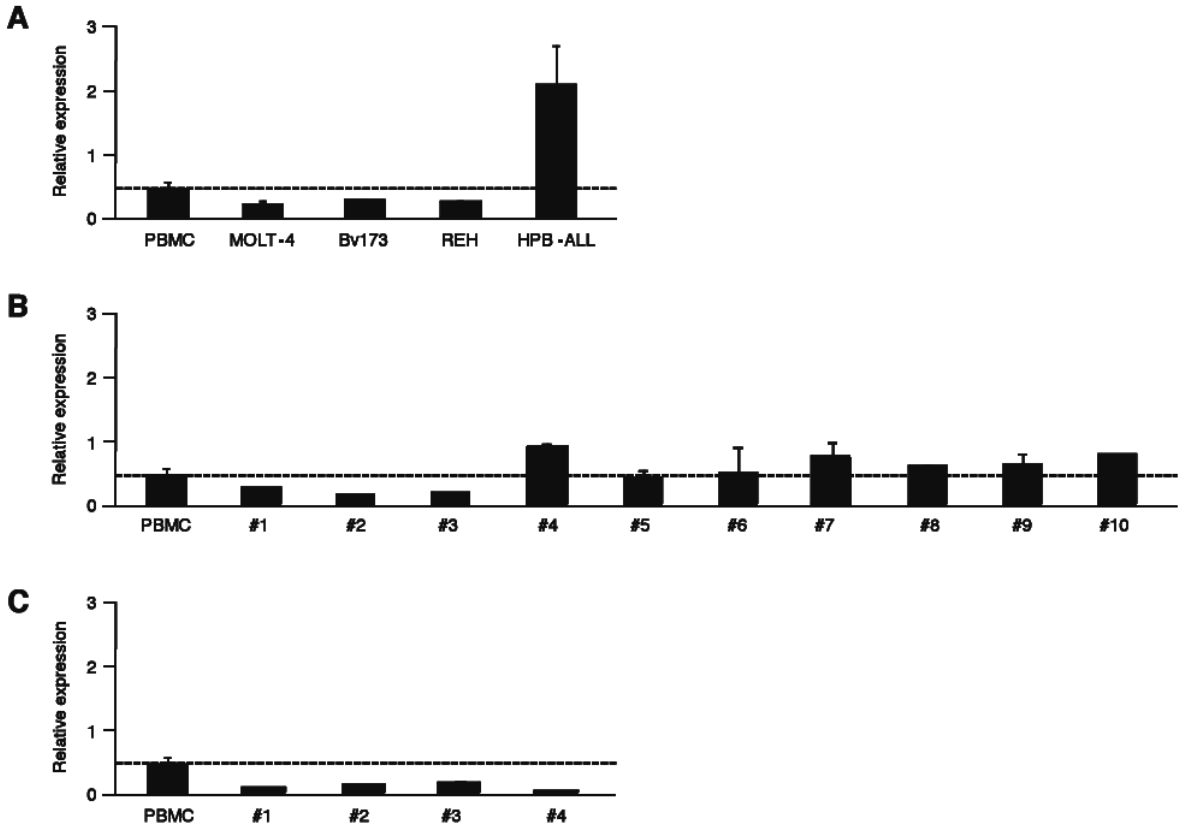
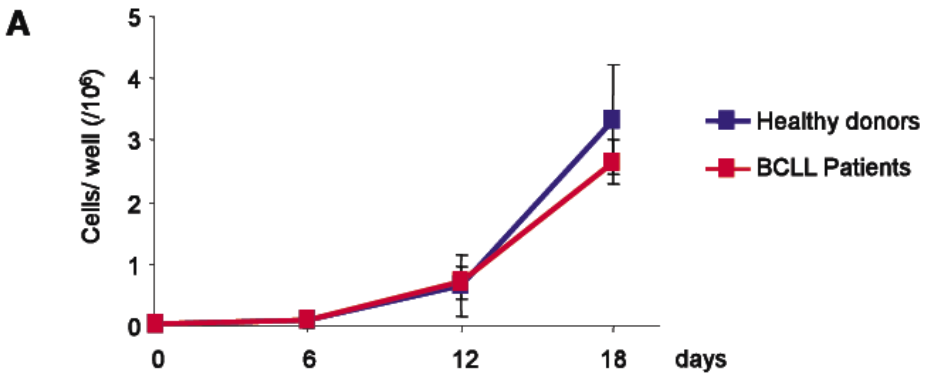


Figure 15. B7h6 is not overexpressed in most lymphoid leukemia samples.

Real-time quantitative PCR data for B7h6 expression on (**A**) acute lymphoblastic leukemia cell lines; (**B**) T-cell acute lymphoblastic leukemia patient samples; (**C**) B-cell chronic lymphocytic leukemia patient samples. Healthy fresh PBMC are shown as reference (dashed line). B7h6 levels were normalized to the housekeeping genes *Gusb* and *Psmb6* and are expressed in arbitrary units. Error bars represent SD of triplicate measurements.



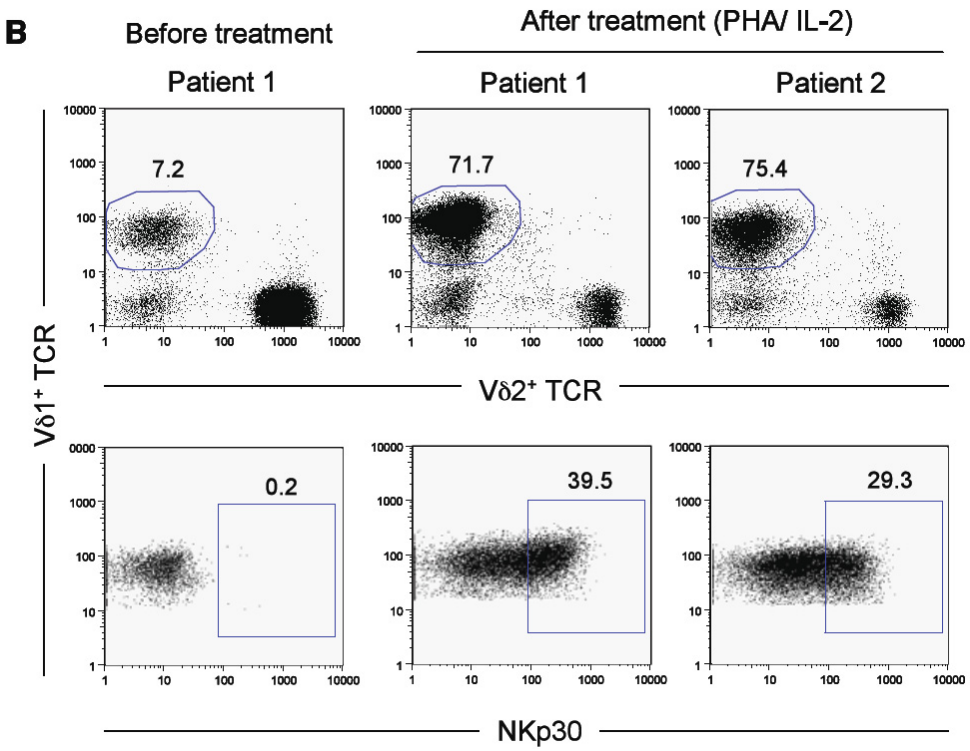


Figure 16. NCR$^+$ Vδ1$^+$ T-cells can be efficiently expanded from PBLs of B-CLL patients.

MACS-sorted γδ PBLs from healthy donors or B-CLL patients were cultured in PHA/ IL-2 (as described in Figure 1), and analyzed for total cell numbers (**A**); and Vδ1 TCR and NKp30 expression at day 18 (**B**). Error bars represent SD (n=3 for each group).

4.5 Discussion

Natural cytotoxicity receptors were identified by A. Moretta and colleagues over a decade ago, and were shown to play critical synergistic roles in the antitumor functions of NK cells[93-96]. In fact, NKp30 and NKp46 are widely considered to be 2 of the most specific NK markers[93-96]. We now show that the combination of cytokine (IL-2 or IL-15) and mitogenic (PHA or OKT3) stimuli induces NCR expression in a sizeable Vδ1$^+$ PBL subset that is endowed with increased cytolytic activity against hematologic tumors. Although PHA is a nonphysiologic T-cell mitogen, we demonstrated that its effect on NCR induction was fully mimicked by crosslinking the TCR-CD3 complex on Vδ1$^+$ PBL. Thus, NCR induction is coupled to TCR-mediated

proliferation of Vδ1$^+$ cells, while also requiring γ_c cytokine signals. This is consistent with previous reports demonstrating that the in vitro acquisition of NK receptors by liver[342] or umbilical cord[102] T cells depends on IL-15.

Among inducible NCRs, NKp30 is clearly the most important for the antitumor activity of Vδ1$^+$ T cells, based on the proportion of cells that express it (Figure 6D), the higher enhancement in Vδ1$^+$ T-cell cytotoxicity on NKp30 triggering (Figure 10A), and the significant reduction in leukemia cell killing on NKp30 blockade (Figure 10B). This notwithstanding, NKp44 (but not NKp46) is also functional in NCR$^+$ Vδ1$^+$ cells (Figure 10A), and appears to synergize with NKp30 for enhanced tumor targeting (Figure 10B). Of note, NKp30 engagement also augments the production of the key antitumor cytokine, interferon-γ, by NCR$^+$ Vδ1$^+$ cells (Figure 14).

Both NKp30[117] and NKp44[123] have been implicated in human NK cell recognition of virus-infected cells. Regarding tumors, antibody-mediated blocking experiments demonstrated important roles for these receptors in myeloma[343] and melanoma[226] cell targeting. Moreover, lack of NCR expression has been clinically correlated with poor survival in AML patients[108].

Interestingly, NKp30 and NKp44 are not encoded in the genome of murine strains (such as C57Bl/6 or Balb/c) widely used for laboratory experimentation[113]. On the other hand, the major Vγ9Vδ2 subset of human PBL, and its reactivity toward phosphoantigens, are also primate-specific[299]. These observations highlight the special functional characteristics of primate γδ T cells; however, they also preclude the direct in vivo study of Vγ9Vδ2 or NKp30$^+$ Vδ1$^+$ T cells in the mouse.

Another aspect that currently limits our understanding of NCR function in immunity is the poor definition of their physiologic ligands, most notably in the context of tumors. In fact, viral hemagglutinin was until recently the only well-established ligand for NKp44[121] and NKp46[128]. This notwithstanding, a novel B7 family member, B7-H6, was lately described to bind NKp30[120]. B7-H6 was not detected in normal human tissues but was expressed on human tumor cells[120], in line with the recognition of "stressed self" by innate and innate-like lymphocytes[22,344]. However, we obtained no evidence for a role of B7-H6 in NCR$^+$ Vδ1$^+$ T-cell recognition of lymphoid leukemias; in fact, many of the NCR$^+$ Vδ1$^+$ T-cell targets expressed lower levels of B7-H6 than healthy control cells (Figure 15). Brandt *et al.* had also previously noted that only 24 of 119 tumor cell lines tested expressed B7-H6[344]. Thus, future lines of research should clarify the repertoire of relevant ligands

expressed by tumors susceptible to NKp30-mediated cytotoxicity. Moreover, although we have thus far concentrated on hematologic malignancies, upcoming work will address whether NKp30$^+$ Vδ1$^+$ lymphocytes also possess enhanced cytotoxicity against solid tumors. Of note, Vδ1$^+$ T cells have previously been shown to be cytolytic against melanoma and various carcinomas[251].

Vδ1$^+$ T cells are the predominant γδ T-cell subset during the fetal stage and early life[345], when they are already able to respond to viral infection[346]. In adults, Vδ1$^+$ T-cell expansions have been associated with CMV infection[154], HIV-1 infection[347], and tumors of either epithelial[54,334,335] or hematopoietic[348,349] origin. An attractive prospect for adoptive transfer of activated Vδ1$^+$ T cells is that they may display particularly good capacity for homing to tissues because, contrary to their circulating Vδ2$^+$ counterparts, Vδ1$^+$ cells are preferentially tissue-associated lymphocytes[54]. Interestingly, the abundance of Vδ1$^+$ T cells at mucosal surfaces has been attributed to IL-15, which induces chromatin modifications that control TCR gene rearrangement[350].

Our key demonstration that NKp30$^+$ Vδ1$^+$ cells are capable of targeting primary lymphoid leukemic cells is particularly relevant when taking into account that Vδ1$^+$ T cells have been previously reported to be inefficient killers of primary leukemia or lymphoma cells[348,349], which has been attributed to their lack of expression of NKG2D ligands[348]. Interestingly, a recent study in which γδ PBLs were activated with concanavalin A demonstrated higher killing ability of expanded Vδ1$^+$ cells against B-CLL–derived cell lines[351]. It will be important to investigate whether this protocol also induces NKp30 expression on Vδ1$^+$ cells. Thus, NKp30$^+$ Vδ1$^+$ cells may provide a valuable layer of intervention against lymphoid malignancies.

A surprising finding that deserves further investigation is the preferential expansion of Vδ1$^+$ T cells (among γδ PBL) on PHA treatment in vitro (Figure 6B). Because this is not due to selective apoptosis of the dominant Vδ2$^+$ counterparts (Figure 4C), it must derive from a proliferative advantage of Vδ1$^+$ cells when receiving PHA-dependent TCR signals (Figure 6A-B). Provocatively, we have previously observed that Vδ1$^+$ T cells express significantly higher levels of the CD27 receptor (compared with Vδ2$^+$ cells)[232]; CD27 costimulation enhances *Bcl2a1* and *Cyclin D2* expression and promotes γδ T-cell survival and proliferation[232].

This study draws important novel (NCR-mediated) insight into the modus operandis of human γδ T cells, and supports the emerging paradigm of γδ T cells recognizing tumors via innate NK receptors rather than using the somatically rearranged TCRγδ (Figure 10B-C)[299]. This notwithstanding, the TCR has a major, albeit indirect, contribution to the antitumor function of NKp30$^+$ Vδ1$^+$ cells. We showed that efficient induction of NKp30 expression on Vδ1$^+$ cells depends on TCR stimulation; in its absence, γ_c cytokines can only effect a very modest up-regulation of NKp30 expression (Figure 8A-B). Thus, TCR signals are upstream of NKp30-mediated tumor cell recognition by NKp30$^+$ Vδ1$^+$ lymphocytes. Interestingly, we have previously showed that, for Vδ2$^+$ cells, TCR signals are essential for cell activation and cytotoxic differentiation, "upstream" of tumor cell recognition via NKG2D.[2] We therefore propose a "2-step" model for human γδ T cells, in which they differentiate and are activated like prototypic T cells (ie, using the TCR), but rely essentially on NK receptors (such as NKG2D, NKp30 or DNAM-1) for tumor cell recognition. This is consistent with the critical role described by the Hayday and Girardi groups for NKG2D ligands in tumor surveillance by mouse γδ T cells[11,332] and fits the general concept of NK receptors being the key molecular recognition determinants of "oncogenic stress"[344].

From a clinical perspective, this study describes a protocol to induce NKp30 ex vivo, which should make it very feasible to expand and inject large numbers of cells into patients. Importantly, we were able to efficiently expand NKp30$^+$ Vδ1$^+$ cells from B-CLL patients (Figure 16). On reinfusion, the activation status of the cells could potentially be maintained via administration of low doses of IL-2, which appears to be sufficient to sustain NKp30 expression. Thus, after this first report describing the differentiation of NKp30$^+$ Vδ1$^+$ lymphocytes, future work should evaluate their potential for adoptive cell immunotherapy of human cancer.

5. CONCLUSIONS AND FUTURE PERSPECTIVES

In this thesis, we have characterized the molecular mechanisms that control human γδ T cell activation and tumor cell recognition, as a key strategy to understand γδ T cell function, and to manipulate it in cancer immunotherapy.

5.1 PHOSPHOANTIGEN RECOGNITION BY Vγ9Vδ2 T CELLS

In chapter 2 we described the molecular mechanisms responsible for HMB-PP-mediated activation of human Vγ9Vδ2 T cells, since their unique responsiveness to non-peptidic prenyl pyrophosphate antigens is not well understood. These pyrophosphate molecules are so distinct from conventional peptides and superantigens, that it was difficult to explain how these substances could induce Vγ9Vδ2 T cell proliferation via TCR[56]. Although early studies reported that transfection of TCR$^-$ Jurkat T cells with cDNA constructs encoding a Vγ9Vδ2 TCR enabled the transfectants to produce IL-2 in response to Daudi cells and mycobacterial extract[166,310], these difficult experiments were not widely reproduced. It seemed clear that phosphoantigen-induced activation of T cells required expression of a Vγ9Vδ2 TCR, as indicated by antibody blocking approaches[352]. However, phosphoantigen recognition by Vγ9Vδ2 T cells required neither the expression of classical MHC class I, MHC class II, MICA/MICB, CD1 molecules, nor MHC class I or class II peptide loading pathways[165,191].

Previous studies had reported a very slow kinetics of activation of TCR-associated signal transduction pathways by IPP[244] and BrH-PP[271], seemingly incompatible with direct binding of these phosphoantigens to the Vγ9Vδ2 TCR. Lafont *et al*. showed that IPP treatment induces, except for the proximal triggering of p56 Lck, a significantly delayed phosphorylation kinetic of the signaling proteins Zap-70, PI3K, LAT, Erk1/2 and p38[244]. In contrast, Cipriani *et al*. described a more rapid activation of signaling proteins such as PKCθ and MAPK on γδ T cells after IPP stimulation[353]. To complicate things further, all attempts to show cognate interactions between recombinant, soluble Vγ9Vδ2 TCRs and phosphoantigens had failed at that time[25,301]. Moreover, most of these experiments were done using phosphoantigens with weak bioactivities. Real-time monitoring of Vγ9Vδ2 T cells following exposition

to weak and mid-range phosphoantigen agonists showed a time- and dose-dependent down-modulation of the γδ TCR, whereas strong phosphoantigen agonists induced little or no TCR down-regulation[185]. This indicated that γδ TCR down-modulation does not match the extent of TCR signaling as assessed by microphysiometry or conventional effector responses (TNF-α production and cytotoxicity)[185]. In any case, there are some contradictory data, since in some studies, IPP (a weak agonist) does not induce down-modulation of TCR/CD3 complex[244], whereas more potent phosphoantigens can induce it (David Vermijlen, personal communication).

We have studied the most potent natural phosphoantigen yet identified, HMB-PP, and examined its effects on γδ T-cell activation and anti-tumor properties. We showed that HMB-PP stimulation recruits the signal transduction machinery employed by the γδ TCR. In fact, HMB-PP activates MEK/Erk and PI-3K/Akt pathways as rapidly as OKT3 (α-CD3ε mAb), and induces an almost identical transcriptional profile in Vγ9$^+$ T-cells. Moreover, MEK/Erk and PI-3K/Akt activities are indispensable for the cellular effects of HMB-PP, including γδ T-cell activation, proliferation and anti-tumor cytotoxicity, which are also abolished upon antibody blockade of the Vγ9$^+$ TCR. Our data is therefore consistent with direct binding of HMB-PP to Vγ9Vδ2 TCR, but a physical interaction was not yet demonstrated.

Our hypothesis for a TCR-dependent mechanism of recognition of phosphoantigens was supported by parallel studies performed by other groups on the extracellular dynamics of HMB-PP signaling. It has been shown that HMB-PP has the capacity to induce the formation of high-density Vγ9Vδ2 TCR nanoclusters on the surface of human γδ T cells[218], that could serve as platforms for intracellular signaling. Additionally, a newly-developed tetramer reagent for the Vγ9Vδ2 TCR of rhesus macaques was reported to bind to HMB-PP loaded on the surface of human APC[302].

Recent data provided novel evidence for the existence of an antigen-presenting molecule for prenyl pyrophosphates[354,355]. Wang *et al.* found that residues in all CDRs of the Vγ9Vδ2 TCR can affect recognition, which is critically dependent on the invariant Vγ9Jγ1.2 chain. Given the large footprint of the required residues, which surpasses the predicted prenyl pyrophosphate-binding site, it is likely that a presenting molecule is required for prenyl pyrophosphate antigens[354]. Prenyl pyrophosphate recognition is primarily defined by germline-encoded regions of the

γδ TCR, allowing a high proportion of Vγ9Vδ2 TCRs to respond[354]. Interestingly, cord-blood –derived human γδ T cells do not respond to phosphoantigens[356], and although HMB-PP reactivity is present at birth[357] it does not elicit proliferation and IFN-γ production in neonatal Vγ9Vδ2 T cells, unless at high concentrations[358]. This could suggest the lack of an antigen-presenting molecule at birth. It was also shown that HMB-PP or photoaffinity analogues of HMB-PP (meta/para-benzophenone-(methylene)-prenyl pyrophosphates), can be crosslinked on the surface of tumor cell lines and be presented as antigens to γδ T cells[359]. The capability of APC to present HMB-PP for recognition by Vγ9Vδ2 TCR was diminished after protease treatment of APC[302]. Adding to this, F1-ATPase was recently shown to display properties characteristic of an antigen presentation molecule for Vγ9Vδ2 T cells[355]. These results do not exclude the possibility that phosphoantigens can both be presented to, and directly bind γδ TCR. The requirement for cell-cell contact should be re-defined in new and carefully designed experiments. In Western Blot experiments (Figure 3, chapter 2), we re-suspended 5 million γδ T cells cells in 1ml PBS before adding 1nM HMB-PP. The cells were highly diluted, and cell-cell contact was thus reduced during the first 7 minutes of stimulation (at this time point, most kinases activated), and this may suggest direct binding of HMB-PP to Vγ9Vδ2 TCR.

It also seems possible that all phosphoantigens are capable of physically interacting, albeit with different affinities, with the Vγ9Vδ2 TCR. The delayed signalling events reported after IPP[244] (Figure 4, chapter 2) and BrH-PP[271] stimulation may occur downstream of the TCR ligation, and could be related with different abilities of these compounds to induce conformational changes on the TCR/CD3 complex.

In our studies, HMB-PP treatment does not induce TCR internalization, and thereby sustains γδ T-cell activation upon re-stimulation. In fact, this was the most obvious difference compared with OKT3-mediated cell activation, and it may explain why 10nM of HMB-PP were able to further increase the abundance of activated $Vγ9^+$ T-cells, to above 80% after 48h (Figure 1A, chapter 2), thus suggesting that phosphoantigens can maximize anti-tumor γδ T-cell responses.

5.2 Vγ9Vδ2 TCR SIGNALING

In recent years we improved our understanding of the pathways involved in Vγ9Vδ2 TCR signaling (reviewed in[29]). The use of pharmacological inhibitors has pointed out the importance of p38, Erk1/2 and PKCα/β pathways during the establishment of TNF-α and IFN-γ responses. Moreover, Erk1/2 and PI3K (which is activated following Vγ9Vδ2 TCR engagement whereas its activation in αβ T cells is mainly under control of the co-receptor CD28) are involved during cytokine production and cytotoxic activity (Nedellec *et al*.[29] and chapter 2).

Our work has thus contributed to these advances. In fact, we have observed that, without the effect of IL-2, all the major cellular effects of HMB-PP, i.e., γδ T cell activation, proliferation, Th1 cytokine secretion and anti-tumor cytotoxicity, are strictly dependent on Erk and Akt-mediated signal transduction.

However, an issue that deserves further analysis is the role played by IL-2 (or IL-2-related cytokines) in these processes. γδ T cells are poor producers of IL-2[360]. As so, phosphoantigen-treated isolated peripheral blood γδ T cells cannot efficiently activate or sustain proliferation and cytokine production, unless they receive exogenous IL-2 (or related cytokines)[304,308]. Cytokine production and cell division are not sustained by treating cells repeatedly with HMB-PP or OKT3, if in the absence of exogenous IL-2 (Figure 2A, chapter 2). In fact, HMB-PP treatment alone can induce transient proliferation only when Vγ9Vδ2 cells are surrounded by IL-2 producing PBL (Figures 1D and 1E, chapter 2). Surprisingly, it seems that IL-2 stimulation alone can induce most of the effector functions of Vγ9Vδ2 T cells, including complete cell activation, proliferation and cytotoxic functions, thus overriding the impact of TCR signaling. In terms of cell activation, IL-2 alone can reproduce the effects of the co-addition of IL-2 and HMB-PP, with 90% of cells expressing $CD69^+$ in 48h (data not shown). The impact of TCR signaling on Vγ9Vδ2 cytotoxicity against tumor cells is also very limited when IL-2 is present (Figure 5D, chapter 2 and data not shown). In fact, even the synergistic effect of HMB-PP and IL-2 on Vγ9Vδ2 T cell proliferation (Figure 1D, chapter 2) is only seen upon stimulation of fresh/resting cells. When cultured with IL-2 alone *in vitro*, γδ T cells go through a lag-phase in cell proliferation rate (for about 8-9 days), but then acquire the ability to proliferate at very high rate levels (data not shown). Thus, while phosphoantigens are critical for Vγ9Vδ2 T cell enrichment *in vitro* and *in vivo* (cancer clinical trials), their functional (effector) differentiation may be mostly driven by IL-2. This deserves further investigation

By contrast to this view, other groups have suggested that IL-2 signaling works merely as a modulator of the critical TCR signals. The expansion of γδ T cells was shown to be dependent on TCR-triggered activation of both PKCα/β and PKCθ, which in turn can upregulate the expression of CD25 (IL2Rα, α-chain unit of IL-2 receptor)[353]. In Table 1 of microarray gene analysis (chapter 2), we can also see that HMB-PP or OKT3 stimulation highly induces CD25 expression. However, low amounts of IL-2 itself are also able to induce IL2Rα expression, without the "help" of TCR signalling. In fact, the upregulation of CD25 at the γδ T cell surface was shown to be required to obtain a strong TCR signal after phosphoantigen/OKT3 stimulation[244].

Surprisingly, in a recent report, IL-2 signals alone were able to activate MEK/Erk and PI-3K/Akt pathways, but failed to induce cytokine expression or cytotoxicity on Vγ9Vδ2 T cells[361]. According to this study, MEK/Erk and PI-3K/Akt activation are necessary but not sufficient to induce effector responses in Vγ9Vδ2 T cells and a TCR-dependent signal is still required for tumor cell killing. A calcium-dependent response, normally triggered by phosphoantigen addition, was required for all effector activities, and the function of IL-2 was to simply increase the magnitude of these responses. It is possible that the observed differences may be consequence of a small delay in IL-2-induced effector responses, compared to phosphoantigen-triggered responses, since cytokine production, γδ cell degranulation and cytotoxicity assays were analysed after a very short period (4h) of IL-2 or IPP-stimulation of fresh Vγ9Vδ2 T cells and 1 week-starved, IL-2 expanded Vγ9Vδ2 T cell lines[361]. In our studies, the effective killing of susceptible tumor cell lines depends, in first place, on sufficient levels of γδ T cell activation. Fresh γδ T cells (and fresh NK cells[92]) do not efficiently kill tumor targets, and the ability to kill these targets increases linearly with time, upon incubation of γδ T cells with IL-2. At least 12h of pre-incubation with IL-2 is needed for Vγ9Vδ2 T cells to kill 90% of DAUDI and MOLT-4 cells in cytotoxicity assays (Figure 3, chapter 3).

In a recent collaboration with Julie Ribot, in our laboratory, we have observed that that IL-2 alone can differentiate unresponsive human γδ thymocytes towards cytotoxic and effector functions *in vitro*. After incubation with IL-2 (in the absence of TCR agonists), γδ thymocytes acquired the capacity to produce IFN-γ, TNF-α, and displayed cytotoxic functions (unpublished data). This notwithstanding, it is important

to consider that TCR-mediated signals during thymic development may "pre-program" human γδ T cells, like they do in mice[362], with IL-2 being subsequently required for functional maturation of human γδ T cells in the periphery. We are currently exploring this hypothesis in our laboratory.

These combined data support the notion that Vγ9Vδ2 TCRs mainly act as biological "sensors" of microbial (and possibly tumoral) metabolites, that can be very important in the immune response, as initiators of γδ T cell proliferation. The initial TCR signals turn the cell more sensitive to exogenous IL-2 (upon CD25 upregulation) and rapidly synergise with IL-2 signals, inducing strong MEK/ERK and PI3K/Akt signalling that translates in immediate cell proliferation. Cell activation and effector functions (which may require lower levels of MEK/ERK and PI3K/AKt phosphorylation) can be further sustained by IL-2 signals alone. This is consistent with the higher number of signalling events triggered upon IL-2 stimulation in Vγ9Vδ2 T cells[363] (and NKT cells[364]), compared to those produced by IL-2 in NK cells and αβ T cells[363]. This hypothesis is also supported by *in vivo* studies. Casetti et al. reported that the sole i.v. administration of phosphoantigens in *Macaca fascicularis* failed to induce cell activation and proliferation. They conclude that IL-2 administration is a strict requirement for Vγ9Vδ2 T cell expansion in vivo[304]. Moreover, only γδ, but not αβ T cells, expressed the CD69 activation marker shortly after IL-2 treatment, suggesting that γδ T lymphocytes may be more sensitive to low doses of s.c. IL-2 than αβ T cells[304]. As mentioned above, we are currently dissecting the roles of IL-2 signaling in human γδ T cell differentiation and activation, from the thymus to the peripheral blood.

5.3 TUMOR CELL RECOGNITION BY Vγ9Vδ2 T CELLS

Phosphoantigen-activated Vγ9Vδ2 T cells can kill various solid tumor cell lines[258] and a particularly large number of hematopoietic cell-derived tumors[255,263,283,317]. However, many tumor cell lines (and most tumor primary biopsies) are resistant to Vγ9Vδ2 T cell-cytotoxicity. Daudi and Raji cell lines derived from Burkitt´s lymphomas have long been used, respectively, as paradigms of susceptible and resistant targets to γδ T cell-mediated killing[183,264-267], both in vitro[265,267,273,277,365] and in vivo[277]. However, the molecular basis of these two distinct susceptibilities has remained unclear. It was first demonstrated a role for Natural Killer inhibitory

receptors (mainly CD94/NKG2A, that binds to HLA-I) in γδ T cell-cytotoxicity[167,268-270]. Contrary to Raji cells, Daudi cells do not express MHC class I on their surface because of the lack of functional β2m. Ditrud (a Daudi-Raji hybrid)[366] and Daudi-β2 (β2m-transfected Daudi)[168] induced a lower stimulation of Vγ9Vδ2 T cells, indicating a negative regulation by HLA-class I antigens on Vγ9Vδ2 T cells. However, the dominant susceptible phenotype of Ditrud and of certain MHC-I$^+$ Raji variants to Vγ9Vδ2 T cell-killing demonstrated that a stimulatory phenotype could be acquired by some tumors independently of the loss of class I antigens, and indicated a role for a non-MHC class I molecule[267]. An alternative explanation for tumor cell recognition was later proposed. According to these studies, Vγ9Vδ2 T cells recognize tumor targets through TCR interaction with phosphoantigens endogenously produced by tumor cells, and use NKR signals to fine-tune the cell activation threshold[148,192,208,271,272]. In fact, the recognition of some tumor cell lines, such as Daudi and K562, by Vγ9Vδ2 T cells, correlated with IPP production[184] and F1-ATPase/ApoA-I expression[207] on tumor cells. Furthermore, Vγ9Vδ2-mediated lysis of B-LCL or Raji cells was not enhanced by MHC class I/class Ib knockdown, but these cells showed very low expression of phosphoantigens and F1-ATPase/ApoA-I[207]. However, this is a controversial issue, since correlations of MHC class I expression levels, or endogenous phosphoantigen production on tumor targets, do not always predict susceptibility to γδ T cell-killing. For example, MHC class I expression can be present in both resistant and susceptible hematopoietic tumors (chapter 3), and the expression of F1-ATP-synthase is very high is some γδ-resistant cell lines and very low in some susceptible cell targets (our data not shown). Moreover, conflicting results have been reported concerning the role of γδTCR and NKG2D in recognition of tumor cell targets. Concerning hematological tumors, TCR was shown to be important for the killing of Daudi[273] cells, but not of MOLT-4 or K562 cell lines[267,273]. However others reported the involvement of TCR for cytotoxicity against MOLT-4[261,273]. Regarding other tumor cell types, both NKG2D and TCR were shown to be involved in recognition of metastatic renal cell carcinoma[367] and colorectal and hepatocellular carcinomas[252,368]. Recently, DNAM-1 and NKG2D were described as co-modulators of Vγ9Vδ2 T cell cytotoxicity against hepatocellular carcinoma[230]. Other studies have shown that heat-shock proteins[174] and ULBP-4[55] can directly bind Vγ9Vδ2 TCR and impact on tumor cell recognition,

although the biological relevance of these interactions was not yet fully demonstrated.

In chapter 3 we have conducted a comprehensive study of gene expression in acute lymphoblastic leukemias and non-Hodgkin's lymphomas, aimed at identifying markers of susceptibility versus resistance to Vγ9Vδ2 T cell-mediated cytotoxicity. Upon careful bioinformatics analysis, we identified a panel of 10 genes encoding cell surface proteins that were statistically differentially expressed between "γδ-susceptible" and "γδ-resistant" hematopoietic tumors. The decision to restrict our analysis to surface-expressed molecules allowed us to perform a practical and fast definition of molecular candidates.

Within our signature profile, 3 genes (*ULBP1*, *TFR2* and *IFITM1*) were associated with increased susceptibility to Vγ9Vδ2 T-cell cytotoxicity, whereas the other 7 (*CLEC2D*, *NRP2*, *SELL*, *PKD2*, *KCNK12*, *ITGA6* and *SLAMF1*) were enriched in resistant tumors. We did not characterize all these candidate ligands further in detail (eg. by performing loss- and gain-of-function studies) because, in my collaboration with Telma Lança, we showed that ULBP-1 was a nonredundant determinant involved in leukemia/lymphoma cell-recognition by Vγ9Vδ2 T cells. In fact, the shRNA-mediated downregulation of ULBP-1 in MOLT-4 and JURKAT cell lines decreased susceptibility of these cells to Vγ9Vδ2 T cell-killing by up to 50% (see chapter 3). One should note that our RNAi strategy resulted in only partial downregulation of ULBP-1, therefore, the impact of ULBP-1 in this process is likely underestimated. Nonetheless, in future work, it may be of interest to assess the role of the other candidate molecules, as they may be modulators of Vγ9Vδ2 T cell cytotoxicity. Our panel of 10 candidate proteins expressed by susceptible or resistant tumor targets, included 3 adhesion molecules: NRP2, SELL and ITGA6. This was not surprising, since the adhesion molecules ICAM-1 and LFA-3 were previously described to modulate NK cell and γδ T cell cytotoxicity, probably by simply promoting physical interactions between effector cells and their targets[252,369]. In fact, several other adhesion molecules (CEACAM-1, TIGIT, cadherins, etc) were found to interact with NK cell receptors, modulating NK cell target recognition (see Table 2 chapter 1).

ULBP-1, IFITM-1 and CLEC2D were shown to interact with receptors expressed on NK cells and to modulate NK cell cytotoxicity[77,98,329,370]. In fact, KLRB1, the ligand for CLEC2D, is highly expressed on phosphoantigen-treated γδ T cells[368]. SLAM-F1

is a high affinity self-ligand, important for positive or negative regulation on bidirectional T-cell to B-cell interactions[371]. It would be interesting to investigate whether SLAM-F1 is also expressed on γδ T cells, since several members of this family of proteins (SLAM-F5, -F6 and -F7) are expressed on NK cells and modulate NK cell cytotoxicity[371] (Table 3, chapter 1). For example, SLAM-F6 (NTBA) and SLAM-F7 (CRACC), which are also self-ligands, have major roles in NK cell-mediated cytotoxicity[372]. As a matter of fact, it was recently shown that the complete gene signature of phosphoantigen-specific Vγ9Vδ2 T cells is a hybrid of those from αβ T and NK cells[373]. These new experiments confirmed that the molecular signature of γδ T cells activated by phosphoantigens corresponds primarily to cytolytic, cytokine and chemokine activities and clonal expansion[373].

Our global results, together with the fact that human NK cells and γδ T cells share many activating/inhibitory receptors (including NKp30, NKp44, NKp46, several KIRs, NKG2D, CD16, CD94-NKG2, CD96, DNAM-1, CD2, ILT2, etc.), ask for a more careful comparative study of NK cell biology in order to understand γδ T cell function and tumor cell recognition in different cancer settings. For example, similarly to NK cells, but contrary to $CD8^+$ T cells, Vγ9Vδ2 T cells can be directly activated by NKG2D-targeted mAb or MICA proteins[256]. In fact, NKG2D can also have co-stimulatory properties in γδ T cells[148], which shows the unique importance of this receptor for these cells. Of note, we described here the first physiologic evidence for lymphocyte requirement of NKG2D-ligand expression on tumors, since previous studies[55,368,374,375] were based on their ectopic expression. Furthermore, our data suggests that NKG2D signals seem strong enough to overcome any potential MHC class I-mediated inhibition of γδ T cells (chapter 3).

Contrary to previous models, our work suggests that γδ T cell-mediated surveillance of hematopoietic tumors is a 2-step process, where effector lymphocyte activation, proliferation and cytokine production, is achieved through TCRγδ stimulation by endogenous phosphoantigens (synergistically with IL-2), but tumor cell recognition is “NK-like”, predominantly mediated by NKG2D and/or other NK cell receptors. This was also observed for the $Vδ1^+$ $NKp30^+$ subset, in which both NKG2D and NCR, but not Vδ1 TCR, were involved in tumor cell recognition (chapter 4). Regarding Vγ9Vδ2 T cells, it is possible that the Vγ9Vδ2 TCR was evolutionary selected to detect microbial phosphoantigens, while the sensing/recognition of self

and “stressed self” is mostly mediated by NKR. This could explain the high number of tumor cell lines that are resistant to fully activated phosphoantigen-treated Vγ9Vδ2 T cells (chapters 2 and 3). The ULBP-1/NKG2D signals thus seemed strong enough to overcome MHC-I inhibition in susceptible cell lines. Our findings have crucial implications for the imunotherapy of hematological malignancies, since it may be possible to modulate ULBP-1 expression in tumors *in vivo*. Recently, it was shown that ULBP-1 can be induced in macrophages by treatment with IL-10, inducing the lysis of macrophages by NK cells[376]. Proteasome inhibitor drugs were also capable of inducing ULBP-1 expression on human tumor cells[377]. Moreover, epidermal growth factor receptor (EGFR) inhibitors predominantly increased the levels of surface ULBP1 protein in various colon cancer cells including KM12, Caco-2, HCT-15 and HT-29, which express EGFR, and increased susceptibility of these colon cancer cells to a NK cell line[378]. However, although the importance of ULBP-1 was demonstrated for the recognition of hematological tumors by Vγ9Vδ2 T cells, we don´t know if this applies to other cancer cell types, since ULBP-1 is not expressed in a considerable number of tumors. For example, susceptible epithelial tumors have been shown to express low or undetectable levels of ULBP1[254]. This either means that ULBP-1 has been drastically repressed (as part of potential immune escape mechanism), or that ULBP-1 is not involved in the process. In the latter case, maybe other NKG2D ligands, or other NK receptor ligands, may play a role. Indeed, tissue-associated $V\delta1^+$ T lymphocytes were also shown to recognize MICA/MICB and ULBP3[258] through NKG2D, and we further showed that they recognize yet unknown NCR ligands (chapter 4).

5.4 NCR EXPRESSION ON γδ T CELLS

In chapter 4, we described the differentiation of a novel subset of $V\delta2^{(-)}$ $V\delta1^+$ PBLs expressing natural cytotoxicity receptors (NCRs) that directly mediate killing of leukemia cell lines and chronic lymphocytic leukemia patient neoplastic cells. We showed that $V\delta1^+$ T-cells can be selectively induced to express NKp30, NKp44 and NKp46, through a process that requires functional PI-3K/ AKT signaling upon stimulation with γc cytokines and TCR agonists. Importantly, this γδ T cell subset is capable of targeting hematological tumors highly resistant to fully-activated Vγ9Vδ2 PBLs, including BV-173, REH, and the prototipic Raji cell line (Figure 1C, chapter 4

and data not shown). Recently, other groups have noticed that γδ T cells expanded with anti-γδTCR mAb have similar or even better anti-tumor properties than phosphoantigen-treated Vγ9Vδ2 T cells (e.g. for lymphoid malignancies, neuroblastoma and lung carcinoma), although those properties could not be entirely explained[379-381]. Compared with phosphoantigen-treated γδ cultures, the most remarkable difference was the presence of both γδ cell subsets ($V\delta1^+$ and $V\delta2^+$) after 2 weeks culture with α-TCR mAbs and IL-2[379]. Another group has shown that only $V\delta1^+$ γδ T lymphocytes (but not $V\delta2^+$ γδ T cells) could inhibit or prevent the s.c. growth of autologous melanoma in SCID mice engrafted with human tumors[276]. This was an important finding, because $V\delta1^+$ T cells do not recognize phosphoantigens, and thus do not benefit from endogenous phosphoantigen presentation by tumor cells. Interestingly, it was known more than 20 years ago that NK cell clones mediate strong cytolysis of both Daudi and Raji targets[273]. In fact, the overall pattern of cytotoxicity mediated by peripheral blood γδ T cells is markedly different from that mediated by NK cells from same individuals. This could suggest that the recognition of Raji cells by NK cells was mediated by NK receptor molecules that were present on NK cells, but not on γδ T cells. However, these authors (and many others) believed that the reason was the influence of the γδ TCR[273]. By showing the role of NKp30 in the recognition of these γδ-resistant targets, our work thus contributed to a change of paradigm in the understanding of the different patterns of anti-tumoral cytotoxicity fulfilled by NK cells and γδ T cells.

Recent studies have shown NCR protein expression on umbilical cord blood (UCB) T cells after IL-15 stimulation[101] (in contrast, the NCRs were not observed when UCB T cells were cultured with IL-4). UCB T cells (essentially $CD3^+$ $CD8^+$ $CD56^+$ T cells, including γδ T cells) acquired NKp30, NKp44, and NKp46 following culture with IL-2 or IL-15; however, only NKp30 was functional. The proximal adaptor protein for NKp44, DAP12, was only minimally expressed by IL-15-expanded UCB T cells, likely explaining the lack of NKp44 signaling. The lack of NKp46 function was more difficult to explain. The proximal adaptor proteins used by NKp46 and NKp30 (CD3ζ and FcεRIγ) were expressed by UCB T cells at levels apparently sufficient to permit signaling via NKp30. The authors suggested that NKp46 could require distinct, and yet undefined, downstream signaling components that are present in NK cells, but not in UCB T cells. This information is important for our work, because

NKp46 seems to be non-functional in Vδ1$^+$ NCR$^+$ T cells. Thus, it would be of interest to analyse the expression of the adaptor proteins used by NKp46 in these cells. Finally, the authors detected small amounts of naive adult peripheral blood T cells expressing NKp30 following IL-15 stimulation, but this receptor was not functional[102]. In these experiments it seems very likely that upon IL-15 stimulation, other human adult peripheral blood T cell subset (e.g. CD8$^+$ αβ T cells) were induced to acquire NKp30 following IL-15 stimulation, but only Vδ1$^+$ T cells (that were very diluted, and thus not detected) were able to sustain NKp30 expression. In fact, it has been recently shown that IL-15 is capable of inducing NKR and CD56 expression, and NK-like cytotoxicity, in purified human CD8$^+$ CD56$^-$ T cells[382]. Together, these data may suggest that peripheral blood Vδ1$^+$ γδ T cells, just like CD8$^+$ CD56$^-$ αβ T cells, could represent a more immature/undifferentiated subset, that retained the ability to express functional NCR. It was shown that Bcl11b gene deletion converted T cells from all developmental stages into "induced T-to-natural killer (ITNK) cells", which were morphologically and genetically similar to conventional NK cells, expressed NCRs, killed tumor cells in vitro, and effectively prevented tumor metastasis in vivo[383]. It would be interesting to know if *Bcl11b* is differentially down-regulated in activated Vδ1$^+$ NKp30$^+$ cells, and if it is involved in NCR expression on Vδ1$^+$ γδ T cells.

Recently, 3 different alternatively spliced NKp30 isoforms were shown to affect the prognosis of gastrointestinal stromal tumors[116]. Future work should elucidate which isoform is present on Vδ1$^+$ cells.

As initially described, NCRs are not detected in fresh human adult γδ T cells (Figure 4, chapter 4). A recent study described that NKp44$^+$ Vγ9Vδ2 T cells could be produced *ex vivo* upon stimulation with complex cytokine cocktails. However, the percentage of NKp44$^+$ γδ T cells in the culture was very low[250], shedding doubts about the biological role of this induced subset in humans. Interestingly, we noticed that the accumulation of NCR$^+$ Vδ1$^+$ cells in PHA/IL-2-activated cultures was associated with very high upregulation of CD11c and CD8 (data not shown), a phenotype reminiscent of intestinal intraepithelial lymphocytes[384]. In fact, while we describe here the *ex vivo* differentiation of NCR$^+$ Vδ1$^+$ γδ T cells upon *ex vivo* activation, our collaborators have collected firm evidence that Vδ1$^+$ NKp46$^+$ IELs can be observed *in vivo*, in the intestinal epithelium (Kelly Hudspeth, and Domenico Mavilio, manuscript in preparation). Therefore, the gut environment may provide the

required cytokines and Vδ1$^+$ TCR ligands, possibly MICA[54,141,251], for physiological NCR induction. Current research is addressing the functional properties of Vδ1$^+$ NKp46$^+$ IELs and their potential roles in gut homeostasis.

5.5 FUTURE PERSPECTIVES

Over the past decade, many studies have reported encouraging results regarding the use of γδ T cells for cancer immunotherapy[299]. However, despite these important findings, various major questions remain unanswered. For instance, it will be very important to decipher the full repertoire of tumor antigens involved in γδ T cell recognition, and to find additional determinants of tumor cell killing. γδ T cells express a very diverse panel of inhibitory and activating receptors that directly impact on their activation state and function (Figure 4, chapter 1). However, we still lack a dynamic picture of the receptors elicited along tumor-induced γδ T cell activation, and a deep understanding of the interplay between the numerous signaling cascades induced upon sequential or concomitant receptor engagement[219]. To understand γδ T cell activation, it will be very important to determine exactly how phosphoantigens interact with the Vγ9Vδ2 TCR. Furthermore, the anergy of repeatedly challenged phosphoantigen-treated Vγ9Vδ2 T cells reported *in vitro* and in clinical trials[289-291] constitutes a serious obstacle to phosphoantigen-based immunotherapies. This acquired anergy may be caused by inhibitory receptors expressed on Vγ9Vδ2 T cells, as it was seen for PD-1 on CD8$^+$ T cells[385], but other mechanisms are also likely to be involved. Moreover, the absolute need for exogenous IL-2 administration in cancer patients has become the major drawback for the later stages of development of phosphoantigen therapies[291]. The *in vivo* administration of IL-2 (a very pleiotropic molecule), has a very deep impact in the patients´ immune system, and unpredictable consequences concerning Vγ9Vδ2 T cell activation. For example, we showed that Tregs (which are highly sensitive to IL-2) can inhibit γδ T cell proinflammatory functions in mice[386] and other studies have shown this in humans[387]. Studies with αβ T cells struggled with the same problem, although only a few trials have omitted IL-2 infusions[388]. As we previously described, phosphoantigens alone cannot sustain Vγ9Vδ2 T cells activation (Chapter 2), and

very low levels of IL-2 lead to incomplete cell activation. Thus, the *ex vivo* activation of γδ T cells for adoptive cellular immunotherapy, avoiding IL-2 infusions, clearly seems to be a more attractive strategy. Still, non-responsive (NR) patients are typically excluded from Vγ9Vδ2 T cell-based adoptive immunotherapy trials, owing to the impossibility of increasing the number of cells *in vivo* or *ex vivo*. The reason for this in not yet understood, although autologous DCs pretreated with zoledronate induced some expansion of Vγ9Vδ2 T cells in NR patients[389].

The tumor itself is a very challenging environment. Reducing the recruitment of tumor infiltrating lymphocytes by laterations in the tumor vasculature represents a first line of defense against cytotoxic T cells. This is even more important for Vγ9Vδ2 T cells, since only a minority of these cells infiltrate established tumors[390]. In this context, the lack of an adequate mouse model for γδ T cell immunotherapy is a big obstacle to a better understanding of the dynamics of γδ T cell infusions.

Probably, the best way to control all these factors would be in a customized treatment of γδ T cells *in vitro*, allowing these cells to become fully functional before being infused in patients. The capacity to overcome the tumor inhibitory environment could eventually be provided by transfecting γδ T cells with new genetic elements, including KARs or genes conferring resistance to several chemicals. Indeed, adoptively transferred γδ T cells should express adhesion molecules that provide strong adhesion to the tumor endothelium, and the capacity to resist immunosupressive cells that are recruited to the tumor environment[391]. For example, immature DCs and myeloid-derived suppressor cells (MDSC) produce arginase I, prostaglandin E2 (PGE_2), reactive oxygen species (ROS) that inhibit T cell function[391]. Mesenchymal stem cells also have a very potent immunosuppressive role, by producing Indoleamine 2,3 dioxygenase (IDO), NO, HGF, as well as TGF-β1 and IL-10 cytokines[392]. Adding to this, Tregs can inhibit phosphoantigen-induced TCR Vγ9Vδ2 T cell proliferation and effector functions. Finally, NKG2D ligands are often proteolytically shed from the surface of tumor cells promoting NKG2D down-regulation in γδ T cells and tumor escape[393].

Thus, although holding great potential, Vγ9Vδ2 T cell-based immunotherapy may be limited by various factors, including poor infiltration into tissues. In chapter 4, we characterized a novel, highly cytotoxic subset of $V\delta1^+$ T cells which, unlike their $V\delta2^+$ counterparts, home preferentially to tissues. In our studies, $V\delta1^+$ T cells were obtained at very high numbers after 19 days of *ex vivo* expansion in cell culture

plates (≈1x10^8 Vδ1$^+$ cells; Figures 1A and 6B, chapter 4), and a high percentage of these cells expressed NKp30$^+$ (Figure 6C), reaching a total of ≈6x10^7 NKp30$^+$ Vδ1$^+$ cells, obtained from 500ml of peripheral blood. However, for clinical purposes, a more efficient protocol, providing larger numbers of NKp30$^+$ Vδ1$^+$ cells, is needed. Along these lines, in the near future we plan to optimize/ maximize our *in vitro* differentiation protocol of Vδ1$^+$ NKp30$^+$ cells, aiming to achieve cell yields (up to 5x10^9 Vδ1$^+$ cells) that may allow adoptive cell therapy for cancer patients. We will develop a method to generate clinical-grade functional NKp30$^+$ γδ cells from the peripheral blood of cancer patients, under good manufacturing practices (GMP). Thus, γδ T cells will be cultured in a fully closed, large-scale, automated bioreactor system, which has been used for large-scale expansion of NK cells[394,395] and other immune cells[396-398]. Several soluble cytokines (IL-12, IL-15, IL-21), soluble NKG2D ligands or coated beads (as artificial feeder cells) will be tested in order to enhance cell proliferation. In fact, although the culture process to generate NKp30$^+$ Vδ1$^+$ cells is more complicated than the Vγ9Vδ2 culture process, it is still far easier than the isolation and expansion of Ag-specific αβ T cells. The major limitation of our current work is the unknown identity of NKp30 ligands on tumor cells. Since B7-H6 does not seem to play a role (chapter 4), further research is needed in order to identify the respective ligands.

The clinical potential of NKp30$^+$ Vδ1$^+$ T cells produced in the bioreactor system will be evaluated in phenotypic and functional assays, both *in vitro* and *in vivo* (e.g., cellular cytotoxicity assays against tumor cells of diverse tissue origin, cytokine production, survival and proliferation assays). After the generation of pre-clinical data, the progress to Phase I clinical trials in ALL patients (the most suitable candidates for this therapy) will depend on attracting the interest of companies or investors specialized in adoptive cell therapy. In Europe and USA, 11.600 new cases of ALL are identified each year. It is the most frequent cancer in children under 15 year of age[399]. With conventional treatments (chemotherapy, or radiotherapy and bone marrow transplant) the death rate after 5 years remains very high (25% in children and 60% in adults). We hope our efforts in developing novel γδ T cell-based immunotherapy protocols will offer an alternative treatment to patients affected by hematological tumors and other cancers, particularly by preventing disease relapse.

6 . REFERENCES

1 Hanahan, D. & Weinberg, R. A. Hallmarks of cancer: the next generation. *Cell* **144**, 646-674 (2011).

2 Ehrlich, P. Ueber den jetzigen stand der Karzinomforschung. *Ned. Tijdschr. Geneeskd* **5**, 273-290 (1909).

3 Burnet, M. Cancer: a biological approach. III. Viruses associated with neoplastic conditions. IV. Practical applications. *Br Med J* **1**, 841-847 (1957).

4 Thomas, L. *Cellular and Humoral Aspects of the Hypersensitive States*. (Hoeber-Harper, 1959).

5 Dighe, A. S., Richards, E., Old, L. J. & Schreiber, R. D. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. *Immunity* **1**, 447-456 (1994).

6 Kaplan, D. H. *et al.* Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice. *Proc Natl Acad Sci U S A* **95**, 7556-7561 (1998).

7 Engel, A. M. *et al.* Methylcholanthrene-induced sarcomas in nude mice have short induction times and relatively low levels of surface MHC class I expression. *Apmis* **104**, 629-639 (1996).

8 Engel, A. M., Svane, I. M., Rygaard, J. & Werdelin, O. MCA sarcomas induced in scid mice are more immunogenic than MCA sarcomas induced in congenic, immunocompetent mice. *Scand J Immunol* **45**, 463-470 (1997).

9 Shankaran, V. *et al.* IFNgamma and lymphocytes prevent primary tumor development and shape tumor immunogenicity. *Nature* **410**, 1107-1111 (2001).

10 Smyth, M. J. *et al.* Perforin-mediated cytotoxicity is critical for surveillance of spontaneous lymphoma. *J Exp Med* **192**, 755-760 (2000).

11 Girardi, M. *et al.* Regulation of cutaneous malignancy by gammadelta T cells. *Science* **294**, 605-609 (2001).

12 De Plaen, E. *et al.* Immunogenic (tum-) variants of mouse tumor P815: cloning of the gene of tum- antigen P91A and identification of the tum- mutation. *Proc Natl Acad Sci U S A* **85**, 2274-2278 (1988).

13 van der Bruggen, P. *et al.* A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* **254**, 1643-1647 (1991).

14 Vesely, M. D., Kershaw, M. H., Schreiber, R. D. & Smyth, M. J. Natural Innate and Adaptive Immunity to Cancer. *Annu Rev Immunol* (2011).

15 Matzinger, P. Tolerance, danger, and the extended family. *Annu Rev Immunol* **12**, 991-1045 (1994).

16 Kumar, H., Kawai, T. & Akira, S. Pathogen recognition by the innate immune system. *International reviews of immunology* **30**, 16-34, doi:10.3109/08830185.2010.529976 (2011).

17 Janeway, C. A., Jr. & Medzhitov, R. Innate immune recognition. *Annu Rev Immunol* **20**, 197-216, doi:10.1146/annurev.immunol.20.083001.084359 (2002).

18 Schenten, D. & Medzhitov, R. The control of adaptive immune responses by the innate immune system. *Adv Immunol* **109**, 87-124, doi:10.1016/B978-0-12-387664-5.00003-0 (2011).

19 Pancer, Z. & Cooper, M. D. The evolution of adaptive immunity. *Annu Rev Immunol* **24**, 497-518, doi:10.1146/annurev.immunol.24.021605.090542 (2006).

20 Rudolph, M. G., Stanfield, R. L. & Wilson, I. A. How TCRs bind MHCs, peptides, and coreceptors. *Annu Rev Immunol* **24**, 419-466, doi:10.1146/annurev.immunol.23.021704.115658 (2006).

21 Stagg, J., Johnstone, R. W. & Smyth, M. J. From cancer immunosurveillance to cancer immunotherapy. *Immunol Rev* **220**, 82-101 (2007).

22 Gomes, A. Q., Correia, D. V. & Silva-Santos, B. Non-classical major histocompatibility complex proteins as determinants of tumor immunosurveillance. *EMBO Rep* **8**, 1024-1030, doi:10.1038/sj.embor.7401090 (2007).

23 Dunn, G. P., Bruce, A. T., Ikeda, H., Old, L. J. & Schreiber, R. D. Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* **3**, 991-998 (2002).

24 Zitvogel, L., Tesniere, A. & Kroemer, G. Cancer despite immunosurveillance: immunoselection and immunosubversion. *Nat Rev Immunol* **6**, 715-727 (2006).

25 Allison, T. J., Winter, C. C., Fournie, J. J., Bonneville, M. & Garboczi, D. N. Structure of a human gammadelta T-cell antigen receptor. *Nature* **411**, 820-824 (2001).

26 Hayday, A. C. [gamma][delta] cells: a right time and a right place for a conserved third way of protection. *Annu Rev Immunol* **18**, 975-1026 (2000).

27 Fooksman, D. R. *et al.* Functional anatomy of T cell activation and synapse formation. *Annu Rev Immunol* **28**, 79-105, doi:10.1146/annurev-immunol-030409-101308 (2010).

28 Smith-Garvin, J. E., Koretzky, G. A. & Jordan, M. S. T cell activation. *Annu Rev Immunol* **27**, 591-619, doi:10.1146/annurev.immunol.021908.132706 (2009).

29 Nedellec, S., Bonneville, M. & Scotet, E. Human Vgamma9Vdelta2 T cells: from signals to functions. *Semin Immunol* **22**, 199-206 (2010).

30 Genot, E. & Cantrell, D. A. Ras regulation and function in lymphocytes. *Curr Opin Immunol* **12**, 289-294 (2000).

31 Hayashi, K. & Altman, A. Protein kinase C theta (PKCtheta): a key player in T cell life and death. *Pharmacological research : the official journal of the Italian Pharmacological Society* **55**, 537-544, doi:10.1016/j.phrs.2007.04.009 (2007).

32 Oh-hora, M. & Rao, A. Calcium signaling in lymphocytes. *Curr Opin Immunol* **20**, 250-258, doi:10.1016/j.coi.2008.04.004 (2008).

33 Appleman, L. J. & Boussiotis, V. A. T cell anergy and costimulation. *Immunol Rev* **192**, 161-180 (2003).

34 Chen, L. Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. *Nat Rev Immunol* **4**, 336-347, doi:10.1038/nri1349 (2004).

35 Croft, M. The role of TNF superfamily members in T-cell function and diseases. *Nat Rev Immunol* **9**, 271-285, doi:10.1038/nri2526 (2009).

36 Trombetta, E. S. & Mellman, I. Cell biology of antigen processing in vitro and in vivo. *Annu Rev Immunol* **23**, 975-1028 (2005).

37 Brandes, M., Willimann, K. & Moser, B. Professional antigen-presentation function by human gammadelta T Cells. *Science* **309**, 264-268 (2005).

38 Petersen, T. R., Dickgreber, N. & Hermans, I. F. Tumor antigen presentation by dendritic cells. *Crit Rev Immunol* **30**, 345-386 (2010).

39 Novellino, L., Castelli, C. & Parmiani, G. A listing of human tumor antigens recognized by T cells: March 2004 update. *Cancer Immunol Immunother* **54**, 187-207 (2005).

40 Van Der Bruggen, P. *et al.* Tumor-specific shared antigenic peptides recognized by human T cells. *Immunol Rev* **188**, 51-64 (2002).

41 Lampen, M. H. & van Hall, T. Strategies to counteract MHC-I defects in tumors. *Curr Opin Immunol* **23**, 293-298, doi:10.1016/j.coi.2010.12.005 (2011).

42 Nestle, F. O. *et al.* Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat Med* **4**, 328-332 (1998).

43 De Smet, C., Lurquin, C., Lethe, B., Martelange, V. & Boon, T. DNA methylation is the primary silencing mechanism for a set of germ line- and tumor-specific genes with a CpG-rich promoter. *Mol Cell Biol* **19**, 7327-7335 (1999).

44 Probst-Kepper, M. *et al.* An alternative open reading frame of the human macrophage colony-stimulating factor gene is independently translated and codes for an antigenic peptide of 14 amino acids recognized by tumor-infiltrating CD8 T lymphocytes. *J Exp Med* **193**, 1189-1198 (2001).

45 Hanada, K., Yewdell, J. W. & Yang, J. C. Immune recognition of a human renal cancer antigen through post-translational protein splicing. *Nature* **427**, 252-256 (2004).

46 van Hall, T. *et al.* Selective cytotoxic T-lymphocyte targeting of tumor immune escape variants. *Nat Med* **12**, 417-424, doi:10.1038/nm1381 (2006).

47 Oliveira, C. C. *et al.* The nonpolymorphic MHC Qa-1b mediates CD8+ T cell surveillance of antigen-processing defects. *J Exp Med* **207**, 207-221, doi:10.1084/jem.20091429 (2010).

48 Koos, D. *et al.* Tumor vaccines in 2010: need for integration. *Cell Immunol* **263**, 138-147, doi:10.1016/j.cellimm.2010.03.019 (2010).

49 Finn, O. J. Cancer vaccines: between the idea and the reality. *Nat Rev Immunol* **3**, 630-641 (2003).

50 Gattinoni, L., Powell, D. J., Jr., Rosenberg, S. A. & Restifo, N. P. Adoptive immunotherapy for cancer: building on success. *Nat Rev Immunol* **6**, 383-393 (2006).

51 Street, S. E. *et al.* Innate immune surveillance of spontaneous B cell lymphomas by natural killer cells and gammadelta T cells. *J Exp Med* **199**, 879-884 (2004).

52 Lanier, L. L. Up on the tightrope: natural killer cell activation and inhibition. *Nat Immunol* **9**, 495-502, doi:10.1038/ni1581 (2008).

53 Cerwenka, A. *et al.* Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. *Immunity* **12**, 721-727 (2000).

54 Groh, V., Steinle, A., Bauer, S. & Spies, T. Recognition of stress-induced MHC molecules by intestinal epithelial gammadelta T cells. *Science* **279**, 1737-1740 (1998).

55 Kong, Y. *et al.* The NKG2D ligand ULBP4 binds to TCRgamma9/delta2 and induces cytotoxicity to tumor cells through both TCRgammadelta and NKG2D. *Blood* **114**, 310-317 (2009).

56 Tanaka, Y. *et al.* Natural and synthetic non-peptide antigens recognized by human gamma delta T cells. *Nature* **375**, 155-158 (1995).

57 Bauer, S. *et al.* Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* **285**, 727-729 (1999).

58 Rodgers, J. R. & Cook, R. G. MHC class Ib molecules bridge innate and acquired immunity. *Nat Rev Immunol* **5**, 459-471 (2005).

59 Moretta, A. Natural killer cells and dendritic cells: rendezvous in abused tissues. *Nat Rev Immunol* **2**, 957-964, doi:10.1038/nri956 (2002).

60 Sun, J. C. & Lanier, L. L. NK cell development, homeostasis and function: parallels with CD8(+) T cells. *Nat Rev Immunol*, doi:10.1038/nri3044 (2011).

61 Kim, S., Iizuka, K., Aguila, H. L., Weissman, I. L. & Yokoyama, W. M. In vivo natural killer cell activities revealed by natural killer cell-deficient mice. *Proc Natl Acad Sci U S A* **97**, 2731-2736, doi:10.1073/pnas.050588297 (2000).

62 Smyth, M. J. *et al.* Activation of NK cell cytotoxicity. *Molecular immunology* **42**, 501-510, doi:10.1016/j.molimm.2004.07.034 (2005).

63 Bryceson, Y. T., March, M. E., Ljunggren, H. G. & Long, E. O. Activation, coactivation, and costimulation of resting human natural killer cells. *Immunol Rev* **214**, 73-91, doi:10.1111/j.1600-065X.2006.00457.x (2006).

64 Mombaerts, P. *et al.* RAG-1-deficient mice have no mature B and T lymphocytes. *Cell* **68**, 869-877 (1992).

65 Shinkai, Y. *et al.* RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* **68**, 855-867 (1992).

66 Kim, S. *et al.* Licensing of natural killer cells by host major histocompatibility complex class I molecules. *Nature* **436**, 709-713, doi:10.1038/nature03847 (2005).

67 Anfossi, N. *et al.* Human NK cell education by inhibitory receptors for MHC class I. *Immunity* **25**, 331-342, doi:10.1016/j.immuni.2006.06.013 (2006).

68 Vivier, E. *et al.* Innate or adaptive immunity? The example of natural killer cells. *Science* **331**, 44-49, doi:10.1126/science.1198687 (2011).

69 Elliott, J. M., Wahle, J. A. & Yokoyama, W. M. MHC class I-deficient natural killer cells acquire a licensed phenotype after transfer into an MHC class I-sufficient environment. *J Exp Med* **207**, 2073-2079, doi:10.1084/jem.20100986 (2010).

70 Joncker, N. T., Shifrin, N., Delebecque, F. & Raulet, D. H. Mature natural killer cells reset their responsiveness when exposed to an altered MHC environment. *J Exp Med* **207**, 2065-2072, doi:10.1084/jem.20100570 (2010).

71 Sun, J. C., Lopez-Verges, S., Kim, C. C., DeRisi, J. L. & Lanier, L. L. NK cells and immune "memory". *J Immunol* **186**, 1891-1897, doi:10.4049/jimmunol.1003035 (2011).

72 Orr, M. T. & Lanier, L. L. Natural killer cell education and tolerance. *Cell* **142**, 847-856, doi:10.1016/j.cell.2010.08.031 (2010).

73 Brodin, P., Karre, K. & Hoglund, P. NK cell education: not an on-off switch but a tunable rheostat. *Trends Immunol* **30**, 143-149, doi:10.1016/j.it.2009.01.006 (2009).

74 Joncker, N. T., Fernandez, N. C., Treiner, E., Vivier, E. & Raulet, D. H. NK cell responsiveness is tuned commensurate with the number of inhibitory receptors for self-MHC class I: the rheostat model. *J Immunol* **182**, 4572-4580, doi:10.4049/jimmunol.0803900 (2009).

75 Lopez-Botet, M., Llano, M., Navarro, F. & Bellon, T. NK cell recognition of non-classical HLA class I molecules. *Semin Immunol* **12**, 109-119, doi:10.1006/smim.2000.0213 (2000).

76 Coles, S. J. *et al.* CD200 expression suppresses natural killer cell function and directly inhibits patient anti-tumor response in acute myeloid leukemia. *Leukemia* **25**, 792-799, doi:10.1038/leu.2011.1 (2011).

77 Roth, P. *et al.* Malignant glioma cells counteract antitumor immune responses through expression of lectin-like transcript-1. *Cancer Res* **67**, 3540-3544 (2007).

78 Rosen, D. B. *et al.* Functional consequences of interactions between human NKR-P1A and its ligand LLT1 expressed on activated dendritic cells and B cells. *J Immunol* **180**, 6508-6517 (2008).

79 Rosen, D. B. *et al.* Cutting edge: lectin-like transcript-1 is a ligand for the inhibitory human NKR-P1A receptor. *J Immunol* **175**, 7796-7799 (2005).

80 Vilches, C. & Parham, P. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu Rev Immunol* **20**, 217-251, doi:10.1146/annurev.immunol.20.092501.134942 (2002).

81 Purdy, A. K. & Campbell, K. S. Natural killer cells and cancer: regulation by the killer cell Ig-like receptors (KIR). *Cancer biology & therapy* **8**, 2211-2220 (2009).

82 Borrego, F., Masilamani, M., Kabat, J., Sanni, T. B. & Coligan, J. E. The cell biology of the human natural killer cell CD94/NKG2A inhibitory receptor. *Molecular immunology* **42**, 485-488, doi:10.1016/j.molimm.2004.07.031 (2005).

83 Le Drean, E. *et al.* Inhibition of antigen-induced T cell response and antibody-induced NK cell cytotoxicity by NKG2A: association of NKG2A with SHP-1 and SHP-2 protein-tyrosine phosphatases. *Eur J Immunol* **28**, 264-276, doi:10.1002/(SICI)1521-4141(199801)28:01<264::AID-IMMU264>3.0.CO;2-O (1998).

84 Lanier, L. L., Corliss, B., Wu, J. & Phillips, J. H. Association of DAP12 with activating CD94/NKG2C NK cell receptors. *Immunity* **8**, 693-701 (1998).

85 Lee, N. *et al.* HLA-E is a major ligand for the natural killer inhibitory receptor CD94/NKG2A. *Proc Natl Acad Sci U S A* **95**, 5199-5204 (1998).

86 Vance, R. E., Jamieson, A. M. & Raulet, D. H. Recognition of the class Ib molecule Qa-1(b) by putative activating receptors CD94/NKG2C and CD94/NKG2E on mouse natural killer cells. *J Exp Med* **190**, 1801-1812 (1999).

87 Derre, L. *et al.* Expression of CD94/NKG2-A on human T lymphocytes is induced by IL-12: implications for adoptive immunotherapy. *J Immunol* **168**, 4864-4870 (2002).

88 Mingari, M. C. *et al.* HLA class I-specific inhibitory receptors in human T lymphocytes: interleukin 15-induced expression of CD94/NKG2A in superantigen- or alloantigen-activated CD8+ T cells. *Proc Natl Acad Sci U S A* **95**, 1172-1177 (1998).

89 Vivier, E. & Anfossi, N. Inhibitory NK-cell receptors on T cells: witness of the past, actors of the future. *Nat Rev Immunol* **4**, 190-198, doi:10.1038/nri1306 (2004).

90 Gooden, M. *et al.* HLA-E expression by gynecological cancers restrains tumor-infiltrating CD8 T lymphocytes. *Proc Natl Acad Sci U S A* **108**, 10656-10661, doi:10.1073/pnas.1100354108 (2011).

91 Moretta, A. *et al.* Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu Rev Immunol* **19**, 197-223, doi:10.1146/annurev.immunol.19.1.197 (2001).

92 Bryceson, Y. T., March, M. E., Ljunggren, H. G. & Long, E. O. Synergy among receptors on resting NK cells for the activation of natural cytotoxicity and cytokine secretion. *Blood* **107**, 159-166, doi:10.1182/blood-2005-04-1351 (2006).

93 Sivori, S. *et al.* p46, a novel natural killer cell-specific surface molecule that mediates cell activation. *J Exp Med* **186**, 1129-1136 (1997).

94 Pessino, A. *et al.* Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. *J Exp Med* **188**, 953-960 (1998).

95 Pende, D. *et al.* Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. *J Exp Med* **190**, 1505-1516 (1999).

96 Vitale, M. *et al.* NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis. *J Exp Med* **187**, 2065-2072 (1998).

97 Cantoni, C. *et al.* NKp44, a triggering receptor involved in tumor cell lysis by activated human natural killer cells, is a novel member of the immunoglobulin superfamily. *J Exp Med* **189**, 787-796 (1999).

98 Raulet, D. H. Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol* **3**, 781-790, doi:10.1038/nri1199 (2003).

99 (!!! INVALID CITATION !!!).

100 Kaifu, T., Escaliere, B., Gastinel, L. N., Vivier, E. & Baratin, M. B7-H6/NKp30 interaction: a mechanism of alerting NK cells against tumors. *Cell Mol Life Sci*, doi:10.1007/s00018-011-0802-7 (2011).

101 Ponnampalam, A. P., Gargett, C. E. & Rogers, P. A. Identification and hormonal regulation of a novel form of NKp30 in human endometrial epithelium. *Eur J Immunol* **38**, 216-226, doi:10.1002/eji.200636429 (2008).

102 Tang, Q. *et al.* Umbilical cord blood T cells express multiple natural cytotoxicity receptors after IL-15 stimulation, but only NKp30 is functional. *J Immunol* **181**, 4507-4515 (2008).

103 Cupedo, T. *et al.* Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+ CD127+ natural killer-like cells. *Nat Immunol* **10**, 66-74, doi:10.1038/ni.1668 (2009).

104 Vivier, E., Spits, H. & Cupedo, T. Interleukin-22-producing innate immune cells: new players in mucosal immunity and tissue repair? *Nat Rev Immunol* **9**, 229-234, doi:10.1038/nri2522 (2009).

105 Della Chiesa, M. *et al.* The tryptophan catabolite L-kynurenine inhibits the surface expression of NKp46- and NKG2D-activating receptors and regulates NK-cell function. *Blood* **108**, 4118-4125, doi:10.1182/blood-2006-03-006700 (2006).

106 Burgess, S. J., Marusina, A. I., Pathmanathan, I., Borrego, F. & Coligan, J. E. IL-21 down-regulates NKG2D/DAP10 expression on human NK and CD8+ T cells. *J Immunol* **176**, 1490-1497 (2006).

107 Castriconi, R. *et al.* Transforming growth factor beta 1 inhibits expression of NKp30 and NKG2D receptors: consequences for the NK-mediated killing of dendritic cells. *Proc Natl Acad Sci U S A* **100**, 4120-4125, doi:10.1073/pnas.0730640100 (2003).

108 Fauriat, C. *et al.* Deficient expression of NCR in NK cells from acute myeloid leukemia: Evolution during leukemia treatment and impact of leukemia cells in NCRdull phenotype induction. *Blood* **109**, 323-330, doi:10.1182/blood-2005-08-027979 (2007).

109 Sanchez-Correa, B. *et al.* Human NK cells in acute myeloid leukaemia patients: analysis of NK cell-activating receptors and their ligands. *Cancer Immunol Immunother* **60**, 1195-1205, doi:10.1007/s00262-011-1050-2 (2011).

110 Mamessier, E. *et al.* Human breast cancer cells enhance self tolerance by promoting evasion from NK cell antitumor immunity. *J Clin Invest* **121**, 3609-3622, doi:10.1172/JCI45816 (2011).

111 Ferlazzo, G. *et al.* Human dendritic cells activate resting natural killer (NK) cells and are recognized via the NKp30 receptor by activated NK cells. *J Exp Med* **195**, 343-351 (2002).

112 Vitale, M. *et al.* NK-dependent DC maturation is mediated by TNFalpha and IFNgamma released upon engagement of the NKp30 triggering receptor. *Blood* **106**, 566-571, doi:10.1182/blood-2004-10-4035 (2005).

113 Hollyoake, M., Campbell, R. D. & Aguado, B. NKp30 (NCR3) is a pseudogene in 12 inbred and wild mouse strains, but an expressed gene in Mus caroli. *Molecular biology and evolution* **22**, 1661-1672, doi:10.1093/molbev/msi162 (2005).

114 Rutjens, E. *et al.* Differential NKp30 inducibility in chimpanzee NK cells and conserved NK cell phenotype and function in long-term HIV-1-infected animals. *J Immunol* **178**, 1702-1712 (2007).

115 Neville, M. J. & Campbell, R. D. A new member of the Ig superfamily and a V-ATPase G subunit are among the predicted products of novel genes close to the TNF locus in the human MHC. *J Immunol* **162**, 4745-4754 (1999).

116 Delahaye, N. F. *et al.* Alternatively spliced NKp30 isoforms affect the prognosis of gastrointestinal stromal tumors. *Nat Med* **17**, 700-707, doi:10.1038/nm.2366 (2011).

117 Arnon, T. I. *et al.* Inhibition of the NKp30 activating receptor by pp65 of human cytomegalovirus. *Nat Immunol* **6**, 515-523, doi:10.1038/ni1190 (2005).

118 Pogge von Strandmann, E. *et al.* Human leukocyte antigen-B-associated transcript 3 is released from tumor cells and engages the NKp30 receptor on natural killer cells. *Immunity* **27**, 965-974, doi:10.1016/j.immuni.2007.10.010 (2007).

119 Byrd, A., Hoffmann, S. C., Jarahian, M., Momburg, F. & Watzl, C. Expression analysis of the ligands for the Natural Killer cell receptors NKp30 and NKp44. *PLoS One* **2**, e1339, doi:10.1371/journal.pone.0001339 (2007).

120 Brandt, C. S. *et al.* The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. *J Exp Med* **206**, 1495-1503, doi:10.1084/jem.20090681 (2009).

121 Arnon, T. I. *et al.* Recognition of viral hemagglutinins by NKp44 but not by NKp30. *Eur J Immunol* **31**, 2680-2689, doi:10.1002/1521-4141(200109)31:9<2680::AID-IMMU2680>3.0.CO;2-A (2001).

122 Hecht, M. L. *et al.* Natural cytotoxicity receptors NKp30, NKp44 and NKp46 bind to different heparan sulfate/heparin sequences. *Journal of proteome research* **8**, 712-720, doi:10.1021/pr800747c (2009).

123 Hershkovitz, O. *et al.* NKp44 receptor mediates interaction of the envelope glycoproteins from the West Nile and dengue viruses with NK cells. *J Immunol* **183**, 2610-2621, doi:10.4049/jimmunol.0802806 (2009).

124 Ho, J. W. *et al.* H5-type influenza virus hemagglutinin is functionally recognized by the natural killer-activating receptor NKp44. *Journal of virology* **82**, 2028-2032, doi:10.1128/JVI.02065-07 (2008).

125 Vieillard, V., Strominger, J. L. & Debre, P. NK cytotoxicity against CD4+ T cells during HIV-1 infection: a gp41 peptide induces the expression of an NKp44 ligand. *Proc Natl Acad Sci U S A* **102**, 10981-10986, doi:10.1073/pnas.0504315102 (2005).

126 Crellin, N. K., Trifari, S., Kaplan, C. D., Cupedo, T. & Spits, H. Human NKp44+IL-22+ cells and LTi-like cells constitute a stable RORC+ lineage distinct from conventional natural killer cells. *J Exp Med* **207**, 281-290, doi:10.1084/jem.20091509 (2010).

127 Gazit, R. *et al.* Lethal influenza infection in the absence of the natural killer cell receptor gene Ncr1. *Nat Immunol* **7**, 517-523, doi:10.1038/ni1322 (2006).

128 Mandelboim, O. *et al.* Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature* **409**, 1055-1060, doi:10.1038/35059110 (2001).

129 Jost, S. *et al.* Expansion of 2B4+ natural killer (NK) cells and decrease in NKp46+ NK cells in response to influenza. *Immunology* **132**, 516-526, doi:10.1111/j.1365-2567.2010.03394.x (2011).

130 Halfteck, G. G. *et al.* Enhanced in vivo growth of lymphoma tumors in the absence of the NK-activating receptor NKp46/NCR1. *J Immunol* **182**, 2221-2230, doi:10.4049/jimmunol.0801878 (2009).

131 Cagnano, E. *et al.* Expression of ligands to NKp46 in benign and malignant melanocytes. *J Invest Dermatol* **128**, 972-979, doi:10.1038/sj.jid.5701111 (2008).

132 Gur, C. *et al.* The activating receptor NKp46 is essential for the development of type 1 diabetes. *Nat Immunol* **11**, 121-128, doi:10.1038/ni.1834 (2010).

133 Gur, C. *et al.* Recognition and Killing of Human and Murine Pancreatic {beta} Cells by the NK Receptor NKp46. *J Immunol*, doi:10.4049/jimmunol.1101269 (2011).

134 Yu, J. *et al.* NKp46 identifies an NKT cell subset susceptible to leukemic transformation in mouse and human. *J Clin Invest* **121**, 1456-1470, doi:10.1172/JCI43242 (2011).

135 Eisenring, M., vom Berg, J., Kristiansen, G., Saller, E. & Becher, B. IL-12 initiates tumor rejection via lymphoid tissue-inducer cells bearing the natural cytotoxicity receptor NKp46. *Nat Immunol* **11**, 1030-1038, doi:10.1038/ni.1947 (2010).

136 Satoh-Takayama, N. *et al.* Microbial flora drives interleukin 22 production in intestinal NKp46+ cells that provide innate mucosal immune defense. *Immunity* **29**, 958-970, doi:10.1016/j.immuni.2008.11.001 (2008).

137 Das, H. *et al.* MICA engagement by human Vgamma2Vdelta2 T cells enhances their antigen-dependent effector function. *Immunity* **15**, 83-93 (2001).

138 Eagle, R. A., Traherne, J. A., Hair, J. R., Jafferji, I. & Trowsdale, J. ULBP6/RAET1L is an additional human NKG2D ligand. *Eur J Immunol* **39**, 3207-3216, doi:10.1002/eji.200939502 (2009).

139 Champsaur, M. & Lanier, L. L. Effect of NKG2D ligand expression on host immune responses. *Immunol Rev* **235**, 267-285, doi:10.1111/j.0105-2896.2010.00893.x (2010).

140 Park, Y. P. *et al.* Complex regulation of human NKG2D-DAP10 cell surface expression: opposing roles of the {gamma}c cytokines and TGF-{beta}1. *Blood*, doi:10.1182/blood-2011-04-346825 (2011).

141 Groh, V., Wu, J., Yee, C. & Spies, T. Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature* **419**, 734-738, doi:10.1038/nature01112 (2002).

142 Jamieson, A. M. *et al.* The role of the NKG2D immunoreceptor in immune cell activation and natural killing. *Immunity* **17**, 19-29 (2002).

143 Vivier, E., Tomasello, E. & Paul, P. Lymphocyte activation via NKG2D: towards a new paradigm in immune recognition? *Curr Opin Immunol* **14**, 306-311 (2002).

144 Sutherland, C. L. *et al.* UL16-binding proteins, novel MHC class I-related proteins, bind to NKG2D and activate multiple signaling pathways in primary NK cells. *J Immunol* **168**, 671-679 (2002).

145 Billadeau, D. D., Upshaw, J. L., Schoon, R. A., Dick, C. J. & Leibson, P. J. NKG2D-DAP10 triggers human NK cell-mediated killing via a Syk-independent regulatory pathway. *Nat Immunol* **4**, 557-564, doi:10.1038/ni929 (2003).

146 Groh, V. *et al.* Costimulation of CD8alphabeta T cells by NKG2D via engagement by MIC induced on virus-infected cells. *Nat Immunol* **2**, 255-260, doi:10.1038/85321 (2001).

147 Barber, A. & Sentman, C. L. NKG2D receptor regulates human effector T-cell cytokine production. *Blood* **117**, 6571-6581, doi:10.1182/blood-2011-01-329417 (2011).

148 Nedellec, S., Sabourin, C., Bonneville, M. & Scotet, E. NKG2D costimulates human V gamma 9V delta 2 T cell antitumor cytotoxicity through protein kinase C theta-dependent modulation of early TCR-induced calcium and transduction signals. *J Immunol* **185**, 55-63, doi:10.4049/jimmunol.1000373 (2010).

149 Maasho, K., Opoku-Anane, J., Marusina, A. I., Coligan, J. E. & Borrego, F. NKG2D is a costimulatory receptor for human naive CD8+ T cells. *J Immunol* **174**, 4480-4484 (2005).

150 Smyth, M. J. *et al.* NKG2D function protects the host from tumor initiation. *J Exp Med* **202**, 583-588, doi:10.1084/jem.20050994 (2005).

151 Heilig, J. S. & Tonegawa, S. Diversity of murine gamma genes and expression in fetal and adult T lymphocytes. *Nature* **322**, 836-840 (1986).

152 Hayday, A. C. *et al.* Structure, organization, and somatic rearrangement of T cell gamma genes. *Cell* **40**, 259-269 (1985).

153 Hayday, A. C. Gammadelta T cells and the lymphoid stress-surveillance response. *Immunity* **31**, 184-196 (2009).

154 Dechanet, J. *et al.* Implication of gammadelta T cells in the human immune response to cytomegalovirus. *J Clin Invest* **103**, 1437-1449 (1999).

155 Bonneville, M., O'Brien, R. L. & Born, W. K. Gammadelta T cell effector functions: a blend of innate programming and acquired plasticity. *Nat Rev Immunol* **10**, 467-478, doi:10.1038/nri2781 (2010).

156 Kuhnlein, P. *et al.* The canonical T cell receptor of dendritic epidermal gamma delta T cells is highly conserved between rats and mice. *Eur J Immunol* **26**, 3092-3097 (1996).

157 Carding, S. R. & Egan, P. J. Gammadelta T cells: functional plasticity and heterogeneity. *Nat Rev Immunol* **2**, 336-345 (2002).

158 Hayday, A. & Tigelaar, R. Immunoregulation in the tissues by gammadelta T cells. *Nat Rev Immunol* **3**, 233-242 (2003).

159 Lefranc, M. P. & Rabbitts, T. H. The human T-cell receptor gamma (TRG) genes. *Trends in biochemical sciences* **14**, 214-218 (1989).

160 Born, W. K. *et al.* Peptide antigens for gamma/delta T cells. *Cell Mol Life Sci* **68**, 2335-2343, doi:10.1007/s00018-011-0697-3 (2011).

161 Adams, E. J., Chien, Y. H. & Garcia, K. C. Structure of a gammadelta T cell receptor in complex with the nonclassical MHC T22. *Science* **308**, 227-231, doi:10.1126/science.1106885 (2005).

162 Adams, E. J., Strop, P., Shin, S., Chien, Y. H. & Garcia, K. C. An autonomous CDR3delta is sufficient for recognition of the nonclassical MHC class I molecules T10 and T22 by gammadelta T cells. *Nat Immunol* **9**, 777-784, doi:10.1038/ni.1620 (2008).

163 Kabelitz, D. Effector functions and control of human gammadelta T-cell activation. *Microbes Infect* **1**, 255-261 (1999).

164 Bukowski, J. F., Morita, C. T. & Brenner, M. B. Human gamma delta T cells recognize alkylamines derived from microbes, edible plants, and tea: implications for innate immunity. *Immunity* **11**, 57-65 (1999).

165 Morita, C. T. *et al.* Direct presentation of nonpeptide prenyl pyrophosphate antigens to human gamma delta T cells. *Immunity* **3**, 495-507 (1995).

166 Bukowski, J. F., Morita, C. T., Band, H. & Brenner, M. B. Crucial role of TCR gamma chain junctional region in prenyl pyrophosphate antigen recognition by gamma delta T cells. *J Immunol* **161**, 286-293 (1998).

167 Fisch, P. *et al.* Control of B cell lymphoma recognition via natural killer inhibitory receptors implies a role for human Vgamma9/Vdelta2 T cells in tumor immunity. *Eur J Immunol* **27**, 3368-3379 (1997).

168 Rothenfusser, S., Buchwald, A., Kock, S., Ferrone, S. & Fisch, P. Missing HLA class I expression on Daudi cells unveils cytotoxic and proliferative responses of human gammadelta T lymphocytes. *Cell Immunol* **215**, 32-44 (2002).

169 Cipriani, B. *et al.* Activation of C-C beta-chemokines in human peripheral blood gammadelta T cells by isopentenyl pyrophosphate and regulation by cytokines. *Blood* **95**, 39-47 (2000).

170 Workalemahu, G., Foerster, M. & Kroegel, C. Expression and synthesis of fibroblast growth factor-9 in human gammadelta T-lymphocytes. Response to isopentenyl pyrophosphate and TGF-beta1/IL-15. *Journal of leukocyte biology* **75**, 657-663, doi:10.1189/jlb.0902471 (2004).

171 Li, H. *et al.* Structure of the Vdelta domain of a human gammadelta T-cell antigen receptor. *Nature* **391**, 502-506, doi:10.1038/35172 (1998).

172 Rock, E. P., Sibbald, P. R., Davis, M. M. & Chien, Y. H. CDR3 length in antigen-specific immune receptors. *J Exp Med* **179**, 323-328 (1994).

173 Shin, S. *et al.* Antigen recognition determinants of gammadelta T cell receptors. *Science* **308**, 252-255, doi:10.1126/science.1106480 (2005).

174 Chen, H. *et al.* Identification of human T cell receptor gammadelta-recognized epitopes/proteins via CDR3delta peptide-based immunobiochemical strategy. *J Biol Chem* **283**, 12528-12537, doi:10.1074/jbc.M708067200 (2008).

175 Pfeffer, K., Schoel, B., Gulle, H., Kaufmann, S. H. & Wagner, H. Primary responses of human T cells to mycobacteria: a frequent set of gamma/delta T cells are stimulated by protease-resistant ligands. *Eur J Immunol* **20**, 1175-1179, doi:10.1002/eji.1830200534 (1990).

176 Belmant, C. *et al.* 3-Formyl-1-butyl pyrophosphate A novel mycobacterial metabolite-activating human gammadelta T cells. *J Biol Chem* **274**, 32079-32084 (1999).

177 Shen, Y. *et al.* Adaptive immune response of Vgamma2Vdelta2+ T cells during mycobacterial infections. *Science* **295**, 2255-2258, doi:10.1126/science.1068819 (2002).

178 Kamath, A. B. *et al.* Antigens in tea-beverage prime human Vgamma 2Vdelta 2 T cells in vitro and in vivo for memory and nonmemory antibacterial cytokine responses. *Proc Natl Acad Sci U S A* **100**, 6009-6014, doi:10.1073/pnas.1035603100 (2003).

179 Kunzmann, V. *et al.* Stimulation of gammadelta T cells by aminobisphosphonates and induction of antiplasma cell activity in multiple myeloma. *Blood* **96**, 384-392 (2000).

180 Carding, S. R. & Egan, P. J. The importance of gamma delta T cells in the resolution of pathogen-induced inflammatory immune responses. *Immunol Rev* **173**, 98-108 (2000).

181 Hintz, M. *et al.* Identification of (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate as a major activator for human gammadelta T cells in Escherichia coli. *FEBS letters* **509**, 317-322 (2001).

182 Morita, C. T. *et al.* Structural features of nonpeptide prenyl pyrophosphates that determine their antigenicity for human gamma delta T cells. *J Immunol* **167**, 36-41 (2001).

183 Constant, P. *et al.* Stimulation of human gamma delta T cells by nonpeptidic mycobacterial ligands. *Science* **264**, 267-270 (1994).

184 Gober, H. J. *et al.* Human T cell receptor gammadelta cells recognize endogenous mevalonate metabolites in tumor cells. *J Exp Med* **197**, 163-168 (2003).

185 Sireci, G. *et al.* Differential activation of human gammadelta cells by nonpeptide phosphoantigens. *Eur J Immunol* **31**, 1628-1635 (2001).

186 Eberl, M. *et al.* Accumulation of a potent gammadelta T-cell stimulator after deletion of the lytB gene in Escherichia coli. *Immunology* **106**, 200-211 (2002).

187 Lange, B. M., Rujan, T., Martin, W. & Croteau, R. Isoprenoid biosynthesis: the evolution of two ancient and distinct pathways across genomes. *Proc Natl Acad Sci U S A* **97**, 13172-13177, doi:10.1073/pnas.240454797 (2000).

188 Jomaa, H. *et al.* Vgamma9/Vdelta2 T cell activation induced by bacterial low molecular mass compounds depends on the 1-deoxy-D-xylulose 5-phosphate pathway of isoprenoid biosynthesis. *FEMS immunology and medical microbiology* **25**, 371-378 (1999).

189 Bonneville, M. & Fournie, J. J. Sensing cell stress and transformation through Vgamma9Vdelta2 T cell-mediated recognition of the isoprenoid pathway metabolites. *Microbes Infect* **7**, 503-509, doi:10.1016/j.micinf.2005.03.004 (2005).

190 Chen, Z. W. & Letvin, N. L. Adaptive immune response of Vgamma2Vdelta2 T cells: a new paradigm. *Trends Immunol* **24**, 213-219 (2003).

191 Kato, Y., Tanaka, Y., Tanaka, H., Yamashita, S. & Minato, N. Requirement of species-specific interactions for the activation of human gamma delta T cells by pamidronate. *J Immunol* **170**, 3608-3613 (2003).

192 Morita, C. T., Jin, C., Sarikonda, G. & Wang, H. Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vgamma2Vdelta2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens. *Immunol Rev* **215**, 59-76 (2007).

193 Yang, K. Y., Liu, Y. & Zhang, S. Activation of a mitogen-activated protein kinase pathway is involved in disease resistance in tobacco. *Proc Natl Acad Sci U S A* **98**, 741-746, doi:10.1073/pnas.98.2.741 (2001).

194 Poupot, M. & Fournie, J. J. Non-peptide antigens activating human Vgamma9/Vdelta2 T lymphocytes. *Immunol Lett* **95**, 129-138, doi:10.1016/j.imlet.2004.06.013 (2004).

195 Shefer, S. *et al.* Is there a relationship between 3-hydroxy-3-methylglutaryl coenzyme a reductase activity and forebrain pathology in the PKU mouse? *Journal of neuroscience research* **61**, 549-563 (2000).

196 Houten, S. M., Schneiders, M. S., Wanders, R. J. & Waterham, H. R. Regulation of isoprenoid/cholesterol biosynthesis in cells from mevalonate kinase-deficient patients. *J Biol Chem* **278**, 5736-5743, doi:10.1074/jbc.M206564200 (2003).

197 Harwood, H. J., Jr., Alvarez, I. M., Noyes, W. D. & Stacpoole, P. W. In vivo regulation of human leukocyte 3-hydroxy-3-methylglutaryl coenzyme A reductase: increased

enzyme protein concentration and catalytic efficiency in human leukemia and lymphoma. *Journal of lipid research* **32**, 1237-1252 (1991).

198 Gueddari, N. *et al.* Evidence for up-regulated low density lipoprotein receptor in human lung adenocarcinoma cell line A549. *Biochimie* **75**, 811-819 (1993).

199 Asslan, R. *et al.* Epidermal growth factor stimulates 3-hydroxy-3-methylglutaryl-coenzyme A reductase expression via the ErbB-2 pathway in human breast adenocarcinoma cells. *Biochem Biophys Res Commun* **260**, 699-706, doi:10.1006/bbrc.1999.0945 (1999).

200 Thompson, K., Rojas-Navea, J. & Rogers, M. J. Alkylamines cause Vgamma9Vdelta2 T-cell activation and proliferation by inhibiting the mevalonate pathway. *Blood* **107**, 651-654, doi:10.1182/blood-2005-03-1025 (2006).

201 Kunzmann, V., Bauer, E. & Wilhelm, M. Gamma/delta T-cell stimulation by pamidronate. *The New England journal of medicine* **340**, 737-738, doi:10.1056/NEJM199903043400914 (1999).

202 Miyagawa, F., Tanaka, Y., Yamashita, S. & Minato, N. Essential requirement of antigen presentation by monocyte lineage cells for the activation of primary human gamma delta T cells by aminobisphosphonate antigen. *J Immunol* **166**, 5508-5514 (2001).

203 Kato, Y., Tanaka, Y., Miyagawa, F., Yamashita, S. & Minato, N. Targeting of tumor cells for human gammadelta T cells by nonpeptide antigens. *J Immunol* **167**, 5092-5098 (2001).

204 Das, H., Wang, L., Kamath, A. & Bukowski, J. F. Vgamma2Vdelta2 T-cell receptor-mediated recognition of aminobisphosphonates. *Blood* **98**, 1616-1618 (2001).

205 Born, W. K., Reardon, C. L. & O'Brien, R. L. The function of gammadelta T cells in innate immunity. *Curr Opin Immunol* **18**, 31-38, doi:10.1016/j.coi.2005.11.007 (2006).

206 Selin, L. K., Stewart, S., Shen, C., Mao, H. Q. & Wilkins, J. A. Reactivity of gamma delta T cells induced by the tumour cell line RPMI 8226: functional heterogeneity of clonal populations and role of GroEL heat shock proteins. *Scand J Immunol* **36**, 107-117 (1992).

207 Scotet, E. *et al.* Tumor recognition following Vgamma9Vdelta2 T cell receptor interactions with a surface F1-ATPase-related structure and apolipoprotein A-I. *Immunity* **22**, 71-80 (2005).

208 Vantourout, P. *et al.* Specific requirements for Vgamma9Vdelta2 T cell stimulation by a natural adenylated phosphoantigen. *J Immunol* **183**, 3848-3857 (2009).

209 Kozbor, D. *et al.* Human TCR-gamma+/delta+, CD8+ T lymphocytes recognize tetanus toxoid in an MHC-restricted fashion. *J Exp Med* **169**, 1847-1851 (1989).

210 Kozbor, D. *et al.* Expression and function of gamma delta- and alpha beta-T cell receptor heterodimers on human somatic T cell hybrids. *J Immunol* **144**, 3677-3683 (1990).

211 Wright, A. *et al.* Cytotoxic T lymphocytes specific for self tumor immunoglobulin express T cell receptor delta chain. *J Exp Med* **169**, 1557-1564 (1989).

212 Sciammas, R. & Bluestone, J. A. HSV-1 glycoprotein I-reactive TCR gamma delta cells directly recognize the peptide backbone in a conformationally dependent manner. *J Immunol* **161**, 5187-5192 (1998).

213 Rust, C. J., Verreck, F., Vietor, H. & Koning, F. Specific recognition of staphylococcal enterotoxin A by human T cells bearing receptors with the V gamma 9 region. *Nature* **346**, 572-574 (1990).

214 Li, L. & Wu, C. Y. CD4+ CD25+ Treg cells inhibit human memory gammadelta T cells to produce IFN-gamma in response to M tuberculosis antigen ESAT-6. *Blood* **111**, 5629-5636, doi:10.1182/blood-2008-02-139899 (2008).

215 Brodin, P., Rosenkrands, I., Andersen, P., Cole, S. T. & Brosch, R. ESAT-6 proteins: protective antigens and virulence factors? *Trends in microbiology* **12**, 500-508, doi:10.1016/j.tim.2004.09.007 (2004).

216 Favier, B. *et al.* Uncoupling between immunological synapse formation and functional outcome in human gamma delta T lymphocytes. *J Immunol* **171**, 5027-5033 (2003).

217 Kato, Y., Tanaka, Y., Hayashi, M., Okawa, K. & Minato, N. Involvement of CD166 in the activation of human gamma delta T cells by tumor cells sensitized with nonpeptide antigens. *J Immunol* **177**, 877-884 (2006).

218 Chen, Y., Shao, L., Ali, Z., Cai, J. & Chen, Z. W. NSOM/QD-based nanoscale immunofluorescence imaging of antigen-specific T-cell receptor responses during an in vivo clonal V{gamma}2V{delta}2 T-cell expansion. *Blood* **111**, 4220-4232 (2008).

219 Ribot, J. C., debarros, A. & Silva-Santos, B. Searching for "signal 2": costimulation requirements of gammadelta T cells. *Cell Mol Life Sci* **68**, 2345-2355, doi:10.1007/s00018-011-0698-2 (2011).

220 Acuto, O. & Michel, F. CD28-mediated co-stimulation: a quantitative support for TCR signalling. *Nat Rev Immunol* **3**, 939-951, doi:10.1038/nri1248 (2003).

221 Diefenbach, A. & Raulet, D. H. Innate immune recognition by stimulatory immunoreceptors. *Curr Opin Immunol* **15**, 37-44 (2003).

222 Chan, C. J. *et al.* DNAM-1/CD155 interactions promote cytokine and NK cell-mediated suppression of poorly immunogenic melanoma metastases. *J Immunol* **184**, 902-911, doi:10.4049/jimmunol.0903225 (2010).

223 Castriconi, R. *et al.* Natural killer cell-mediated killing of freshly isolated neuroblastoma cells: critical role of DNAX accessory molecule-1-poliovirus receptor interaction. *Cancer Res* **64**, 9180-9184, doi:10.1158/0008-5472.CAN-04-2682 (2004).

224 Carlsten, M. *et al.* DNAX accessory molecule-1 mediated recognition of freshly isolated ovarian carcinoma by resting natural killer cells. *Cancer Res* **67**, 1317-1325, doi:10.1158/0008-5472.CAN-06-2264 (2007).

225 El-Sherbiny, Y. M. *et al.* The requirement for DNAM-1, NKG2D, and NKp46 in the natural killer cell-mediated killing of myeloma cells. *Cancer Res* **67**, 8444-8449, doi:10.1158/0008-5472.CAN-06-4230 (2007).

226 Lakshmikanth, T. *et al.* NCRs and DNAM-1 mediate NK cell recognition and lysis of human and mouse melanoma cell lines in vitro and in vivo. *J Clin Invest* **119**, 1251-1263, doi:10.1172/JCI36022 (2009).

227 Sanchez-Correa, B. *et al.* Decreased expression of DNAM-1 on NK cells from acute myeloid leukemia patients. *Immunology and cell biology*, doi:10.1038/icb.2011.15 (2011).

228 Carlsten, M. *et al.* Reduced DNAM-1 expression on bone marrow NK cells associated with impaired killing of CD34+ blasts in myelodysplastic syndrome. *Leukemia* **24**, 1607-1616, doi:10.1038/leu.2010.149 (2010).

229 Tahara-Hanaoka, S. *et al.* Tumor rejection by the poliovirus receptor family ligands of the DNAM-1 (CD226) receptor. *Blood* **107**, 1491-1496, doi:10.1182/blood-2005-04-1684 (2006).

230 Toutirais, O. *et al.* DNAX accessory molecule-1 (CD226) promotes human hepatocellular carcinoma cell lysis by Vgamma9Vdelta2 T cells. *Eur J Immunol* **39**, 1361-1368, doi:10.1002/eji.200838409 (2009).

231 Ribot, J. C. *et al.* CD27 is a thymic determinant of the balance between interferon-gamma- and interleukin 17-producing gammadelta T cell subsets. *Nat Immunol* **10**, 427-436, doi:10.1038/ni.1717 (2009).

232 DeBarros, A., Chaves-Ferreira, M., d'Orey, F., Ribot, J. C. & Silva-Santos, B. CD70-CD27 interactions provide survival and proliferative signals that regulate T cell receptor-driven activation of human gammadelta peripheral blood lymphocytes. *Eur J Immunol* **41**, 195-201, doi:10.1002/eji.201040905 (2011).

233 Ribot, J. C. *et al.* Cutting edge: adaptive versus innate receptor signals selectively control the pool sizes of murine IFN-gamma- or IL-17-producing gammadelta T cells upon infection. *J Immunol* **185**, 6421-6425, doi:10.4049/jimmunol.1002283 (2010).

234 Dieli, F. *et al.* Differentiation of effector/memory Vdelta2 T cells and migratory routes in lymph nodes or inflammatory sites. *J Exp Med* **198**, 391-397, doi:10.1084/jem.20030235 (2003).

235 Takeda, K. & Akira, S. Toll-like receptors in innate immunity. *International immunology* **17**, 1-14, doi:10.1093/intimm/dxh186 (2005).

236 Beetz, S. *et al.* Innate immune functions of human gammadelta T cells. *Immunobiology* **213**, 173-182 (2008).

237 Deetz, C. O. *et al.* Gamma interferon secretion by human Vgamma2Vdelta2 T cells after stimulation with antibody against the T-cell receptor plus the Toll-Like receptor 2 agonist Pam3Cys. *Infection and immunity* **74**, 4505-4511, doi:10.1128/IAI.00088-06 (2006).

238 Wesch, D. *et al.* Direct costimulatory effect of TLR3 ligand poly(I:C) on human gamma delta T lymphocytes. *J Immunol* **176**, 1348-1354 (2006).

239 Pietschmann, K. *et al.* Toll-like receptor expression and function in subsets of human gammadelta T lymphocytes. *Scand J Immunol* **70**, 245-255, doi:10.1111/j.1365-3083.2009.02290.x (2009).

240 Kunzmann, V., Kretzschmar, E., Herrmann, T. & Wilhelm, M. Polyinosinic-polycytidylic acid-mediated stimulation of human gammadelta T cells via CD11c dendritic cell-derived type I interferons. *Immunology* **112**, 369-377, doi:10.1111/j.1365-2567.2004.01908.x (2004).

241 Devilder, M. C., Allain, S., Dousset, C., Bonneville, M. & Scotet, E. Early triggering of exclusive IFN-gamma responses of human Vgamma9Vdelta2 T cells by TLR-activated myeloid and plasmacytoid dendritic cells. *J Immunol* **183**, 3625-3633, doi:10.4049/jimmunol.0901571 (2009).

242 De Maria, A., Bozzano, F., Cantoni, C. & Moretta, L. Revisiting human natural killer cell subset function revealed cytolytic CD56(dim)CD16+ NK cells as rapid producers of abundant IFN-gamma on activation. *Proc Natl Acad Sci U S A* **108**, 728-732, doi:10.1073/pnas.1012356108 (2011).

243 Angelini, D. F. *et al.* FcgammaRIII discriminates between 2 subsets of Vgamma9Vdelta2 effector cells with different responses and activation pathways. *Blood* **104**, 1801-1807 (2004).

244 Lafont, V., Liautard, J., Sable-Teychene, M., Sainte-Marie, Y. & Favero, J. Isopentenyl pyrophosphate, a mycobacterial non-peptidic antigen, triggers delayed and highly sustained signaling in human gamma delta T lymphocytes without inducing eown-modulation of T cell antigen receptor. *J Biol Chem* **276**, 15961-15967, doi:10.1074/jbc.M008684200 (2001).

245 Angelini, D. F. *et al.* NKG2A inhibits NKG2C effector functions of gammadelta T cells: implications in health and disease. *Journal of leukocyte biology* **89**, 75-84, doi:10.1189/jlb.0710413 (2011).

246 Diefenbach, A., Jensen, E. R., Jamieson, A. M. & Raulet, D. H. Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature* **413**, 165-171 (2001).

247 Lopez-Larrea, C., Suarez-Alvarez, B., Lopez-Soto, A., Lopez-Vazquez, A. & Gonzalez, S. The NKG2D receptor: sensing stressed cells. *Trends Mol Med* **14**, 179-189, doi:10.1016/j.molmed.2008.02.004 (2008).

248 Trichet, V. *et al.* Complex interplay of activating and inhibitory signals received by Vgamma9Vdelta2 T cells revealed by target cell beta2-microglobulin knockdown. *J Immunol* **177**, 6129-6136 (2006).

249 Lesport, E. *et al.* Inhibition of human Vgamma9Vdelta2 T-cell antitumoral activity through HLA-G: implications for immunotherapy of cancer. *Cell Mol Life Sci* **68**, 3385-3399, doi:10.1007/s00018-011-0632-7 (2011).

250 von Lilienfeld-Toal, M. *et al.* Activated gammadelta T cells express the natural cytotoxicity receptor natural killer p 44 and show cytotoxic activity against myeloma cells. *Clin Exp Immunol* **144**, 528-533, doi:10.1111/j.1365-2249.2006.03078.x (2006).

251 Groh, V. *et al.* Broad tumor-associated expression and recognition by tumor-derived gamma delta T cells of MICA and MICB. *Proc Natl Acad Sci U S A* **96**, 6879-6884 (1999).

252 Corvaisier, M. *et al.* V gamma 9V delta 2 T cell response to colon carcinoma cells. *J Immunol* **175**, 5481-5488 (2005).

253 Todaro, M. *et al.* Efficient killing of human colon cancer stem cells by gammadelta T lymphocytes. *J Immunol* **182**, 7287-7296 (2009).

254 Wrobel, P. *et al.* Lysis of a broad range of epithelial tumour cells by human gamma delta T cells: involvement of NKG2D ligands and T-cell receptor- versus NKG2D-dependent recognition. *Scand J Immunol* **66**, 320-328 (2007).

255 Kunzmann, V. & Wilhelm, M. Anti-lymphoma effect of gammadelta T cells. *Leuk Lymphoma* **46**, 671-680 (2005).

256 Rincon-Orozco, B. *et al.* Activation of V gamma 9V delta 2 T cells by NKG2D. *J Immunol* **175**, 2144-2151 (2005).

257 Poggi, A. *et al.* Differential survival of gammadeltaT cells, alphabetaT cells and NK cells upon engagement of NKG2D by NKG2DL-expressing leukemic cells. *Int J Cancer* **129**, 387-396, doi:10.1002/ijc.25682 (2011).

258 Kabelitz, D., Wesch, D. & He, W. Perspectives of gammadelta T cells in tumor immunology. *Cancer Res* **67**, 5-8 (2007).

259 Ensslin, A. S. & Formby, B. Comparison of cytolytic and proliferative activities of human gamma delta and alpha beta T cells from peripheral blood against various human tumor cell lines. *J Natl Cancer Inst* **83**, 1564-1569 (1991).

260 Guo, B. L., Liu, Z., Aldrich, W. A. & Lopez, R. D. Innate anti-breast cancer immunity of apoptosis-resistant human gammadelta-T cells. *Breast Cancer Res Treat* **93**, 169-175 (2005).

261 Zheng, B. J. *et al.* Anti-tumor effects of human peripheral gammadelta T cells in a mouse tumor model. *Int J Cancer* **92**, 421-425 (2001).

262 Kabelitz, D., Wesch, D., Pitters, E. & Zoller, M. Characterization of tumor reactivity of human V gamma 9V delta 2 gamma delta T cells in vitro and in SCID mice in vivo. *J Immunol* **173**, 6767-6776 (2004).

263 Sicard, H., Al Saati, T., Delsol, G. & Fournie, J. J. Synthetic phosphoantigens enhance human Vgamma9Vdelta2 T lymphocytes killing of non-Hodgkin's B lymphoma. *Mol Med* **7**, 711-722 (2001).

264 Fisch, P. *et al.* Recognition by human V gamma 9/V delta 2 T cells of a GroEL homolog on Daudi Burkitt's lymphoma cells. *Science* **250**, 1269-1273 (1990).

265 Sturm, E. *et al.* Human V gamma 9-V delta 2 T cell receptor-gamma delta lymphocytes show specificity to Daudi Burkitt's lymphoma cells. *J Immunol* **145**, 3202-3208 (1990).

266 Davodeau, F. *et al.* Close correlation between Daudi and mycobacterial antigen recognition by human gamma delta T cells and expression of V9JPC1 gamma/V2DJC delta-encoded T cell receptors. *J Immunol* **151**, 1214-1223 (1993).

267 L'Faqihi, F. E. *et al.* Acquisition of a stimulatory activity for Vgamma9/Vdelta2 T cells by a Burkitt's lymphoma cell line without loss of HLA class I expression. *Hum Immunol* **60**, 928-938 (1999).

268 Halary, F. *et al.* Control of self-reactive cytotoxic T lymphocytes expressing gamma delta T cell receptors by natural killer inhibitory receptors. *Eur J Immunol* **27**, 2812-2821, doi:10.1002/eji.1830271111 (1997).

269 Carena, I., Shamshiev, A., Donda, A., Colonna, M. & Libero, G. D. Major histocompatibility complex class I molecules modulate activation threshold and early signaling of T cell antigen receptor-gamma/delta stimulated by nonpeptidic ligands. *J Exp Med* **186**, 1769-1774 (1997).

270 Poccia, F. *et al.* CD94/NKG2 inhibitory receptor complex modulates both anti-viral and anti-tumoral responses of polyclonal phosphoantigen-reactive V gamma 9V delta 2 T lymphocytes. *J Immunol* **159**, 6009-6017 (1997).

271 Thedrez, A. *et al.* Self/non-self discrimination by human gammadelta T cells: simple solutions for a complex issue? *Immunol Rev* **215**, 123-135 (2007).

272 Li, J. *et al.* Reduced expression of the mevalonate pathway enzyme farnesyl pyrophosphate synthase unveils recognition of tumor cells by Vgamma9Vdelta2 T cells. *J Immunol* **182**, 8118-8124 (2009).

273 Fisch, P. *et al.* Gamma/delta T cell clones and natural killer cell clones mediate distinct patterns of non-major histocompatibility complex-restricted cytolysis. *J Exp Med* **171**, 1567-1579 (1990).

274 Qin, Z. *et al.* A critical requirement of interferon gamma-mediated angiostasis for tumor rejection by CD8+ T cells. *Cancer Res* **63**, 4095-4100 (2003).

275 Santini, D. *et al.* Pamidronate induces modifications of circulating angiogenetic factors in cancer patients. *Clin Cancer Res* **8**, 1080-1084 (2002).

276 Lozupone, F. *et al.* Effect of human natural killer and gammadelta T cells on the growth of human autologous melanoma xenografts in SCID mice. *Cancer Res* **64**, 378-385 (2004).

277 Malkovska, V., Cigel, F. K., Armstrong, N., Storer, B. E. & Hong, R. Antilymphoma activity of human gamma delta T-cells in mice with severe combined immune deficiency. *Cancer Res* **52**, 5610-5616 (1992).

278 Yuasa, T. *et al.* Intravesical administration of gammadelta T cells successfully prevents the growth of bladder cancer in the murine model. *Cancer Immunol Immunother* **58**, 493-502 (2009).

279 Otto, M. *et al.* Combination immunotherapy with clinical-scale enriched human gammadelta T cells, hu14.18 antibody, and the immunocytokine Fc-IL7 in disseminated neuroblastoma. *Clin Cancer Res* **11**, 8486-8491 (2005).

280 Beck, B. H. *et al.* Adoptively transferred ex vivo expanded gammadelta-T cells mediate in vivo antitumor activity in preclinical mouse models of breast cancer. *Breast Cancer Res Treat* (2009).

281 Simoni, D. *et al.* Design, synthesis, and biological evaluation of novel aminobisphosphonates possessing an in vivo antitumor activity through a gammadelta-T lymphocytes-mediated activation mechanism. *J Med Chem* **51**, 6800-6807 (2008).

282 Malkovska, V., Cigel, F. & Storer, B. E. Human T cells in hu-PBL-SCID mice proliferate in response to Daudi lymphoma and confer anti-tumour immunity. *Clin Exp Immunol* **96**, 158-165 (1994).

283 Tokuyama, H. *et al.* V gamma 9 V delta 2 T cell cytotoxicity against tumor cells is enhanced by monoclonal antibody drugs--rituximab and trastuzumab. *Int J Cancer* **122**, 2526-2534 (2008).

284 Chen, J., Niu, H., He, W. & Ba, D. Antitumor activity of expanded human tumor-infiltrating gammadelta T lymphocytes. *International archives of allergy and immunology* **125**, 256-263 (2001).

285 Kondo, M. *et al.* Zoledronate facilitates large-scale ex vivo expansion of functional gammadelta T cells from cancer patients for use in adoptive immunotherapy. *Cytotherapy* **10**, 842-856 (2008).

286 Abe, Y. *et al.* Clinical and immunological evaluation of zoledronate-activated Vgamma9gammadelta T-cell-based immunotherapy for patients with multiple myeloma. *Exp Hematol* **37**, 956-968 (2009).

287 Kobayashi, H. *et al.* Safety profile and anti-tumor effects of adoptive immunotherapy using gamma-delta T cells against advanced renal cell carcinoma: a pilot study. *Cancer Immunol Immunother* **56**, 469-476 (2007).

288 Dieli, F. *et al.* Induction of gammadelta T-lymphocyte effector functions by bisphosphonate zoledronic acid in cancer patients in vivo. *Blood* **102**, 2310-2311 (2003).

289 Wilhelm, M. *et al.* Gammadelta T cells for immune therapy of patients with lymphoid malignancies. *Blood* **102**, 200-206 (2003).

290 Dieli, F. *et al.* Targeting human {gamma}delta} T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. *Cancer Res* **67**, 7450-7457, doi:10.1158/0008-5472.CAN-07-0199 (2007).

291 Bennouna, J. *et al.* Phase-I study of Innacell gammadelta, an autologous cell-therapy product highly enriched in gamma9delta2 T lymphocytes, in combination with IL-2, in patients with metastatic renal cell carcinoma. *Cancer Immunol Immunother* **57**, 1599-1609 (2008).

292 Ali, Z. *et al.* Prolonged (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate-driven antimicrobial and cytotoxic responses of pulmonary and systemic Vgamma2Vdelta2 T cells in macaques. *J Immunol* **179**, 8287-8296 (2007).

293 Sicard, H. *et al.* In vivo immunomanipulation of V gamma 9V delta 2 T cells with a synthetic phosphoantigen in a preclinical nonhuman primate model. *J Immunol* **175**, 5471-5480 (2005).

294 Li, B. *et al.* Disease-specific changes in gammadelta T cell repertoire and function in patients with pulmonary tuberculosis. *J Immunol* **157**, 4222-4229 (1996).

295 Poccia, F. *et al.* Peripheral V gamma 9/V delta 2 T cell deletion and anergy to nonpeptidic mycobacterial antigens in asymptomatic HIV-1-infected persons. *J Immunol* **157**, 449-461 (1996).

296 Jin, H. T., Ahmed, R. & Okazaki, T. Role of PD-1 in regulating T-cell immunity. *Current topics in microbiology and immunology* **350**, 17-37, doi:10.1007/82_2010_116 (2011).

297 Zhou, Q. *et al.* Coexpression of Tim-3 and PD-1 identifies a CD8+ T-cell exhaustion phenotype in mice with disseminated acute myelogenous leukemia. *Blood* **117**, 4501-4510, doi:10.1182/blood-2010-10-310425 (2011).

298 Iwasaki, M. *et al.* Expression and function of PD-1 in human gammadelta T cells that recognize phosphoantigens. *Eur J Immunol* **41**, 345-355, doi:10.1002/eji.201040959 (2011).

299 Gomes, A. Q., Martins, D. S. & Silva-Santos, B. Targeting gammadelta T lymphocytes for cancer immunotherapy: from novel mechanistic insight to clinical application. *Cancer Res* **70**, 10024-10027, doi:10.1158/0008-5472.CAN-10-3236 (2010).

300 Kobayashi, H., Tanaka, Y., Shimmura, H., Minato, N. & Tanabe, K. Complete remission of lung metastasis following adoptive immunotherapy using activated autologous gammadelta T-cells in a patient with renal cell carcinoma. *Anticancer Res* **30**, 575-579 (2010).

301 Bonneville, M. & Scotet, E. Human Vgamma9Vdelta2 T cells: promising new leads for immunotherapy of infections and tumors. *Curr Opin Immunol* **18**, 539-546 (2006).

302 Wei, H. *et al.* Definition of APC presentation of phosphoantigen (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate to Vgamma2Vdelta2 TCR. *J Immunol* **181**, 4798-4806 (2008).

303 Barata, J. T. *et al.* Activation of PI3K is indispensable for interleukin 7-mediated viability, proliferation, glucose use, and growth of T cell acute lymphoblastic leukemia cells. *J Exp Med* **200**, 659-669 (2004).

304 Casetti, R. *et al.* Drug-induced expansion and differentiation of V gamma 9V delta 2 T cells in vivo: the role of exogenous IL-2. *J Immunol* **175**, 1593-1598 (2005).

305 Yamashita, S. *et al.* Analysis of mechanism for human gammadelta T cell recognition of nonpeptide antigens. *Biochem Biophys Res Commun* **334**, 349-360 (2005).

306 Dietrich, J. *et al.* Ligand-induced TCR down-regulation is not dependent on constitutive TCR cycling. *J Immunol* **168**, 5434-5440 (2002).

307 Naramura, M. *et al.* c-Cbl and Cbl-b regulate T cell responsiveness by promoting ligand-induced TCR down-modulation. *Nat Immunol* **3**, 1192-1199 (2002).

308 Vermijlen, D. *et al.* Distinct cytokine-driven responses of activated blood gammadelta T cells: insights into unconventional T cell pleiotropy. *J Immunol* **178**, 4304-4314 (2007).

309 Shu, S. T. *et al.* A novel bioluminescent mouse model and effective therapy for adult T-cell leukemia/lymphoma. *Cancer Res* **67**, 11859-11866 (2007).

310 Bukowski, J. F. *et al.* V gamma 2V delta 2 TCR-dependent recognition of non-peptide antigens and Daudi cells analyzed by TCR gene transfer. *J Immunol* **154**, 998-1006 (1995).

311 Devilder, M. C. *et al.* Potentiation of antigen-stimulated V gamma 9V delta 2 T cell cytokine production by immature dendritic cells (DC) and reciprocal effect on DC maturation. *J Immunol* **176**, 1386-1393 (2006).

312 Ismaili, J., Olislagers, V., Poupot, R., Fournie, J. J. & Goldman, M. Human gamma delta T cells induce dendritic cell maturation. *Clin Immunol* **103**, 296-302 (2002).

313 Eberl, M. *et al.* A rapid crosstalk of human gammadelta T cells and monocytes drives the acute inflammation in bacterial infections. *PLoS Pathog* **5**, e1000308, doi:10.1371/journal.ppat.1000308 (2009).

314 Pennington, D. J. *et al.* The integration of conventional and unconventional T cells that characterizes cell-mediated responses. *Adv Immunol* **87**, 27-59 (2005).

315 Gao, Y. *et al.* Gamma delta T cells provide an early source of interferon gamma in tumor immunity. *J Exp Med* **198**, 433-442 (2003).

316 Liu, Z. *et al.* Protective immunosurveillance and therapeutic antitumor activity of gammadelta T cells demonstrated in a mouse model of prostate cancer. *J Immunol* **180**, 6044-6053 (2008).

317 Correia, D. V. *et al.* Highly active microbial phosphoantigen induces rapid yet sustained MEK/Erk- and PI-3K/Akt-mediated signal transduction in anti-tumor human gammadelta T-cells. *PLoS One* **4**, e5657, doi:10.1371/journal.pone.0005657 (2009).

318 Gertner-Dardenne, J. *et al.* Bromohydrin pyrophosphate enhances antibody-dependent cell-mediated cytotoxicity induced by therapeutic antibodies. *Blood* **113**, 4875-4884 (2009).

319 Lanca, T. *et al.* The MHC class Ib protein ULBP1 is a nonredundant determinant of leukemia/lymphoma susceptibility to gammadelta T-cell cytotoxicity. *Blood* **115**, 2407-2411, doi:10.1182/blood-2009-08-237123 (2010).

320 Moffat, J. *et al.* A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. *Cell* **124**, 1283-1298, doi:10.1016/j.cell.2006.01.040 (2006).

321 R: A Language and Environment for Statistical Computing (Vienna, Austria, 2009).

322 Gentleman, R. C. *et al.* Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* **5**, R80 (2004).

323 Gautier, L., Cope, L., Bolstad, B. M. & Irizarry, R. A. affy--analysis of Affymetrix GeneChip data at the probe level. *Bioinformatics* **20**, 307-315 (2004).

324 Smyth, G. K. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* **3**, Article3 (2004).

325 Smyth, G. K. in *Bioinformatics and Computational Biology Solutions using R and Bioconductor* (ed W. Huber R. Gentleman and V. Carey and S. Dudoit and R. Irizarry) 397-420 (Springer, 2005).

326 Benjamini, Y. H., Y. Controlling the false discovery rate: a pratical and powerful approach to multiple testing. *Journal of the Royal Statistical Society Series B* **57**, 289-300 (1995).

327 Ringner, M. What is principal component analysis? *Nat Biotechnol* **26**, 303-304 (2008).

328 Sole, X. *et al.* Biological convergence of cancer signatures. *PLoS One* **4**, e4544 (2009).

329 Yang, Y. *et al.* The interferon-inducible 9-27 gene modulates the susceptibility to natural killer cells and the invasiveness of gastric cancer cells. *Cancer Lett* **221**, 191-200 (2005).

330 Pellet-Many, C., Frankel, P., Jia, H. & Zachary, I. Neuropilins: structure, function and role in disease. *Biochem J* **411**, 211-226 (2008).

331 Guerra, N. *et al.* NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. *Immunity* **28**, 571-580 (2008).

332 Strid, J. *et al.* Acute upregulation of an NKG2D ligand promotes rapid reorganization of a local immune compartment with pleiotropic effects on carcinogenesis. *Nat Immunol* **9**, 146-154 (2008).

333 Gomes, A. Q. *et al.* Identification of a panel of ten cell surface protein antigens associated with immunotargeting of leukemias and lymphomas by peripheral blood gammadelta T cells. *Haematologica* **95**, 1397-1404, doi:10.3324/haematol.2009.020602 (2010).

334 Maeurer, M. J. *et al.* Human intestinal Vdelta1+ lymphocytes recognize tumor cells of epithelial origin. *J Exp Med* **183**, 1681-1696 (1996).

335 Choudhary, A. *et al.* Selective lysis of autologous tumor cells by recurrent gamma delta tumor-infiltrating lymphocytes from renal carcinoma. *J Immunol* **154**, 3932-3940 (1995).

336 Greaves, M., Janossy, G. & Doenhoff, M. Selective triggering of human T and B lymphocytes in vitro by polyclonal mitogens. *J Exp Med* **140**, 1-18 (1974).

337 Lee, K. M. *et al.* Requirement of homotypic NK-cell interactions through 2B4(CD244)/CD48 in the generation of NK effector functions. *Blood* **107**, 3181-3188, doi:10.1182/blood-2005-01-0185 (2006).

338 Behr, C., Couzi, L., Taupin, J.-L. & Déchanet-Merville, J. Vdelta2neg gamma delta T Cells, a Multi-Reactive Tissue Subset: from Innate to Adaptive Altered-Self Surveillance. *The Open Immunology Journal* **2**, 106-118 (2009).

339 Chilson, O. P. & Kelly-Chilson, A. E. Mitogenic lectins bind to the antigen receptor on human lymphocytes. *Eur J Immunol* **19**, 389-396, doi:10.1002/eji.1830190225 (1989).

340 Cantrell, D. Protein kinase B (Akt) regulation and function in T lymphocytes. *Semin Immunol* **14**, 19-26, doi:10.1006/smim.2001.0338 (2002).

341 Alexander, A. A. *et al.* Isopentenyl pyrophosphate-activated CD56+ {gamma}{delta} T lymphocytes display potent antitumor activity toward human squamous cell carcinoma. *Clin Cancer Res* **14**, 4232-4240 (2008).

342 Correia, M. P. *et al.* Hepatocytes and IL-15: a favorable microenvironment for T cell survival and CD8+ T cell differentiation. *J Immunol* **182**, 6149-6159, doi:10.4049/jimmunol.0802470 (2009).

343 Carbone, E. *et al.* HLA class I, NKG2D, and natural cytotoxicity receptors regulate multiple myeloma cell recognition by natural killer cells. *Blood* **105**, 251-258, doi:10.1182/blood-2004-04-1422 (2005).

344 Raulet, D. H. & Guerra, N. Oncogenic stress sensed by the immune system: role of natural killer cell receptors. *Nat Rev Immunol* **9**, 568-580, doi:10.1038/nri2604 (2009).

345 De Rosa, S. C. *et al.* Ontogeny of gamma delta T cells in humans. *J Immunol* **172**, 1637-1645 (2004).

346 Vermijlen, D. *et al.* Human cytomegalovirus elicits fetal gammadelta T cell responses in utero. *J Exp Med* **207**, 807-821, doi:10.1084/jem.20090348 (2010).

347 Boullier, S., Cochet, M., Poccia, F. & Gougeon, M. L. CDR3-independent gamma delta V delta 1+ T cell expansion in the peripheral blood of HIV-infected persons. *J Immunol* **154**, 1418-1431 (1995).

348 Poggi, A. *et al.* Vdelta1 T lymphocytes from B-CLL patients recognize ULBP3 expressed on leukemic B cells and up-regulated by trans-retinoic acid. *Cancer Res* **64**, 9172-9179 (2004).

349 Catellani, S. *et al.* Expansion of Vdelta1 T lymphocytes producing IL-4 in low-grade non-Hodgkin lymphomas expressing UL-16-binding proteins. *Blood* **109**, 2078-2085, doi:10.1182/blood-2006-06-028985 (2007).

350 Zhao, H., Nguyen, H. & Kang, J. Interleukin 15 controls the generation of the restricted T cell receptor repertoire of gamma delta intestinal intraepithelial lymphocytes. *Nat Immunol* **6**, 1263-1271, doi:10.1038/ni1267 (2005).

351 Siegers, G. M. *et al.* Human Vdelta1 gammadelta T cells expanded from peripheral blood exhibit specific cytotoxicity against B-cell chronic lymphocytic leukemia-derived cells. *Cytotherapy* **13**, 753-764, doi:10.3109/14653249.2011.553595 (2011).

352 Miyagawa, F. *et al.* Essential contribution of germline-encoded lysine residues in Jgamma1.2 segment to the recognition of nonpeptide antigens by human gammadelta T cells. *J Immunol* **167**, 6773-6779 (2001).

353 Cipriani, B., Knowles, H., Chen, L., Battistini, L. & Brosnan, C. F. Involvement of classical and novel protein kinase C isoforms in the response of human V gamma 9V delta 2 T cells to phosphate antigens. *J Immunol* **169**, 5761-5770 (2002).

354 Wang, H., Fang, Z. & Morita, C. T. Vgamma2Vdelta2 T Cell Receptor recognition of prenyl pyrophosphates is dependent on all CDRs. *J Immunol* **184**, 6209-6222, doi:10.4049/jimmunol.1000231 (2010).

355 Mookerjee-Basu, J. *et al.* F1-adenosine triphosphatase displays properties characteristic of an antigen presentation molecule for Vgamma9Vdelta2 T cells. *J Immunol* **184**, 6920-6928, doi:10.4049/jimmunol.0904024 (2010).

356 Placido, R. *et al.* Characterization of the immune response of human cord-blood derived gamma/delta T cells to stimulation with aminobisphosphonate compounds. *International journal of immunopathology and pharmacology* **24**, 101-110 (2011).

357 Puan, K. J. *et al.* Preferential recognition of a microbial metabolite by human Vgamma2Vdelta2 T cells. *International immunology* **19**, 657-673, doi:10.1093/intimm/dxm031 (2007).

358 Moens, E. *et al.* IL-23R and TCR signaling drives the generation of neonatal Vgamma9Vdelta2 T cells expressing high levels of cytotoxic mediators and producing IFN-gamma and IL-17. *Journal of leukocyte biology* **89**, 743-752, doi:10.1189/jlb.0910501 (2011).

359 Sarikonda, G. *et al.* Photoaffinity antigens for human gammadelta T cells. *J Immunol* **181**, 7738-7750 (2008).

360 Kabelitz, D. Human gammadelta T lymphocytes for immunotherapeutic strategies against cancer. *F1000 medicine reports* **2**, doi:10.3410/M2-45 (2010).

361 Li, H. & David Pauza, C. Interplay of T-cell receptor and interleukin-2 signalling in Vgamma2Vdelta2 T-cell cytotoxicity. *Immunology* **132**, 96-103, doi:10.1111/j.1365-2567.2010.03343.x (2011).

362 Jensen, K. D. *et al.* Thymic selection determines gammadelta T cell effector fate: antigen-naive cells make interleukin-17 and antigen-experienced cells make interferon gamma. *Immunity* **29**, 90-100, doi:10.1016/j.immuni.2008.04.022 (2008).

363 Lafont, V. *et al.* Specific signaling pathways triggered by IL-2 in human V gamma 9V delta 2 T cells: an amalgamation of NK and alpha beta T cell signaling. *J Immunol* **171**, 5225-5232 (2003).

364 Bessoles, S. *et al.* IL-2 triggers specific signaling pathways in human NKT cells leading to the production of pro- and anti-inflammatory cytokines. *Journal of leukocyte biology* **84**, 224-233, doi:10.1189/jlb.1007669 (2008).

365 Hacker, G. *et al.* V delta 1+ subset of human gamma delta T cells responds to ligands expressed by EBV-infected Burkitt lymphoma cells and transformed B lymphocytes. *J Immunol* **149**, 3984-3989 (1992).

366 Zeuthen, J. *et al.* Human lymphoma-lymphoma hybrids and lymphoma-leukemia hybrids. I. Isolation, characterization, cell surface markers, and B-cell markers. *J Natl Cancer Inst* **68**, 179-195 (1982).

367 Viey, E. *et al.* Phosphostim-activated gamma delta T cells kill autologous metastatic renal cell carcinoma. *J Immunol* **174**, 1338-1347 (2005).

368 Bouet-Toussaint, F. *et al.* Vgamma9Vdelta2 T cell-mediated recognition of human solid tumors. Potential for immunotherapy of hepatocellular and colorectal carcinomas. *Cancer Immunol Immunother* **57**, 531-539, doi:10.1007/s00262-007-0391-3 (2008).

369 Wang, P. & Malkovsky, M. Different roles of the CD2 and LFA-1 T-cell co-receptors for regulating cytotoxic, proliferative, and cytokine responses of human V gamma 9/V delta 2 T cells. *Mol Med* **6**, 196-207 (2000).

370 Kamishikiryo, J., Fukuhara, H., Okabe, Y., Kuroki, K. & Maenaka, K. Molecular basis for LLT1 protein recognition by human CD161 protein (NKRP1A/KLRB1). *J Biol Chem* **286**, 23823-23830, doi:10.1074/jbc.M110.214254 (2011).

371 Detre, C., Keszei, M., Romero, X., Tsokos, G. C. & Terhorst, C. SLAM family receptors and the SLAM-associated protein (SAP) modulate T cell functions. *Seminars in immunopathology* **32**, 157-171, doi:10.1007/s00281-009-0193-0 (2010).

372 Veillette, A. NK cell regulation by SLAM family receptors and SAP-related adapters. *Immunol Rev* **214**, 22-34, doi:10.1111/j.1600-065X.2006.00453.x (2006).

373 Pont, F. *et al.* The gene expression profile of phosphoantigen-specific human gammadelta T lymphocytes is a blend of alphabeta T cell and NK cell signatures. *Eur J Immunol*, doi:10.1002/eji.201141870 (2011).

374 Cerwenka, A., Baron, J. L. & Lanier, L. L. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. *Proc Natl Acad Sci U S A* **98**, 11521-11526, doi:10.1073/pnas.201238598 (2001).

375 Sutherland, C. L. *et al.* ULBPs, human ligands of the NKG2D receptor, stimulate tumor immunity with enhancement by IL-15. *Blood* **108**, 1313-1319, doi:10.1182/blood-2005-11-011320 (2006).

376 Schulz, U. *et al.* Interleukin-10 promotes NK cell killing of autologous macrophages by stimulating expression of NKG2D ligands. *Scand J Immunol* **72**, 319-331, doi:10.1111/j.1365-3083.2010.02435.x (2010).

377 Butler, J. E. *et al.* Proteasome regulation of ULBP1 transcription. *J Immunol* **182**, 6600-6609, doi:10.4049/jimmunol.0801214 (2009).

378 Bae, J. H. *et al.* The susceptibility to NK cell-mediated lysis of colon cancer cells is enhanced by treatment with EGFR inhibitors through induction of ULBP1. *Cancer science*, doi:10.1111/j.1349-7006.2011.02109.x (2011).

379 Kang, N. *et al.* Adoptive immunotherapy of lung cancer with immobilized anti-TCRgammadelta antibody-expanded human gammadelta T-cells in peripheral blood. *Cancer biology & therapy* **8**, 1540-1549 (2009).

380 Zhou, J., Kang, N., Cui, L., Ba, D. & He, W. Anti-gammadelta TCR antibody-expanded gammadelta T cells: a better choice for the adoptive immunotherapy of lymphoid malignancies. *Cellular & molecular immunology*, doi:10.1038/cmi.2011.16 (2011).

381 Schilbach, K., Frommer, K., Meier, S., Handgretinger, R. & Eyrich, M. Immune response of human propagated gammadelta-T-cells to neuroblastoma recommend the Vdelta1+ subset for gammadelta-T-cell-based immunotherapy. *J Immunother* **31**, 896-905, doi:10.1097/CJI.0b013e31818955ad (2008).

382 Correia, M. P., Costa, A. V., Uhrberg, M., Cardoso, E. M. & Arosa, F. A. IL-15 induces CD8+ T cells to acquire functional NK receptors capable of modulating cytotoxicity and cytokine secretion. *Immunobiology* **216**, 604-612, doi:10.1016/j.imbio.2010.09.012 (2011).

383 Li, P. *et al.* Reprogramming of T cells to natural killer-like cells upon Bcl11b deletion. *Science* **329**, 85-89, doi:10.1126/science.1188063 (2010).

384 Deusch, K. *et al.* A major fraction of human intraepithelial lymphocytes simultaneously expresses the gamma/delta T cell receptor, the CD8 accessory molecule and preferentially uses the V delta 1 gene segment. *Eur J Immunol* **21**, 1053-1059, doi:10.1002/eji.1830210429 (1991).

385 Fourcade, J. *et al.* Upregulation of Tim-3 and PD-1 expression is associated with tumor antigen-specific CD8+ T cell dysfunction in melanoma patients. *J Exp Med* **207**, 2175-2186, doi:10.1084/jem.20100637 (2010).

386 Goncalves-Sousa, N. *et al.* Inhibition of murine gammadelta lymphocyte expansion and effector function by regulatory alphabeta T cells is cell-contact-dependent and sensitive to GITR modulation. *Eur J Immunol* **40**, 61-70, doi:10.1002/eji.200939715 (2010).

387 Kunzmann, V., Kimmel, B., Herrmann, T., Einsele, H. & Wilhelm, M. Inhibition of phosphoantigen-mediated gammadelta T-cell proliferation by CD4+ CD25+ FoxP3+ regulatory T cells. *Immunology* **126**, 256-267, doi:10.1111/j.1365-2567.2008.02894.x (2009).

388 Pule, M. A. *et al.* Virus-specific T cells engineered to coexpress tumor-specific receptors: persistence and antitumor activity in individuals with neuroblastoma. *Nat Med* **14**, 1264-1270, doi:10.1038/nm.1882 (2008).

389 Cabillic, F. *et al.* Aminobisphosphonate-pretreated dendritic cells trigger successful Vgamma9Vdelta2 T cell amplification for immunotherapy in advanced cancer

patients. *Cancer Immunol Immunother* **59**, 1611-1619, doi:10.1007/s00262-010-0887-0 (2010).

390 Mariani, S. *et al.* Effector gammadelta T cells and tumor cells as immune targets of zoledronic acid in multiple myeloma. *Leukemia* **19**, 664-670, doi:10.1038/sj.leu.2403693 (2005).

391 Capietto, A. H., Martinet, L. & Fournie, J. J. How tumors might withstand gammadelta T-cell attack. *Cell Mol Life Sci* **68**, 2433-2442, doi:10.1007/s00018-011-0705-7 (2011).

392 Uccelli, A., Moretta, L. & Pistoia, V. Immunoregulatory function of mesenchymal stem cells. *Eur J Immunol* **36**, 2566-2573, doi:10.1002/eji.200636416 (2006).

393 Marten, A., von Lilienfeld-Toal, M., Buchler, M. W. & Schmidt, J. Soluble MIC is elevated in the serum of patients with pancreatic carcinoma diminishing gammadelta T cell cytotoxicity. *Int J Cancer* **119**, 2359-2365, doi:10.1002/ijc.22186 (2006).

394 Sutlu, T. *et al.* Clinical-grade, large-scale, feeder-free expansion of highly active human natural killer cells for adoptive immunotherapy using an automated bioreactor. *Cytotherapy* **12**, 1044-1055, doi:10.3109/14653249.2010.504770 (2010).

395 Siegler, U. *et al.* Good manufacturing practice-compliant cell sorting and large-scale expansion of single KIR-positive alloreactive human natural killer cells for multiple infusions to leukemia patients. *Cytotherapy* **12**, 750-763, doi:10.3109/14653241003786155 (2010).

396 Spanholtz, J. *et al.* Clinical-grade generation of active NK cells from cord blood hematopoietic progenitor cells for immunotherapy using a closed-system culture process. *PLoS One* **6**, e20740, doi:10.1371/journal.pone.0020740 (2011).

397 Di Ianni, M. *et al.* Immunoselection and clinical use of T regulatory cells in HLA-haploidentical stem cell transplantation. *Best practice & research. Clinical haematology* **24**, 459-466, doi:10.1016/j.beha.2011.05.005 (2011).

398 Keever-Taylor, C. A. *et al.* Characteristics of CliniMACS((R)) System CD34-Enriched T Cell-Depleted Grafts in a Multicenter Trial for Acute Myeloid Leukemia-Blood and Marrow Transplant Clinical Trials Network (BMT CTN) Protocol 0303. *Biol Blood Marrow Transplant*, doi:10.1016/j.bbmt.2011.08.017 (2011).

399 Hayat, M. J., Howlader, N., Reichman, M. E. & Edwards, B. K. Cancer statistics, trends, and multiple primary cancer analyses from the Surveillance, Epidemiology, and End Results (SEER) Program. *The oncologist* **12**, 20-37, doi:10.1634/theoncologist.12-1-20 (2007).

APPENDIX - Peer-reviewed articles associated to this thesis:

1. Correia DV*****, d'Orey F*****, Cardoso BA, Lança T, Grosso AR, deBarros A, Martins LR, Barata T, Silva-Santos B. **Highly active microbial phosphoantigen induces rapid yet sustained MEK/Erk- and PI-3K/Akt-mediated signal transduction in anti-tumor human γδ T-cells.** PLoS One. **2009** ; 4(5):e5657. *co-first authors

2. Gomes AQ*****, Correia DV*****, Grosso AR, Lança T, Ferreira C, Lacerda JF, Barata T, Gomes da Silva M, Silva-Santos B. **Identification of a panel of ten cell surface protein antigens associated with immunotargeting of leukemias and lymphomas by peripheral blood gamma-delta T cells.** Haematologica. **2010** ; 95(8):1397-404. Epub 2010/03/12. *co-first authors

3. Correia DV, Fogli M, Hudspeth K, da Silva MG, Mavilio D, Silva-Santos B. **Differentiation of human peripheral blood Vδ1^{+} T cells expressing the natural cytotoxicity receptor NKp30 for recognition of lymphoid leukemia cells.** Blood. **2011** ; 118(4):992-1001. Epub 2011/06/03.

4. Lança T, Correia DV, Moita CF, Raquel H, Neves-Costa A, Ferreira C, Ramalho JS, Barata JT, Moita LF, Gomes AQ, Silva-Santos B. **The MHC class Ib protein ULBP1 is a nonredundant determinant of leukemia/lymphoma susceptibility to gammadelta T-cell cytotoxicity**. Blood. **2010** ; **115**(12): 2407-11.

5. Goncalves-Sousa N, Ribot JC, deBarros A, Correia DV, Caramalho I, Silva-Santos B. **Inhibition of murine gammadelta lymphocyte expansion and effector function by regulatory alphabeta T cells is cell-contact-dependent and sensitive to GITR modulation**. Eur J Immunol. **2010** ; **40**(1): 61-70.

6. Gomes AQ, Correia DV, Silva-Santos B. **Non-classical major histocompatibility complex proteins as determinants of tumour immunosurveillance.** EMBO Rep. **2007** ; **8**(11): 1024-30.

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Highly Active Microbial Phosphoantigen Induces Rapid yet Sustained MEK/Erk- and PI-3K/Akt-Mediated Signal Transduction in Anti-Tumor Human γδ T-Cells

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Abstract

Background: The unique responsiveness of Vγ9Vδ2 T-cells, the major γδ subset of human peripheral blood, to non-peptidic prenyl pyrophosphate antigens constitutes the basis of current γδ T-cell-based cancer immunotherapy strategies. However, the molecular mechanisms responsible for phosphoantigen-mediated activation of human γδ T-cells remain unclear. In particular, previous reports have described a very slow kinetics of activation of T-cell receptor (TCR)-associated signal transduction pathways by isopentenyl pyrophosphate and bromohydrin pyrophosphate, seemingly incompatible with direct binding of these antigens to the Vγ9Vδ2 TCR. Here we have studied the most potent natural phosphoantigen yet identified, (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), produced by *Eubacteria* and *Protozoa*, and examined its γδ T-cell activation and anti-tumor properties.

Methodology/Principal Findings: We have performed a comparative study between HMB-PP and the anti-CD3ε monoclonal antibody OKT3, used as a reference inducer of *bona fide* TCR signaling, and followed multiple cellular and molecular γδ T-cell activation events. We show that HMB-PP activates MEK/Erk and PI-3K/Akt pathways as rapidly as OKT3, and induces an almost identical transcriptional profile in $V\gamma9^+$ T-cells. Moreover, MEK/Erk and PI-3K/Akt activities are indispensable for the cellular effects of HMB-PP, including γδ T-cell activation, proliferation and anti-tumor cytotoxicity, which are also abolished upon antibody blockade of the $V\gamma9^+$ TCR Surprisingly, HMB-PP treatment does not induce down-modulation of surface TCR levels, and thereby sustains γδ T-cell activation upon re-stimulation. This ultimately translates in potent human γδ T-cell anti-tumor function both *in vitro* and *in vivo* upon transplantation of human leukemia cells into lymphopenic mice,

Conclusions/Significance: The development of efficient cancer immunotherapy strategies critically depends on our capacity to maximize anti-tumor effector T-cell responses. By characterizing the intracellular mechanisms of HMB-PP-mediated activation of the highly cytotoxic $V\gamma9^+$ T-cell subset, our data strongly support the usage of this microbial antigen in novel cancer clinical trials.

Citation: Correia DV, d'Orey F, Cardoso BA, Lança T, Grosso AR, et al. (2009) Highly Active Microbial Phosphoantigen Induces Rapid yet Sustained MEK/Erk- and PI-3K/Akt-Mediated Signal Transduction in Anti-Tumor Human γδ T-Cells. PLoS ONE 4(5): e5657. doi:10.1371/journal.pone.0005657

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Introduction

The capacity to recognize and eliminate transformed cells is common to several lymphocyte subsets of both the adaptive and the innate immune systems that are being targeted in cancer immunotherapy [1,2]. One population that appears to bridge these two systems in humans is characterized by the expression of a Vγ9Vδ2 T-cell receptor and represents 1–10% of peripheral blood lymphocytes (PBL) of healthy individuals, but expands up to 30–50% upon bacterial or protozoan infection [3].

In line with the cancer susceptibility phenotype of mice devoid of γδ T-cells [4], human Vγ9Vδ2 T-cells are endowed with notable anti-tumor activity toward a large spectrum of malignant cell lines of diverse tissue origin, particularly among lymphomas and leukemias [5], but also including melanomas and carcinomas [6], and are being explored in various clinical trials [7,8]. Unexpectedly, Vγ9Vδ2 cells were shown to respond to self- and foreign *non*-peptidic low molecular weight antigens with phosphate moieties ("phosphoantigens"), in what turns out to be an exclusive property of this lymphocyte subset [9,10,11]. Indeed, no other

human T-cell subset (namely Vδ1 cells), or any of the murine γδ populations, respond to phosphoantigens such as prenyl pyrophosphates [3].

From its early isolation from mycobacteria, isopentenyl pyrophosphate (IPP) [10] became the model phosphoantigen for studies on Vγ9Vδ2 activation. However, it is now clear that this class of compounds contains multiple members, either naturally occurring or synthetic, which span an extremely diverse range of bioactivities, up to 10^{10} fold differences. To date, the natural phosphoantigen with highest bioactivity known (32 picomolar) is (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), an intermediate of the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway employed by *Eubacteria* and apicomplexan *Protozoa* but not by eukaryotes [12]. Although HMB-PP is respectively 30,000 and 100 times more potent than IPP and bromohydrin pyrophosphate (BrH-PP, also known as "Phosphostim"), most of the studies on phosphoantigens have been performed with these compounds (already applied in the clinic) due to their historical precedence [3]. Such studies revealed a very slow kinetics of activation of TCR-associated signal transduction pathways, and conflicting results regarding their potential interactions with the Vγ9Vδ2 TCR [12,13,14]. This, added to the consistent failure to demonstrate cognate interactions between Vγ9Vδ2 TCRs and phosphoantigens in acellular systems [15], has shed some skepticism regarding the action of phosphoantigens as direct TCRγδ agonists. As HMB-PP is considered for γδ T-cell-based cancer clinical trials, hoping to improve the performance of previous phosphoantigens [7,8], it is crucial to clarify its own molecular/cellular mechanisms of action, including its potential capacity to trigger *bona fide* Vγ9Vδ2 TCR signaling. Consistent with such potential, it has been recently shown that HMB-PP has the capacity to induce the formation of high-density TCR nanoclusters on the surface of human γδ T-cells [16], and a newly-developed tetramer reagent for the Vγ9Vδ2 TCR of rhesus macaques was reported to bind to HMB-PP loaded on the surface of human antigen presenting cells (APC) [17].

In this study we have analyzed the intracellular effects of HMB-PP stimulation of human γδ T-cells. Our data show that HMB-PP induces the activation of MEK/Erk and PI-3K/Akt signaling pathways with similar kinetics to direct cross-linking of the TCR complex in human γδ T-cells, and requires those activities to mediate effective γδ T-cell activation, including a full repertoire of TCR-associated transcriptional signatures and the secretion of pro-inflammatory cytokines IFN-γ and TNF-α. Although TCR accessibility is required for HMB-PP activity, this phosphoantigen does not lead to ligand-induced TCR internalization, which appears to be advantageous for sustaining the cells' activation status upon re-stimulation. Finally, very low amounts of HMB-PP in conjugation with interleukin-2 (IL-2) confers human γδ T-cells with very potent anti-lymphoma/leukemia activity both *in vitro* and in a human/SCID mouse model for the transplantation of human tumors, thus attesting the therapeutic potential of HMB-PP for cancer immunotherapy.

Results

Nanomolar amounts of HMB-PP replicate saturating TCR/CD3 ligation for activation of Vγ9$^+$ T-cells

In this study we used the anti-CD3ε monoclonal antibody (αCD3 mAb) OKT3 as a control for canonical T-cell activation through the TCR/CD3 complex, for direct comparison with HMB-PP. We began by testing the effect of several doses of each stimulating compound on human γδ T-cell activation, proliferation and survival. Concentrations of 1 nM HMB-PP and 1 μg/ml OKT3 produced identical profiles of expression of the activation marker CD69 in the Vγ9$^+$ T-cell population (Figure 1A), and displayed strikingly similar kinetics of activation without significant differences in cell viability (Figure 1B); they were therefore used in all subsequent experiments. Interestingly, whereas αCD3 mAb treatment reached a plateau of 60% CD69$^+$ cells at 1–10 μg/ml OKT3, 10 nM of HMB-PP were able to further increase the abundance of activated Vγ9$^+$ T-cells, to above 80% (Figure 1A).

Activated Vγ9Vδ2 T-cells are known to secrete large amounts of IFNγ and TNFα, very potent anti-tumor mediators *in vivo*. In accordance, treatment of sorted γδ PBL (80–95% Vγ9$^+$) with HMB-PP induced a typical Th1 cytokine profile, characterized by the preferential production of TNFα, IFNγ and LTα, in the absence of significant IL-4 or IL-10 (Figure 1C). Notably, the levels of Th1 cytokines produced after 1 nM HMB-PP treatment were similar to those induced by saturating amounts of αCD3 mAb (Figure 1C and data not shown), suggesting that low amounts of this phosphoantigen are able to fully exploit the TCR-mediated functional potential of Vγ9Vδ2 T-cells.

For the selective expansion of Vγ9$^+$ T-cells, HMB-PP has the advantage of not inducing αβ T-cell proliferation. Thus, HMB-PP treatment promoted the specific proliferation of Vγ9$^+$ T-cells within human PBL (Figure 1D). Importantly, this effect was completely abolished upon addition of a blocking antibody to the Vγ9$^+$ TCR (Figure 1E), demonstrating the TCR-dependence of HMB-PP activity.

While HMB-PP alone promoted up to 5 divisions of Vγ9$^+$ T-cells over 4 days, further proliferation required the co-administration of IL-2 (Figure 1D). A cooperative effect between phosphoantigens and IL-2 has been previously described [18,19], and in this study translated into a Vγ9$^+$ T-cell expansion of 30-fold within one week and 45-fold within two weeks of stimulation (Figure S1A). Moreover, addition of 100 units/mL IL-2 to HMB-PP cultures dramatically increased the total amounts of Th1 cytokines secreted by γδ T-cells by 20–80 fold (Figure S1B), which correlated with the induction of key transcription factor *t-bet* in cells stimulated with IL-2 or IL-2/HMB-PP combination (Figure S1C)

HMB-PP rapidly triggers MEK/Erk and PI-3K/Akt signaling required for Vγ9$^+$ T-cell activation and anti-tumor function

Having characterized the cellular behavior of HMB-PP-stimulated Vγ9$^+$ T-cells, we next investigated the intracellular signaling mechanisms downstream of HMB-PP. Previous studies with less active phosphoantigens [3] reported a significant delay in the activation of kinase cascades when compared to direct TCR/CD3 complex ligation with OKT3 mAb [12,13]. Instead, for HMB-PP, we observed a very rapid (peaking around 7 min of stimulation), and absolutely identical to OKT3, kinetics of phosphorylation of the major signaling pathways implicated in TCR signal transduction: JNK, Erk and p38 MAPK; and PI-3K-associated Akt and GSK3β (Figure 2A, left panel). The same was valid in the presence of IL-2, in which kinase phosphorylation peaked earlier (immediately after 1 min of stimulation) but was still identical for HMB-PP or OKT3 combinations (Figure 2A, right panel). Of note, we verified that IPP could not replicate these signaling properties of HMB-PP, as illustrated by its failure to induce Akt phosphorylation within 60 minutes of stimulation (Figure S2). Furthermore, IPP treatment (even when used at 10^5 fold higher concentrations than HMB-PP) resulted in a modest production of TNFα and IFNγ within the first 6 hours of stimulation, when compared to HMB-PP (Figure 2B). These data reveal a thus far unique capacity of HMB-PP to trigger very rapid

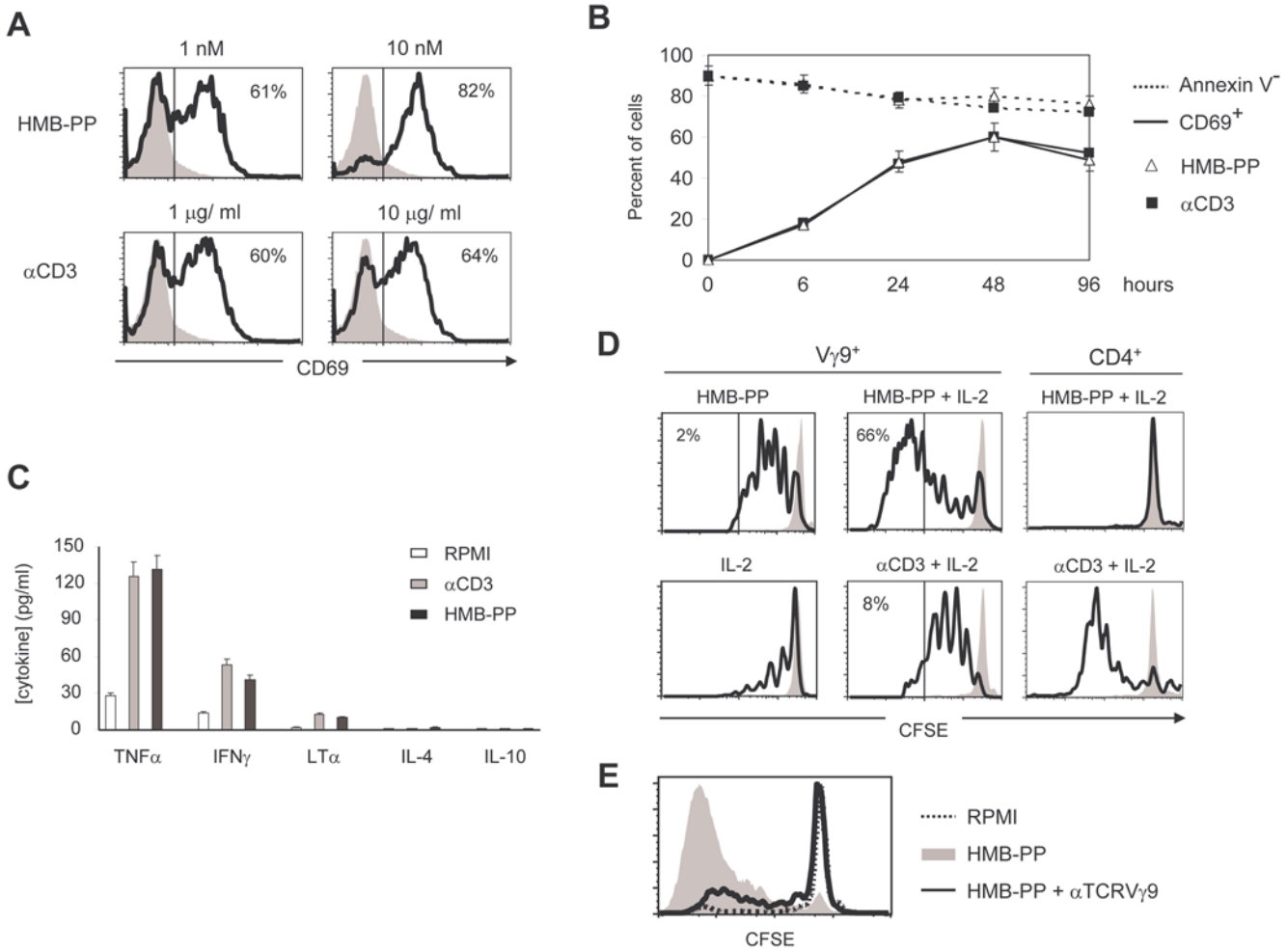

Figure 1. Nanomolar HMB-PP replicates saturating TCR/CD3 ligation for Vγ9⁺ T-cell activation. (**A**) Flow cytometry analysis for the expression of the activation marker CD69 in MACS-sorted (97–98% purity) γδ PBL, stimulated for 48 hours with the indicated amounts of HMB-PP or anti-CD3 mAb (OKT3). Shaded are non-stimulated Vγ9⁺ T-cells. Percentages refer to cells above the threshold bar. (**B**) Time-course of the experiment described in (A) for 1 nM HMB-PP and 1 μg/ml OKT3; cells were also stained with Annexin V to assess their viability (Annexin V⁻). (**C**) Cytokine bead array analysis of supernatants of MACS-sorted γδ PBL (of which 80–90% Vγ9⁺) cultures after 24 hours of stimulation with HMB-PP or OKT3. RPMI refers to cells kept in media not supplemented with activating compounds. (**D**) CFSE dilution assays to monitor T-cell proliferation in total PBMC cultures supplemented with HMB-PP (1 nM) or OKT3 (1 μg/ml), with or without 100 U/mL rhIL-2. Cells (gated on Vγ9⁺ or CD4⁺) were analyzed by flow cytometry after 4 days in culture; shaded are non-divided cells. Percentages indicate cells that have undergone more than 5 rounds of division. (**E**) CFSE dilution in gated Vγ9⁺ T-cells within 6-day cultures of total PBMC activated with 1 nM HMB-PP in the presence or absence of blocking anti-TcRVγ9 antibody. Dashed is a control incubated in 10% RPMI without HMB-PP. Results shown in this figure are representative of 3 independent experiments.

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TCR-associated signaling, compatible with direct binding of the phosphoantigen to the TCR complex.

We next tested the requirement on intact PI-3K and MAPK pathways for γδ T-cell activation and anti-tumor function induced by HMB-PP. We pre-treated γδ T-cells with chemical inhibitors that specifically block those pathways and then analyzed the effects on cell activation, proliferation, TNFα secretion and tumor cell killing. Inhibition of PI-3K/Akt pathway using LY294002 resulted in approximately half of the cells losing their responsiveness to HMB-PP after 24–46 hours of stimulation (Figure 3A). Inhibition of the MEK/Erk pathway by UO126 produced even more dramatic effects, precluding HMB-PP-activation of approximately two thirds of Vγ9⁺ T-cells. Moreover, inhibition of PI-3K/Akt and MEK/Erk signaling reduced TNFα production by HMB-PP-activated γδ T-cells to around 20% and 10% of control levels, respectively, both in the absence and in the presence of IL-2 (Figure 3B). These effects were remarkably mirrored in cultures supplied with αCD3 mAb, further demonstrating the similarity of these two activation regimens (Figures 3A and 3B).

In what regards γδ T-cell proliferation induced either by HMB-PP or by OKT3 (in the presence of IL-2), this was mostly dependent on intact PI-3K/Akt signaling, since UO126 had a more modest effect when compared with the severe block produced by LY294002 treatment, which reduced the proportion of γδ cells that divided twice or more over 4 days in culture, from over 80% to approximately 20% (Figure 3C).

Finally, the anti-tumor function of sorted γδ PBL (80–95% Vγ9⁺) was assessed through *in vitro* killing of the Jurkat leukemic target cell line. HMB-PP pre-treatment augmented γδ T-cell-mediated tumor cell death from around 20% (non-activated γδ) to 40% (1 nM HMB-PP) or 70% (10 nM HMB-PP) in a 6 hour assay (Figure 3D and data not shown). However, the addition of UO126

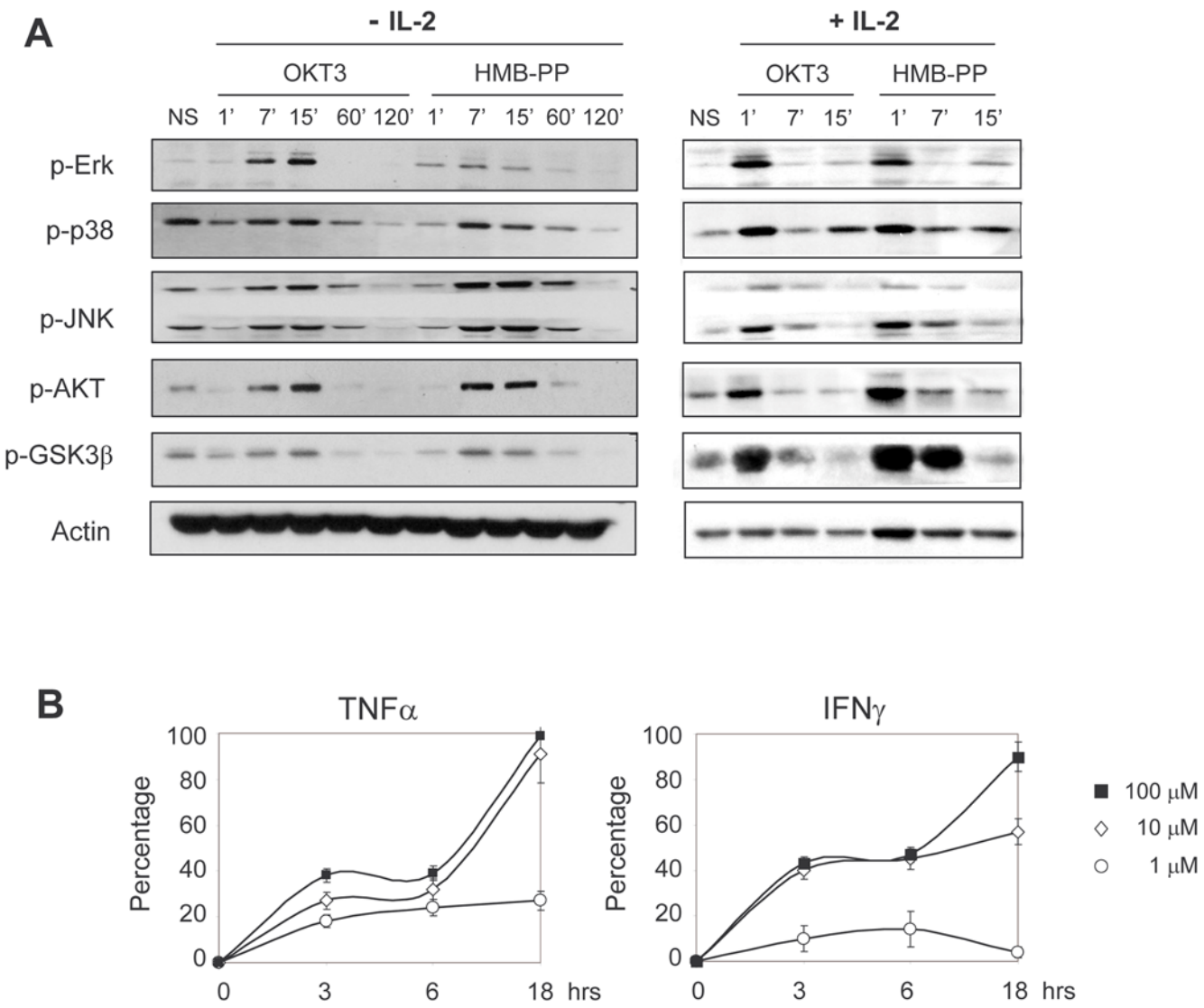

Figure 2. HMB-PP stimulation kinetically mimics Vγ9$^+$ TCR/CD3 signal transduction. (**A**) Phosphoimmunoblotting for kinases implicated in TCR signaling. MACS-sorted γδ PBL (of which 80–95% Vγ9$^+$) were incubated with OKT3 (1 μg/ml) or HMB-PP (1 nM), in the absence (*left panel*) or presence (*right panel*) of 100 U/mL rhIL-2, for the times indicated, or kept in control media (NS, non-stimulated). Results shown in this figure are representative of 4 independent experiments. (**B**) TNFα and IFNγ levels were measured by CBA in the culture supernatants of MACS-sorted γδ PBL. Results were compared with the total amounts present in parallel cultures stimulated with 1 nM HMB-PP, and were expressed as percentages (IPP/HMB-PP).
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or/and LY294002 to the treatment reduced posterior leukemia targeting to basal (20–30%) levels; this was also the case for the more efficient (over 80% killing) combination of HMB-PP with IL-2 (Figure 3D). Collectively, these data demonstrate an absolute requirement of PI-3K/Akt- and MEK/Erk-mediated signal transduction for HMB-PP-induced activation of anti-tumor Vγ9Vδ2 T-cells.

HMB-PP signaling mimics the transcriptional events downstream of TCR ligation

Signaling cascades ultimately produce alterations in gene transcription, which can be effectively tracked by microarray analysis. We employed this technology to compare the transcriptomes of Vγ9Vδ2 T-cells activated with either HMB-PP or OKT3. Both stimuli produced dramatic transcriptional changes: when compared to non-stimulated cells, HMB-PP and OKT3 treatment resulted in 1359 and 1080 differences in gene expression of 4-fold or above, respectively (Figure 4A; full microarray data available on ArrayExpress via http://www.ebi.ac.uk/; accession E-MEXP-1601). These were consistent across 3 individual microarray experiments (Figure S2). Strikingly, a direct comparison of the two stimuli revealed that they affected essentially the same genes, as only 6 were differentially expressed (>4-fold) between them (Table 1). Therefore, the transcriptional program downstream HMB-PP appears to be extremely similar to that induced by *bona fide* TCR signaling, as clearly illustrated by the Volcano plots of Figure 4A.

The gene expression program shared by HMB-PP treatment and direct TCR cross-linking involves, among many others targets (E-MEXP-1601), the very high (above 16-fold) up-regulation of pro-inflammatory genes IFNγ and LTα, chemokines CCL8, CCL2, CXCL9 and CXCL10, cell cycle mediator cyclin D2,

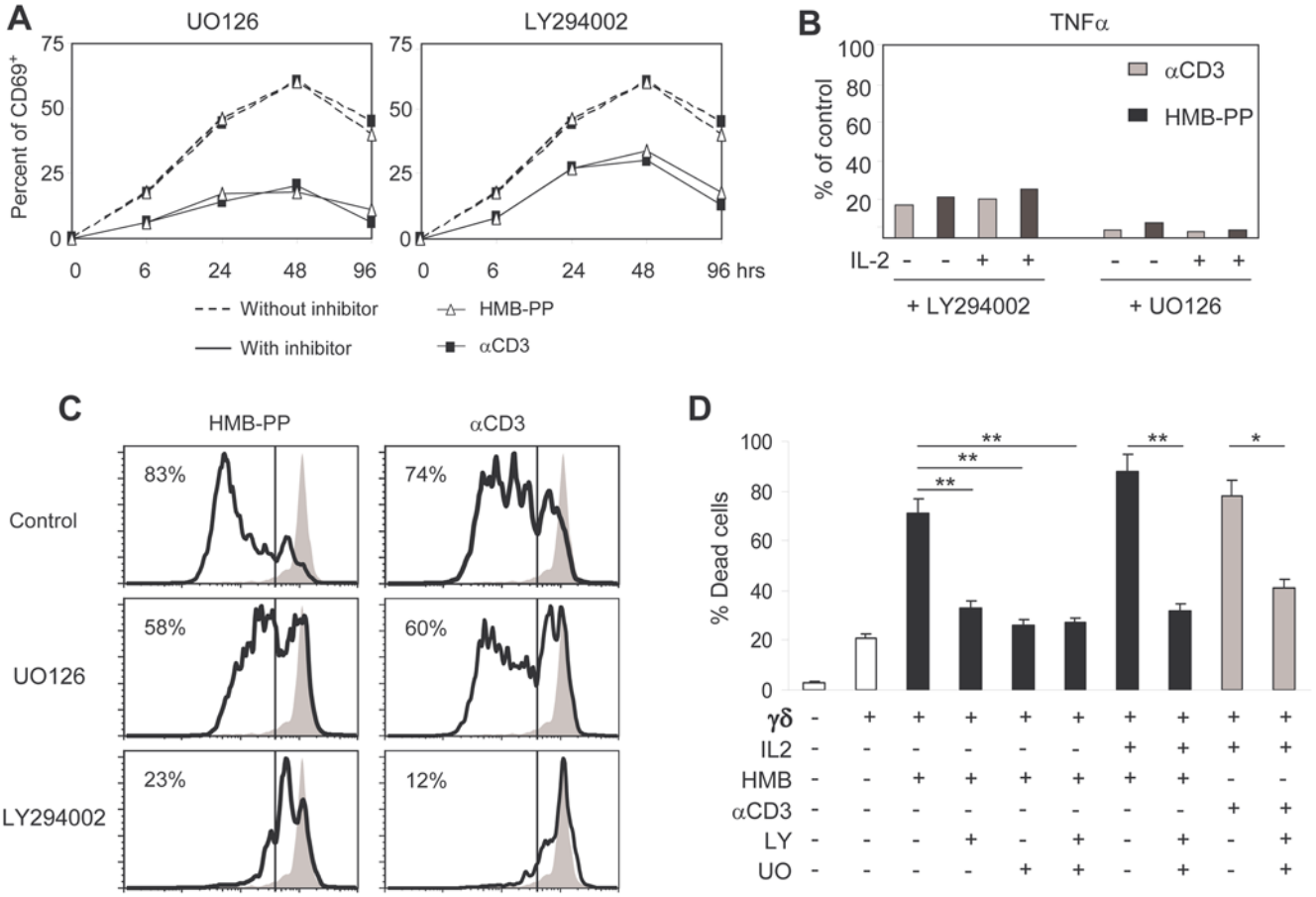

Figure 3. HMB-PP-mediated Vγ9$^+$ T-cell activation requires functional MEK/Erk and PI-3K/Akt signaling pathways. Effects of MEK/Erk inhibitor UO126 and PI-3K/Akt inhibitor LY294002 on the activation and function of MACS-sorted γδ PBL (of which 85–95% Vγ9$^+$). (**A**) Expression of activation marker CD69, assessed by flow cytometry. (**B**) secretion of TNFα after 24 hours of stimulation, measured by CBA. (**C**) Cell proliferation, assessed by CFSE dilution after 4 days in culture (percentages indicate cells that have undergone 2 or more rounds of division). (**D**) Jurkat leukemia cell killing, assessed by Annexin V staining and flow cytometry analysis after 6 hrs of co-incubation with pre-activated (for 3 days) γδ PBL. Results shown in this figure are representative of 3 independent experiments. Error bars represent SD and significant differences refer to controls without addition of chemical inhibitors (n = 3, *$p<0.05$ and **$p<0.01$).
doi:10.1371/journal.pone.0005657.g003

activation co-receptor ICOS, cytolysis mediator Fas ligand (Fas-L), and components of cytokine receptors IL-2Rα (CD25) and IL-15Rα (Table 1), many of which are also induced by related phosphoantigens [20]. These results were validated by quantitative real-time PCR (qPCR), as shown on Figure 4B for a selection of genes.

Although we have concentrated here on genes upregulated upon stimulation, the profile of downregulated genes was also almost identical between the two treatments (E-MEXP-1601). Our results collectively suggest that HMB-PP essentially recapitulates the transcriptional program associated with *bona fide* TCR signaling. This phenomenon is further illustrated by a heatmap representation of gene expression levels across the samples, as depicted in Figure S3.

HMB-PP does not induce Vγ9$^+$ TCR internalization and sustains the production of anti-tumor cytokines

Although our previous data demonstrated a striking parallel between HMB-PP- and OKT3-mediated γδ T-cell activation, previous reports on various phosphoantigens (other than HMB-PP) had revealed contradictory data on the modulation of surface Vγ9Vδ2 TCR levels [13,14]. This, added to recent data on the properties of HMB-PP interactions with TCR/CD3 complexes [16,17], prompted our investigation on whether HMB-PP stimulation induced TCR internalization in human γδ PBLs. In αβ T cells, activation by cognate antigen or anti-TCR/CD3 antibodies typically induces TCR internalization and consequently the down-modulation of its surface levels independently of the constitutive recycling of the complex [21,22]. Using two independent approaches, based on flow cytometry (Figure 5A) or confocal microscopy (Figure 5B), we consistently observed that HMB-PP-stimulated γδ T-cells maintained their high TCR surface expression, in stark contrast with the extensive down-regulation seen in OKT3-treated cells. This was the case both in the absence and in the presence of IL-2 (data not shown).

We next asked whether the lack of TCR internalization upon HMB-PP treatment could be associated with sustained activation of γδ T-cells. We tested the capacity of cells that had been treated for 2 days with either HMB-PP or OKT3, to respond to a second boost of stimulation (Figure 5C). Whereas HMB-PP-treated cells, which maintained high TCR levels on the cell surface after the initial 48 hour treatment (Figure 5A), produced high amounts (similar to primary activation) of anti-tumor Th1 cytokines in response to the secondary 24 hour stimulation with HMB-PP (Figure 5D), OKT3-treated cells failed to do so, presumably due to

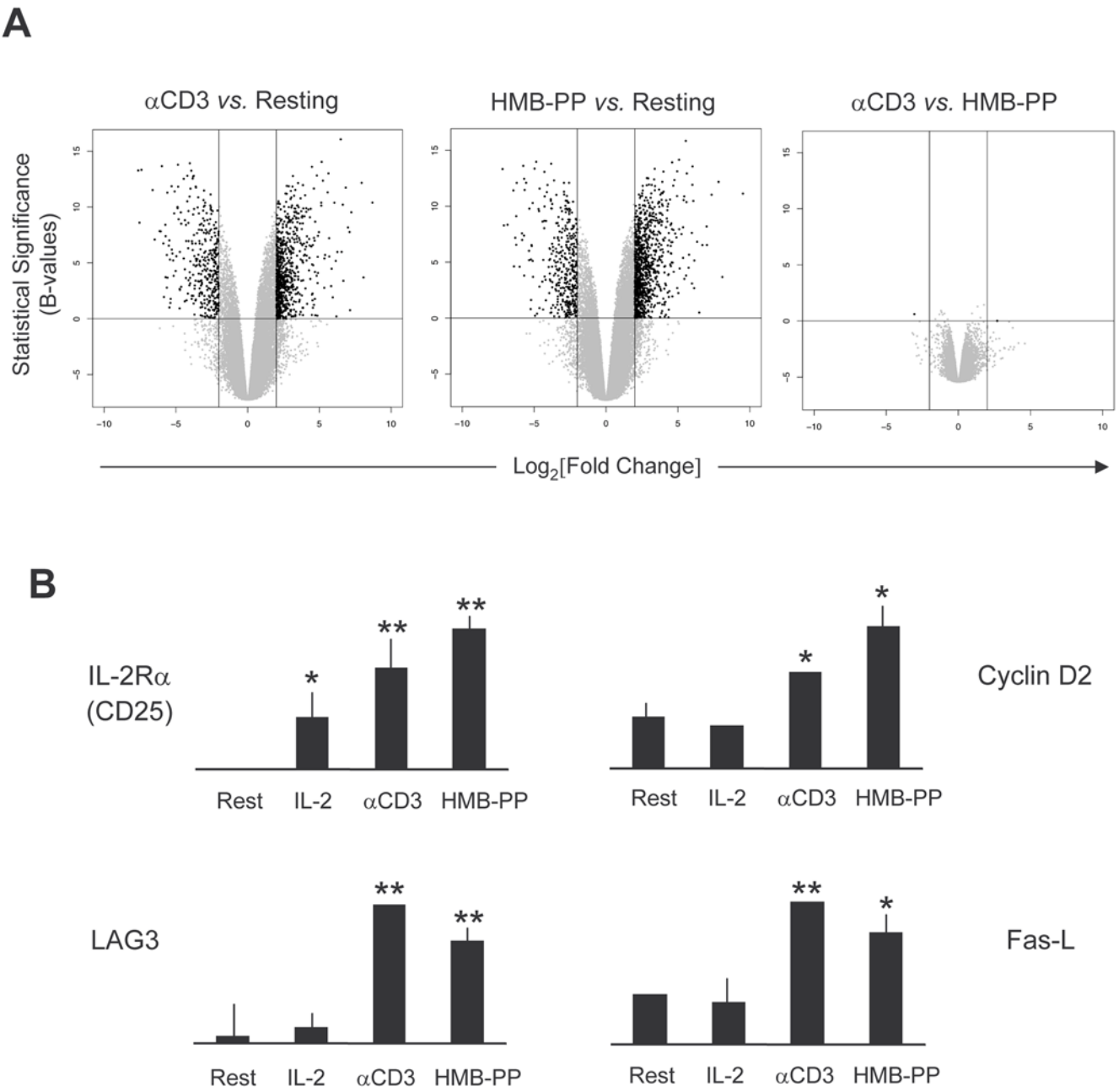

Figure 4. HMB-PP treatment reproduces the transcriptional alterations induced by TCR/CD3 ligation on γδ T-cells. (**A**) Volcano plots of DNA microarray comparisons between αCD3 (OKT3) mAb-treated, HMB-PP-treated and non-stimulated ("resting") MACS-sorted γδ PBL (of which 85–95% $V\gamma9^+$). After 18 hours of incubation with the stimuli, RNA was extracted and submitted to Affymetrix GeneChip analysis. Represented are Fold-changes ("biological significance") versus statistical significance (*B*-values). Black dots represent genes over 4-fold differentially expressed (DE) between samples; all other probed genes are depicted in grey. Genes selected as differentially expressed had adjusted *p*-values lower than 0.005. Results are representative of 3 independent microarray experiments (see Figure S2). (**B**) Real-time PCR validation of microarray results for a selection of genes similarly induced by OKT3 and HMB-PP (from Table 1). Gene expression was quantified in independent samples of control and treated cells, also including an IL-2-treated sample. Error bars represent SD and significant differences refer to "resting" cells (n = 3, $*p<0.05$ and $**p<0.01$).
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their inability to respond to the mAb once their TCR complexes have been internalized (Figure 5A–B). Although upon restimulation with HMB-PP, IFNγ became more abundant than TNFα (Figure 5D), contrary to the primary activation data (Figure 1C and Figure S1B), the cytokine profile of the two HMB-PP-based protocols were qualitatively very similar and consistently Th1-biased (Figure 5D and data not shown). These data show that HMB-PP is remarkably capable of sustaining Vγ9Vδ2 T-cell activation and the production of anti-tumor cytokines, which are critical parameters in immunotherapy protocols.

HMB-PP plus IL-2 treatment promotes leukemia cell killing *in vitro* and *in vivo*

Having characterized the intracellular mechanisms of HMB-PP-mediated γδ T-cell activation, we next evaluated the anti-tumor potential of HMB-PP-based regimens. We selected

Table 1. Transcriptional changes induced by HMB-PP or OKT3 (anti-CD3 mAb) in Vγ9Vδ2 T cells.

Similarly induced by HMB-PP and OKT3[a]

Link[b]	Gene	Description	Function	HMB[c]	OKT3[c]	Differ[d]
3458	*IFNγ*	Interferon-γ	Cytokine	9.53	8.70	0.83
6355	*CCL8*	Chemokine CC motif 8	Chemokine	8.09	8.08	0.01
114614	*MIRN155*	MicroRNA 155	MicroRNA	7.83	7.95	−0.12
4049	*LTα*	Lymphotoxin-α	Cytokine	7.35	6.64	0.71
6347	*CCL2*	Chemokine CC motif 2	Chemokine	6.49	6.19	0.30
3559	*IL2Rα*	IL-2R α chain	Cytokine-R	6.34	7.10	−0.76
4283	*CXCL9*	Chemokine CXC motif 9	Chemokine	6.14	6.14	0.00
3627	*CXCL10*	Chemokine CXC motif 10	Chemokine	5.75	4.87	0.88
894	*CCND2*	Cyclin D2	Cell cycle	5.62	5.25	0.37
3902	*LAG3*	Lymphocyte activation gene	Activation-R	4.46	4.08	0.38
29851	*ICOS*	Inducible T cell costimulator	Activation-R	4.46	5.40	−0.94
6504	*SLAMF1*	Signal transducer SLAM-1	Signaling	4.23	4.34	−0.11

Differentially induced by HMB-PP or OKT3

Link[b]	Gene	Description	Function	HMB[c]	OKT3[c]	Differ[d]
7412	*VCAM1*	Vascular cell adhesion-R	Adhesion	4.50	1.45	3.05
6373	*CXCL11*	Chemokine CXC motif 11	Chemokine	5.45	2.59	2.86
1493	*CTLA4*	Co-receptor CTLA-4	Activation-R	4.53	7.23	−2.70
112744	*IL17F*	IL-17 isoform F	Cytokine	1.29	4.81	−3.52
4094	*MAF*	Transcription factor Maf	Signaling	0.04	3.83	−3.79
6374	*CXCL5*	Chemokine CXC motif 5	Chemokine	0.04	7.14	−7.10

Values are $\log_2$[fold change] compared to non-stimulated cells, based on triplicate microarray experiments. (-R, receptor).
[a]Listed is a selection of genes implicated in T cell activation. Full cDNA microarray data available on ArrayExpress (E-MEXP-1601).
[b]Locus link gene ID (for unequivocal gene identification).
[c]$\log_2$[fold change] relative to non-stimulated cells.
[d]Difference in fold induction between HMB-PP-treated and OKT3-treated cells.
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leukemias as model tumors to employ in both *in vitro* and *in vivo* assays. The *in vitro* system previously used with Jurkat cells (Figure 3D) was applied to a larger panel of leukemia cell lines: Molt-4 (T-cell), RCH-ACV (pre-B cell) and HL-60 (myeloid) (Figures 6A–B). γδ PBL (80–95% $V\gamma9^+$) were treated with the different stimulating agents for 72 hours, and then transferred to plain media in co-culture with the leukemia cells. In just 3 hours, more than 80% of leukemia cells were killed by the γδ T-cells that had been stimulated with a combination of HMB-PP with IL-2 (compared to less than 20% by non-activated γδ T-cells), and such a regimen was at least as effective as saturating αCD3 plus IL-2 (Figures 6A–B). Of note, αCD3 mAb or HMB-PP used in isolation produced more modest increases in target-cell lysis (Figures 6A–B), highlighting the importance of exogenous IL-2 for the full activation of Vγ9Vδ2 T-cells [18,23] (Figure S1).

Taking into account the added relevance of pre-clinical *in vivo* systems for the evaluation of the anti-tumor potential of immunotherapy strategies, we adapted a model of transplantation of human tumors into lymphopenic SCID mice, previously used with human γδ T-cells by Kabelitz and colleagues [24], and added bioluminescent analysis of tumor development, which allows early detection of tumors and temporal evaluation throughout the course of treatment, in live animals and in real-time [25]. Four weeks after tumor injection, mice that had received HMB-PP plus IL-2-treated (activated and expanded over 12 days) γδ PBL showed significantly reduced tumor load (derived from Molt-4 leukemia cells) compared to control mice that did not receive γδ T-cells (Figures 6C–E). Furthermore, while most control had to be sacrificed at week 4 due to excessive body weight loss, γδ-treated animals resisted wasting for longer, up to week 6 (Figures 6C–D and data not shown). These results attest the capacity of HMB-PP-expanded and activated γδ T-cells to induce anti-tumor responses *in vivo*, and support the application of this phosphoantigen in conjugation with low amounts of IL-2 in clinical cancer settings.

Discussion

The stimulatory effect prenyl pyrophosphates have on Vγ9Vδ2 T-cells has been well documented and seems to require TCR expression, as indicated by antibody blocking and gene transfer experiments [26,27]. However, some of these experiments have been difficult to reproduce, and all attempts at showing cognate interactions between Vγ9Vδ2 TCRs and phosphoantigens in acellular systems (including surface plasmon resonance and X-ray crystallography of isolated complexes) have failed [15], probably due to the requirement of an unknown phosphoantigen-presenting molecule [12]. This has raised some skepticism on phosphoantigens as TCRγδ agonists, also stemming from the lack of precedent for such type of compounds interacting with any other variable region molecule, including all other γδ TCRs in humans or mice. However, recent data have highlighted particular properties of HMB-PP within the large family of phophoantigens. Namely, HMB-PP induces the formation of high-density Vγ9Vδ2 TCR nanoclusters on the membrane of human γδ T-cells [16], and is

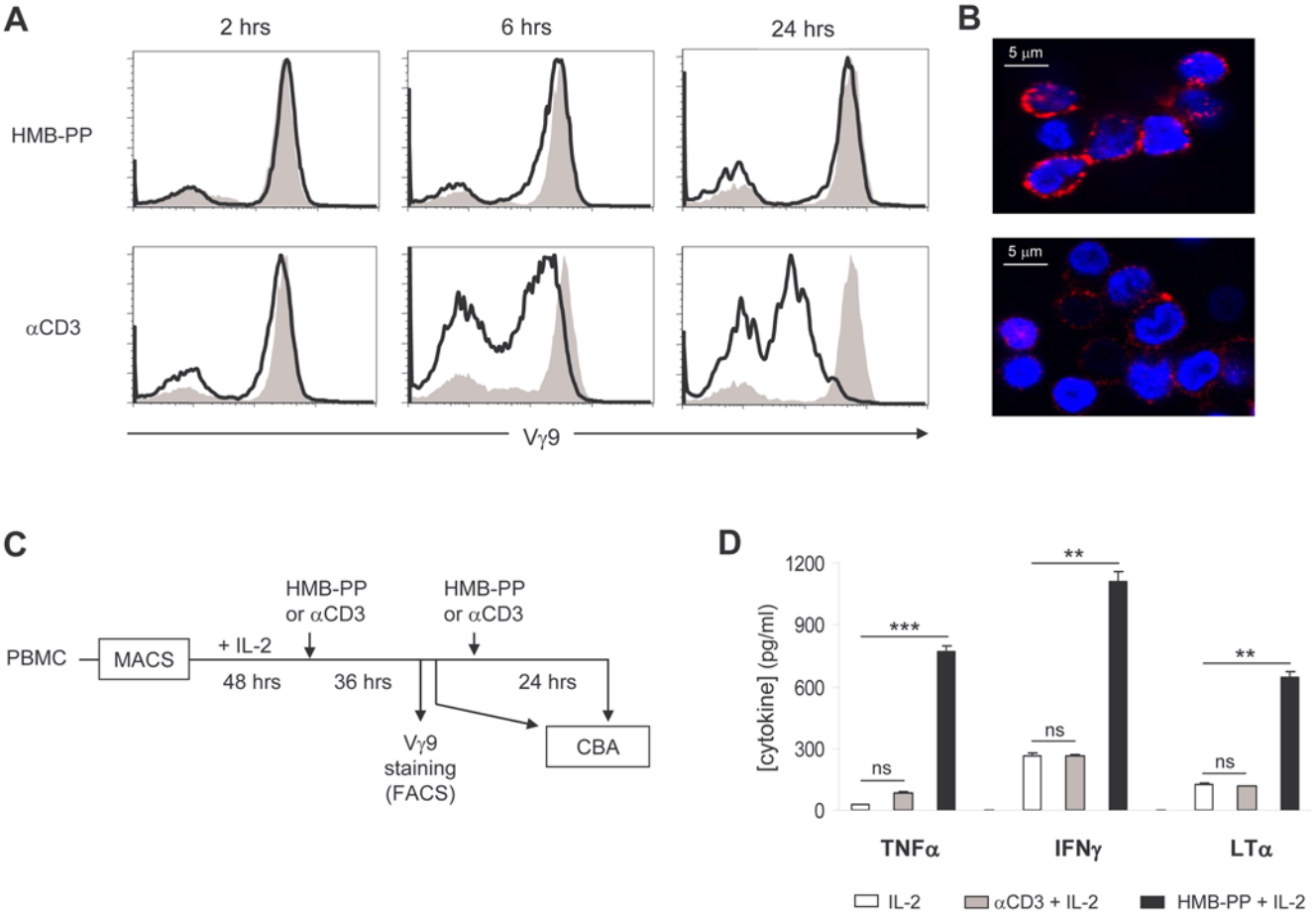

Figure 5. HMB-PP does not induce down-modulation of surface Vγ9$^+$ TCR and sustains cytokine production upon re-stimulation. (**A**) MACS-sorted γδ PBL (of which 80–90% Vγ9$^+$) were incubated for the indicated times with HMB-PP or OKT3, and stained with anti-Vγ9 mAb for flow cytometry analysis. Bold lines represent treated cells, while shaded are non-stimulated Vγ9$^+$ cells (time = 0 hrs). (**B**) Confocal microscopy photos of γδ T-cells cultured for 24 hrs as in (A) and then stained for Vγ9$^+$ TCR. (**C–D**) Experimental design (C) and CBA analysis (D) of the re-stimulation response of MACS-sorted γδ PBL. After 36 hrs of stimulation, cells were re-plated for secondary activation during 24 hrs, when supernatants were collected and analyzed for Th1 cytokines by CBA. Error bars represent SD and differences refer to IL-2 controls (ns, non-significant; $^{**}p<0.01$; $^{***}p<0.001$). Results shown in this figure are representative of 2–5 independent experiments.
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bound on the surface of human APC by a tetramer reagent for the Vγ9Vδ2 TCR of rhesus macaques [17].

Following from these results on the extracellular dynamics of HMB-PP, our study aimed at clarifying the intracellular mechanisms of γδ T-cell activation mediated by HMB-PP. Our results show that very low amounts of HMB-PP are able to mimic the major effects of saturating ligation of the TCRγδ/CD3 complex, including the very rapid activation of MEK/Erk and PI-3K/Akt pathways to set up a transcriptional program, further enhanced by IL-2 signaling, that upregulates crucial target genes such as IFNγ or TNFα and endows cells with potent anti-tumor capacity. Interestingly, HMB-PP can produce all these intracellular events without down-modulating surface TCR levels, and this may be advantageous for sustaining the cells' activation status upon re-stimulation, as suggested by our cytokine secretion data. The crucial effect of HMB-PP on Vγ9Vδ2 T-cells may thus be the formation of high-density surface TCR nanoclusters [16] that may serve as platforms for intracellular signaling.

Our kinetic data on signal transduction further suggest that the interaction between HMB-PP and the Vγ9Vδ2 TCR is much more direct/stable than those of previously studied phosphoantigens, since downstream Erk phosphorylation, for example, peaks simultaneously for HMB-PP and OKT3 treatments, in stark contrast with the delays of 115 min and 60 min, also relative to OKT3, observed respectively for the "pioneer" (naturally-occurring) IPP [13] and the more recent (synthetic) BrH-PP [12], currently in clinical trials as "Phosphostim". Of note, the concentration of HMB-PP we used was 50,000-fold and 3,000-fold *lower* than those used for IPP and BrH-PP, respectively. These data reveal a thus far unique capacity of HMB-PP to trigger very rapid TCR-associated signaling, compatible with direct binding to the Vγ9Vδ2 TCR, which remains to be formally shown and may require the assistance of an antigen-presenting molecule yet to identify [12,17]. This notwithstanding, we show for the first time, using a chemical inhibition strategy, that the major cellular effects of HMB-PP - γδ T cell activation, proliferation, Th1 cytokine secretion and anti-tumor cytotoxicity - are strictly dependent on Erk- and Akt-mediated signal transduction. HMB-PP stimulation therefore recruits the same signal transduction machinery employed by the γδ TCR, and is capable of doing so at minimal concentrations and within a strikingly short temporal scale that distinguish it from less potent phosphoantigens, whose stimulating effects on γδ T-cells most probably derive from their structural similarities with HMB-PP [3].

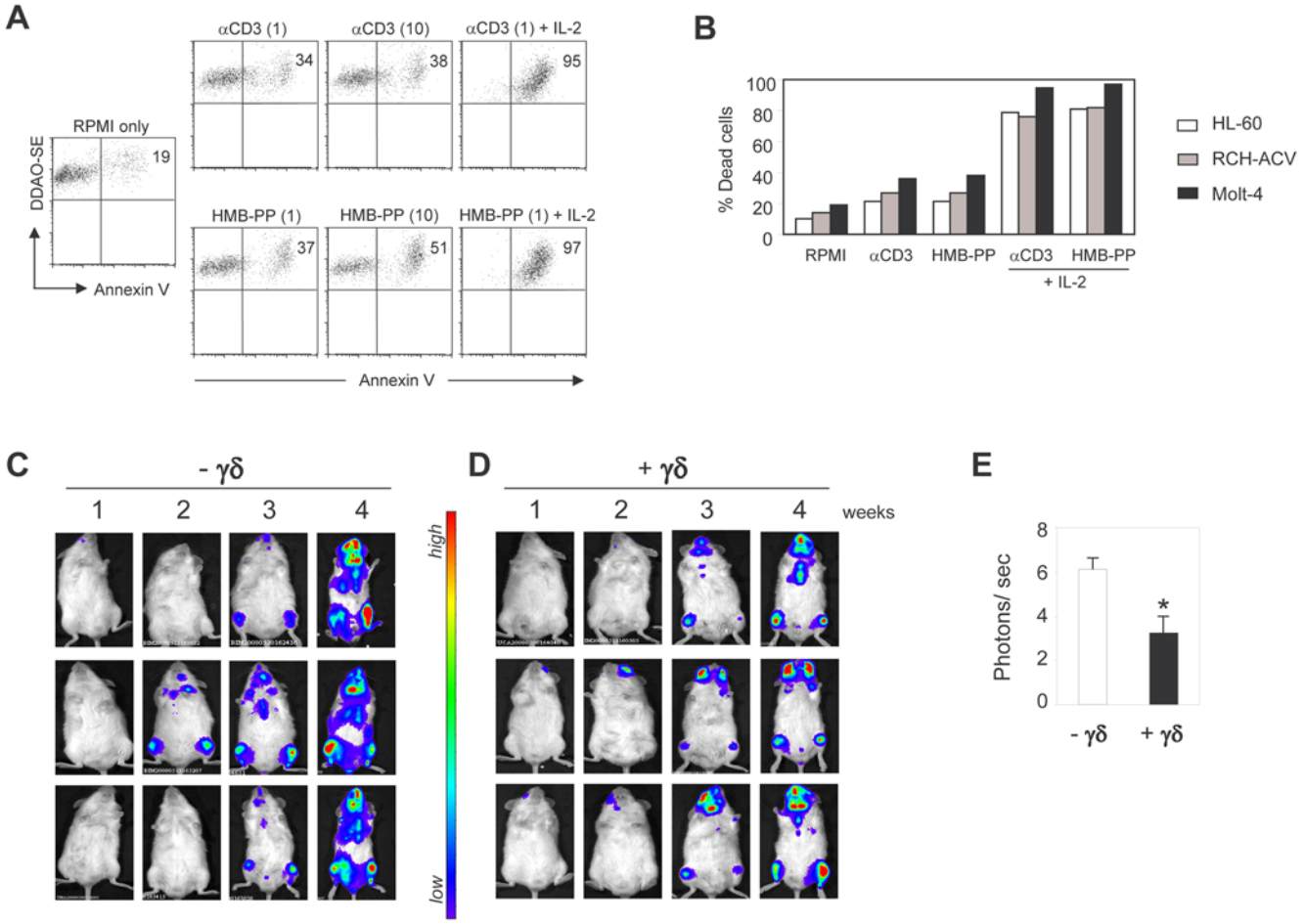

Figure 6. Leukemia cell killing by HMB-PP-activated γδ T-cells. (**A**) *In vitro* lysis of Molt-4 leukemia cells. MACS-sorted γδ PBL (of which 85–95% Vγ9$^+$) were pre-activated for 72 hours with 1 or 10 μg/ml αCD3 mAb (OKT3), or 1 or 10 nM HMB-PP in the absence of IL-2, and also combined at the lower concentrations with IL-2 (100 U/ml). For the killing assay, DDAO-SE-labelled Molt-4 cells and pre-activated γδ PBL were co-incubated for 3 hours in media devoid of activating compounds. Samples were then stained with Annexin V to identify dying (Annevin V$^+$) tumor (DDAO-SE$^+$) cells by flow cytometry. (**B**) Data summary for killing assays (as in A) performed with three distinct leukemia cell lines. (**C–D**) Bioluminescent imaging of NOD/SCID mice inoculated with luciferase$^+$ Molt-4 leukemic cells, with (D) or without (C) co-injection of pre-activated γδ PBL, analyzed weekly as described in Materials and Methods. (**E**) LivingImage quantification of photon signals (tumor load) collected at day 28 of the experiment illustrated in (C–D). Comparison of γδ-treated and control animals ($n = 5$, $p < 0.05$). Data in this figure are representative of 3 (A–B) or 2 (C–E) independent experiments.

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The use of a microbial compound for the activation of human anti-tumor lymphocytes fits the overall strategy of providing immune adjuvants (like viral nucleic acids for CD8$^+$ T-cells) for cancer therapy. Compared to other T-cell agonists, HMB-PP offers the advantage of specifically activating a T-cell population with overt effector function, devoid of known immune suppressive (“regulatory”) subsets. Moreover, Vγ9Vδ2 T-cells are broadly reactive to tumors, potentially allowing them to be used to treat a variety of cancers.

The data presented in this report provide a framework for designing novel immunotherapy protocols using γδ T-cells, and encourage the use of HMB-PP in clinical settings. γδ T-cell-mediated tumor surveillance should evidently be seen as complementary to the adaptive component provided by MHC-restricted αβ T-cells upon priming by dendritic cells. Importantly, Vγ9Vδ2 T-cells can also induce monocyte and DC maturation [28,29,30], on one hand; and even act as CD80/86-expressing antigen-presenting cells that prime αβ T-cells, on the other [31]. Furthermore, γδ T-cells are prototypic representatives of unconventional lymphocytes with innate anti-tumor capacity, alike NK and NKT-cells, all of which recognize tumors independently of classical MHC presentation [2]. We believe the success of cancer immunotherapy will critically depend on the integration of conventional and unconventional lymphocyte responses [32] to tackle the multiple immune evasion strategies developed by tumors.

Materials and Methods

Ethics statement

All experiments involving animals (rodents) were performed in compliance with the relevant laws and institutional guidelines and have been approved by the Instituto de Medicina Molecular animal ethics committee.

In vitro cultures of human peripheral blood lymphocytes

Peripheral blood was collected from anonymous healthy volunteers, diluted 1:1 (v/v) with PBS(1×) (Invitrogen Gibco)

and centrifuged in LSM Lymphocyte Separation Medium (MP Biomedicals) in a volume ratio of 3:4 (3 parts of LSM for 4 of diluted blood) for 15 minutes at 1500 rpm and 25°C. The interfase containing PBMC was collected, washed in PBS (1×) and cultured at 1×10^6 cells/mL at 37°C, 5% CO2 in round-bottom 96 well plates with RPMI 1640 with 2 mM L-Glutamine (Invitrogen Gibco) supplemented with 10% foetal bovine serum (Invitrogen Gibco), 1 mM Sodium Pyruvate (Invitrogen Gibco), 50 mg/mL of penicillin/streptomycin (Invitrogen Gibco), in the presence or absence of 100 U/mL of rhIL-2 (Roche Applied Science), 1–10 nM of HMB-PP (4-hydroxy-3-methyl-but-2-enyl pyrophosphate) (a kind gift from H. Jomaa and M. Eberl), and 1-10 ug/ml of soluble anti-CD3 antibody (eBioscience, clone OKT3).

For TCR blockade, freshly-isolated PBMC were CFSE-labeled and then incubated for 6 days with anti- TcRVγ9 (Beckman Coulter, clone IMMU360) diluted 1:20 in complete medium supplemented with 1 nM HMB-PP.

For the phophoimmunoblotting experiments, MACS-isolated γδ T cells were expanded with 100 U/mL rhIL-2 for 15 days.

To study the effects of chemical inhibitors of signal transduction, the MEK inhibitor UO126 and the PI-3K inhibitor LY294002 (both from Calbiochem) were added at 10 μM for a 2-hour incubation period, and then transferred to fresh medium (without inhibitors).

Magnetic cell sorting and flow cytometry analysis

γδ T-cells were isolated (to above 95% purity) from PBMC by magnetic cell sorting via positive selection with a FITC-labeled anti-TCRγδ antibody (Miltenyi Biotec). For flow cytometry analysis (on a FACSCalibur, BD Biosciences), cells were labelled with fluorescent monoclonal antibodies: anti-CD69-PE (BD Pharmingen), anti-TcRVγ9-PC5 (Beckman Coulter) and anti-CD4-PerCP (BD Pharmingen). In all cultures the percentage of $V\gamma9^+$ T-cells was evaluated by flow cytometry. Cell proliferation was measured by following a standard CFSE staining protocol (CellTrace CFSE Cell Proliferation Kit, Invitrogen; final concentration 0.5 μM), while apoptosis was assessed by AnnexinV-FITC (BD Pharmingen) staining. Cells were counted in Mossbauer chambers using 0.4% Trypan Blue solution (Sigma-Aldrich) for viability control.

Cytometric Bead Array (CBA)

Cytokine secretion was measured using Cytometric Bead Array (CBA) technology (BD Biosciences). Cells were seeded with the respective activators at 2×10^5 cells/well, culture supernatants were collected at different time points and analyzed on a FACSCanto (BD Biosciences) using a custom-made Flex Set with five different cytokine capture beads: LT-α, IL-10, IL-4, TNF-α and IFN-γ. Data were analyzed using the FCAP Array Software v1.0.1 running on BD FACSDiva (BD Biosciences).

Protein isolation and phosphoimmunoblotting

Cells were incubated at 37°C with pre-warmed PBS alone or with HMB-PP (1 nM) or OKT3 (1 μg/mL). Reactions were stopped by placing samples on ice and adding ice-cold PBS. Cell lysates were prepared and equal amounts of protein were analyzed by 10% SDS-PAGE electrophoresis, transferred onto nitrocellulose membranes, and immunoblotted with the following mAbs or antisera: Actin, phospho-Erk (Y204) (Santa Cruz Biotechnology), ZAP-70 and phospho-STAT5A/B (Y694/Y699) (Upstate Biotechnology), phospho-Akt (S473), phospho-GSK-3β (S9), phospho-JNK/SAPK (Y183/185), phospho-p38 MAPK (Y180/182) (Cell Signalling Technology), and phospho-LCK (Y505) (Transduction Laboratories). Immunodetection was performed with horseradish peroxidase-conjugated secondary antibody and developed by chemiluminescence as described [33]. Whenever necessary membranes were striped using 15 mM TRIS pH 6.8 plus 2% SDS and β-Mercaptoethanol (100 mM) for 40 minutes at 57°C.

RNA isolation and Affymetrix GeneChip analysis

RNA labeling, hybridization to the Affymetrix GeneChip Human Genome U133 plus 2.0 Arrays and scanning was performed by the Affymetrix Core Facility, Instituto Gulbenkian de Ciencia, Portugal as described below.

Total RNA was extracted using the RNeasy Mini Kit according to manufacture's protocol (Qiagen, Hilden, Germany). Concentration and purity was determined by spectrophotometry and integrity was confirmed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA). RNA was processed for use on Affymetrix (Santa Clara, CA, USA) GeneChip Human Genome U133 Plus 2.0 Arrays, according to the manufacturer's One-Cycle Target Labeling Assay. Arrays were scanned on an Affymetrix GeneChip scanner 3000 7G.

All the microarray data analysis was done using R and several packages available from CRAN (R Development Core Team, 2008) and Bioconductor. The raw data (CEL files) was normalized and summarized with the Robust MultiArray Average method from the *affy* package.

The differentially expressed genes were selected using linear models and empirical Bayes methods as implemented in *limma* package, verifying the *p*-values corresponding to moderated F-statistics, and selecting as differentially expressed genes those that had adjusted *p*-values lower than 0.005.

Real-time quantitative PCR

Total RNA was reverse-transcribed into cDNA using random hexamers and Superscript II first strand synthesis reagents (Invitrogen). qPCR was performed on ABI Prism 7700 Sequence Detection System using SYBR Green detection system (both from PE Applied Biosystems). Primers were designed using Primer3 v.0.4.0 online program (http://primer3.sourceforge.net). Primer sequences are available upon request. For each transcript, quantification was done using the calibration curve method. β2-microglobulin was used as the internal control for normalization. All samples were run in triplicate and repeated three times. Analysis of the qPCR results was performed using the ABI SDS v1.1 sequence analysis software (Applied Biosystems).

Tumor cell cultures and *in vitro* killing assays

All tumor cell lines were cultured in complete 10% RPMI 1640 (as above), maintained at 1×10^5 up to 2×10^6 cells/mL by dilution and splitting 1:3 every 3–4 days.

For cytotoxicity assays, magnetically purified γδ PBL were pre-activated for 72 hours with 1–10 μg/mL αCD3 mAb (OKT3) or 1–10 nM HMB-PP either in the absence or presence of IL-2 (100 U/mL). Tumor cell lines were stained with CellTracer Far Red DDAO-SE (1 μM) (Molecular Probes, Invitrogen) and each 3×10^4 tumour cells were incubated with 3×10^5 γδ T-cells in RPMI devoid of activating compounds, for 3 hours at 37°C and 5% CO2 on a round-bottom 96 well plate. Cells were then stained with Annexin V-FITC and analyzed by flow cytometry.

Confocal microscopy

Cells were stained at 4°C with mouse anti-human TCR Vgamma9-PC5 (Beckman Coulter) primary antibody, and with

anti-mouse Alexa Fluor 633 (Invitrogen, Molecular Probes) secondary antibody. Cells were then fixed with 4% Paraformaldeheyde for 15 minutes at 4°C. Nuclear DNA content was stained for with DAPI Fluoromount G (Southern Biotech). Immunofluorescence microscopy was performed with a LSM 510 META confocal microscope (Zeiss). Separate images were collected with a 63× objective for each fluorochrome and then overlaid to obtain a multicolor image.

Bioluminescent imaging of transplanted leukemia development in SCID mice

10^7 Molt-4 T-cell leukemia cells stably expressing firefly luciferase and GFP were injected i.v. in groups of 6 NOD/SCID mice per experiment, either in isolation or together with 5×10^7 γδ PBL (>80% $V\gamma9^+$), previously expanded and activated *in vitro* with 1 nM HMB-PP for 12 days. Treated mice received boosts of 5×10^7 γδ PBL i.v. on day 14 and 10,000 U IL-2 i.p. twice every week, whereas control mice received only IL-2. All mice were analyzed on a weekly basis by *in vivo* imaging (IVIS, Caliper Lifesciences) upon intra-peritoneal injection of luciferin. Photon signals were quantified with LivingImage software (Caliper Lifesciences). Mouse body weight was measured weekly, and animals suffering from wasting (loss of over 20% of initial body weight) were sacrificed.

Statistical analysis

Statistical significance of differences between subpopulations was assessed using Student's t-test and is indicated when significant as *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

Supporting Information

Figure S1 Exogenous IL-2 expands HMB-PP-activated Vg9+ T-cells and up-regulates their Th1 cytokine profile. (A) Absolute numbers of Vg9+ cells in PBMC cultures stimulated with HMB-PP (1 nM) or OKT3 (1 ug/ml), supplemented or not with IL-2 (100 U/ml). Cells were analyzed by flow cytometry and light microscopy (Mossbauer chamber cell counts). (B) Cytokine bead array (CBA) analysis of supernatants of MACS-sorted gd PBL (of which 80–90% Vg9+) after 24 hours of stimulation with HMB-PP or anti-CD3 mAb (OKT3). Represented is the ratio between the cytokine amounts produced in the presence (100 U/ml) and in the absence of IL-2. (C) Real-time PCR quantification of t-bet mRNA expression in activated Vg9Vd2 T-cells, normalized with Beta2-microglobulin. Cells were pre-incubated for 6 hours with the activating compounds (or kept in RPMI as control). Significant differences refer to cells cultured in RPMI in the absence of IL-2 ($n = 3$, *$p<0.05$ and **$p<0.01$).
Found at: doi:10.1371/journal.pone.0005657.s001 (0.07 MB TIF)

Figure S2 Akt phosphorylation in response to IPP versus HMB-PP stimulation of gd PBL. MACS-sorted gd PBL were activated with 10 uM IPP or 1 nM HMB-PP for the indicated times. Cell lysates were analyzed by SDS-PAGE and immunoblotted for Phospho-Akt (P-Akt) or Beta-Actin on nitrocellulose membranes. Densitometry for P-Akt bands was normalized with Beta-Actin loading controls. Data correspond to the induction of Akt phosphorylation above basal levels, i.e., after subtraction of the unstimulated control levels.
Found at: doi:10.1371/journal.pone.0005657.s002 (0.05 MB TIF)

Figure S3 Heatmap of non-stimulated, HMB-PP-treated and anti-CD3 mAb (OKT3)-treated gd T-cells. The DNA microarray expression value for each gene is normalized across the samples; levels greater than the mean in a given sample are colored in red, and those below the mean are depicted in blue. Exp1-3 are triplicate independent microarray experiments. Note the striking similarity between HMB-PP-treated and anti-CD3-treated samples.
Found at: doi:10.1371/journal.pone.0005657.s003 (0.34 MB TIF)

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Author Contributions

Conceived and designed the experiments: DVC FSd JTB BSS. Performed the experiments: DVC FSd BAC TL Ad. Analyzed the data: DVC FSd ARG JTB BSS. Contributed reagents/materials/analysis tools: LRM. Wrote the paper: BSS.

References

1. Stagg J, Johnstone RW, Smyth MJ (2007) From cancer immunosurveillance to cancer immunotherapy. Immunol Rev 220: 82–101.
2. Gomes AQ, Correia DV, Silva-Santos B (2007) Non-classical major histocompatibility complex proteins as determinants of tumour immunosurveillance. EMBO Rep 8: 1024–1030.
3. Morita CT, Jin C, Sarikonda G, Wang H (2007) Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vgamma2V-delta2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens. Immunol Rev 215: 59–76.
4. Girardi M, Oppenheim DE, Steele CR, Lewis JM, Glusac E, et al. (2001) Regulation of cutaneous malignancy by gammadelta T cells. Science 294: 605–609.
5. Kunzmann V, Wilhelm M (2005) Anti-lymphoma effect of gammadelta T cells. Leuk Lymphoma 46: 671–680.
6. Corvaisier M, Moreau-Aubry A, Diez E, Bennouna J, Mosnier JF, et al. (2005) V gamma 9V delta 2 T cell response to colon carcinoma cells. J Immunol 175: 5481–5488.
7. Wilhelm M, Kunzmann V, Eckstein S, Reimer P, Weissinger F, et al. (2003) Gammadelta T cells for immune therapy of patients with lymphoid malignancies. Blood 102: 200–206.
8. Dieli F, Vermijlen D, Fulfaro F, Caccamo N, Meraviglia S, et al. (2007) Targeting human {gamma}delta} T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. Cancer Res 67: 7450–7457.
9. Constant P, Davodeau F, Peyrat MA, Poquet Y, Puzo G, et al. (1994) Stimulation of human gamma delta T cells by nonpeptidic mycobacterial ligands. Science 264: 267–270.
10. Tanaka Y, Morita CT, Tanaka Y, Nieves E, Brenner MB, et al. (1995) Natural and synthetic non-peptide antigens recognized by human gamma delta T cells. Nature 375: 155–158.
11. Gober HJ, Kistowska M, Angman L, Jeno P, Mori L, et al. (2003) Human T cell receptor gammadelta cells recognize endogenous mevalonate metabolites in tumor cells. J Exp Med 197: 163–168.
12. Thedrez A, Sabourin C, Gertner J, Devilder MC, Allain-Maillet S, et al. (2007) Self/non-self discrimination by human gammadelta T cells: simple solutions for a complex issue? Immunol Rev 215: 123–135.
13. Lafont V, Liautard J, Sable-Teychene M, Sainte-Marie Y, Favero J (2001) Isopentenyl pyrophosphate, a mycobacterial non-peptidic antigen, triggers delayed and highly sustained signaling in human gamma delta T lymphocytes without inducing eown-modulation of T cell antigen receptor. J Biol Chem 276: 15961–15967.
14. Sireci G, Espinosa E, Di Sano C, Dieli F, Fournie JJ, et al. (2001) Differential activation of human gammadelta cells by nonpeptide phosphoantigens. Eur J Immunol 31: 1628–1635.
15. Bonneville M, Scotet E (2006) Human Vgamma9Vdelta2 T cells: promising new leads for immunotherapy of infections and tumors. Curr Opin Immunol 18: 539–546.
16. Chen Y, Shao L, Ali Z, Cai J, Chen ZW (2008) NSOM/QD-based nanoscale immunofluorescence imaging of antigen-specific T-cell receptor responses during an in vivo clonal V{gamma}2V{delta}2 T-cell expansion. Blood 111: 4220–4232.
17. Wei H, Huang D, Lai X, Chen M, Zhong W, et al. (2008) Definition of APC presentation of phosphoantigen (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate to Vgamma2Vdelta2 TCR. J Immunol 181: 4798–4806.

18. Casetti R, Perretta G, Taglioni A, Mattei M, Colizzi V, et al. (2005) Drug-induced expansion and differentiation of V gamma 9V delta 2 T cells in vivo: the role of exogenous IL-2. J Immunol 175: 1593–1598.
19. Ali Z, Shao L, Halliday L, Reichenberg A, Hintz M, et al. (2007) Prolonged (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate-driven antimicrobial and cytotoxic responses of pulmonary and systemic Vgamma2Vdelta2 T cells in macaques. J Immunol 179: 8287–8296.
20. Yamashita S, Tanaka Y, Tsutsumi S, Aburatani H, Minato N, et al. (2005) Analysis of mechanism for human gammadelta T cell recognition of nonpeptide antigens. Biochem Biophys Res Commun 334: 349–360.
21. Dietrich J, Menne C, Lauritsen JP, von Essen M, Rasmussen AB, et al. (2002) Ligand-induced TCR down-regulation is not dependent on constitutive TCR cycling. J Immunol 168: 5434–5440.
22. Naramura M, Jang IK, Kole H, Huang F, Haines D, et al. (2002) c-Cbl and Cbl-b regulate T cell responsiveness by promoting ligand-induced TCR down-modulation. Nat Immunol 3: 1192–1199.
23. Vermijlen D, Ellis P, Langford C, Klein A, Engel R, et al. (2007) Distinct cytokine-driven responses of activated blood gammadelta T cells: insights into unconventional T cell pleiotropy. J Immunol 178: 4304–4314.
24. Kabelitz D, Wesch D, Pitters E, Zoller M (2004) Characterization of tumor reactivity of human V gamma 9V delta 2 gamma delta T cells in vitro and in SCID mice in vivo. J Immunol 173: 6767–6776.
25. Shu ST, Nadella MV, Dirksen WP, Fernandez SA, Thudi NK, et al. (2007) A novel bioluminescent mouse model and effective therapy for adult T-cell leukemia/lymphoma. Cancer Res 67: 11859–11866.
26. Bukowski JF, Morita CT, Band H, Brenner MB (1998) Crucial role of TCR gamma chain junctional region in prenyl pyrophosphate antigen recognition by gamma delta T cells. J Immunol 161: 286–293.
27. Bukowski JF, Morita CT, Tanaka Y, Bloom BR, Brenner MB, et al. (1995) V gamma 2V delta 2 TCR-dependent recognition of non-peptide antigens and Daudi cells analyzed by TCR gene transfer. J Immunol 154: 998–1006.
28. Ismaili J, Olislagers V, Poupot R, Fournie JJ, Goldman M (2002) Human gamma delta T cells induce dendritic cell maturation. Clin Immunol 103: 296–302.
29. Devilder MC, Maillet S, Bouyge-Moreau I, Donnadieu E, Bonneville M, et al. (2006) Potentiation of antigen-stimulated V gamma 9V delta 2 T cell cytokine production by immature dendritic cells (DC) and reciprocal effect on DC maturation. J Immunol 176: 1386–1393.
30. Eberl M, Roberts GW, Meuter S, Williams JD, Topley N, et al. (2009) A rapid crosstalk of human gammadelta T cells and monocytes drives the acute inflammation in bacterial infections. PLoS Pathog 5: e1000308.
31. Brandes M, Willimann K, Moser B (2005) Professional antigen-presentation function by human gammadelta T Cells. Science 309: 264–268.
32. Pennington DJ, Vermijlen D, Wise EL, Clarke SL, Tigelaar RE, et al. (2005) The integration of conventional and unconventional T cells that characterizes cell-mediated responses. Adv Immunol 87: 27–59.
33. Barata JT, Silva A, Brandao JG, Nadler LM, Cardoso AA, et al. (2004) Activation of PI3K is indispensable for interleukin 7-mediated viability, proliferation, glucose use, and growth of T cell acute lymphoblastic leukemia cells. J Exp Med 200: 659–669.

Identification of a panel of ten cell surface protein antigens associated with immunotargeting of leukemias and lymphomas by peripheral blood γδ T cells

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background

Vγ9Vδ2 T lymphocytes are regarded as promising mediators of cancer immunotherapy due to their capacity to eliminate multiple experimental tumors, particularly within those of hematopoietic origin. However, Vγ9Vδ2 T-cell based lymphoma clinical trials have suffered from the lack of biomarkers that can be used as prognostic of therapeutic success.

Design and Methods

We have conducted a comprehensive study of gene expression in acute lymphoblastic leukemias and non-Hodgkin's lymphomas, aimed at identifying markers of susceptibility versus resistance to Vγ9Vδ2 T cell-mediated cytotoxicity. We employed cDNA microarrays and quantitative real-time PCR to screen 20 leukemia and lymphoma cell lines, and 23 primary hematopoietic tumor samples. These data were analyzed using state-of-the-art bioinformatics, and gene expression patterns were correlated with susceptibility to Vγ9Vδ2 T cell mediated cytolysis *in vitro*.

Results

We identified a panel of 10 genes encoding cell surface proteins that were statistically differentially expressed between "γδ-susceptible" and "γδ-resistant" hematopoietic tumors. Within this panel, 3 genes (ULBP1, TFR2 and IFITM1) were associated with increased susceptibility to Vγ9Vδ2 T-cell cytotoxicity, whereas the other 7 (CLEC2D, NRP2, SELL, PKD2, KCNK12, ITGA6 and SLAMF1) were enriched in resistant tumors. Furthermore, some of these candidates displayed a striking variance of expression among primary follicular lymphomas and T-cell acute lymphoblastic leukemias.

Conclusions

Our results suggest that hematopoietic tumors display a highly variable repertoire of surface proteins that can impact on Vγ9Vδ2 cell-mediated immunotargeting. The prognostic value of the proposed markers can now be evaluated in upcoming Vγ9Vδ2 T cell-based lymphoma/leukemia clinical trials.

Key words: biomarkers, Vγ9Vδ2 T-lymphocytes, hematopoietic tumors, lymphoma cell lines.

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Introduction

γδ T lymphocytes display potent innate anti-tumor activity in both humans[1] and mice.[2,3] For example, mice genetically devoid of γδ T cells displayed increased susceptibility to skin tumor development induced experimentally by carcinogens,[2,3] and to transgenic adenocarcinoma of the mouse prostate model (TRAMP).[4] More importantly, murine γδ T cells were shown to prevent (through perforin-mediated cytotoxicity) the development of spontaneous B-cell lymphomas.[5]

The major γδ T-cell subset in human peripheral blood, Vγ9Vδ2 T lymphocytes, exert potent cytotoxicity towards tumor cell lines upon activation with small non-peptidic prenyl pyrophosphate intermediates of isoprenoid biosynthesis.[6] We and others have shown that, among such "phosphoantigens", 4-hydroxy-3-methyl-but-2-enylpyrophosphate (HMB-PP), a metabolite found in *Eubacteria* and *Protozoa*, is a very potent agonist of the Vγ9Vδ2 T-cell receptor (TCR) that promotes cytotoxicity and the secretion of anti-tumor cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor α (TNF-α).[6,7]

Phosphoantigen-activated Vγ9Vδ2 T cells can kill various solid tumor cell lines,[1] and a particularly large number of hematopoietic cell-derived tumors,[7-9] as well as freshly isolated tumor cells from patients with follicular B-cell lymphoma or chronic lymphocytic leukemia (CLL).[10]

The well-established anti-tumor activity of Vγ9Vδ2 T cells has been recently explored in clinical trials for solid/epithelial[11-13] or liquid/hematopoietic tumors (14-16), which were collectively promising even though they showed limited success. The lack of response to therapy of some patients was attributed to deficient expansion of effector Vγ9Vδ2 T cells.[11,13,14] However, a large proportion of patients exhibiting significant and sustained *in vivo* activation and proliferation of Vγ9Vδ2 T cells also failed to respond to treatment. Thus, in both prostate carcinoma[11] and non-Hodgkin's lymphoma,[14] objective responses (partial remissions) were observed in just 33% of the patients who activated/expanded their Vγ9Vδ2 T cells. These data emphasize the need for tumor biomarkers with prognostic value for γδ peripheral blood lymphocyte (γδ-PBL)-mediated immunotherapy.

Here we have conducted a comprehensive genome-wide expression study aimed at identifying lymphoma/leukemia markers of susceptibility or resistance to γδ-PBL cytotoxicity. We set up an experimental system consisting of lymphoma/leukemia cell lines with various degrees of susceptibility to γδ-PBL-mediated lysis, and performed comparative cDNA microarray analyses to characterize their gene expression profiles. These were validated through bioinformatics and quantitative real-time PCR (RT-qPCR), allowing us to define a panel of 10 candidate biomarkers whose expression displayed very marked variability among non-Hodgkin's lymphoma and acute lymphoblastic leukemia patients.

Design and Methods

In vitro *cultures of human γδ-PBL and tumor cell lines*

Peripheral blood was collected from healthy volunteers and peripheral blood mononuclear cells (PBMCs) were isolated as previously described.[7] γδ–PBL were expanded from isolated PBMCs for 12 days in RPMI 1640 complete media[7] supplemented with 100 U/mL of rhIL-2 (Roche Applied Science) and 1 nM HMB-PP (4-hydroxy-3-methyl-but-2-enylpyrophosphate) (Sup-RPMI). The percentage of $V\gamma9^+$ T cells in peripheral blood increased from 3-14% at day 0 to 90-98% at day 12 (*Online Supplementary Figure S1*). All tumor cell lines were cultured in complete 10% RPMI-1640 as previously described.[7]

Leukemia and lymphoma primary samples

Pediatric B- or T-cell acute lymphoblastic leukemia cells containing high (> 80%) leukemia involvement were obtained from the peripheral blood and/or the bone marrow of patients at presentation after informed consent and institutional review board approval (Instituto Português de Oncologia, Lisbon, Portugal) had been obtained. Fresh leukemia samples were enriched by density centrifugation over Ficoll-Paque and then washed twice in 10% RPMI-1640 medium supplemented with 2 mM L-glutamine (Sup-RPMI). For lymphoma biopsies, lymph nodes were surgically removed, immediately frozen in liquid nitrogen and kept at -80°C until further use (Department of Pathology, Hospital de Santa Maria, CHLN, Lisbon, Portugal). Upon diagnosis, we selected lymph nodes from lymphoma cases and reactive lymph nodes for our studies.

In vitro *killing assays*

For cytotoxicity assays, tumor cells (cell lines or primary samples) were stained with DDAO-SE (Molecular Probes, Invitrogen) and incubated at a ratio of 1:10 with γδ T cells in Sup-RPMI. Typically, 3×10^5 HMB-PP-activated γδ-PBL (>90% $V\gamma9^+$) were co-incubated with 3×10^4 tumor cells (pre-labeled with 1 μM DDAO-SE) for 3-4h, then stained with Annexin V-FITC (BD Biosciences) and analyzed by flow cytometry.

RNA isolation, RT-qPCR and Affymetrix Microarrays

Total RNA from tumor cell lines was extracted using the RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). RNA from leukemia cells and samples was extracted with TRIzol Reagent (Invitrogen) and purified with RNeasy Mini Kit according to the manufacturer's instructions. Concentration and purity was determined by spectrophotometry and integrity was confirmed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA, USA). Total RNA was reverse-transcribed into cDNA as previously described.[7] qPCR was performed on Rotor-Gene 6000 (Corbett) using SYBR Green detection system (PE Applied Biosystems). Glucoronidase beta (GUSB) and proteasome subunit beta type 6 (PSMB6) were used as endogenous controls in relative quantification using the standard curve method. Primers were designed using the Roche Design Centre (for sequences see *Online Supplementary Table S1*).

For genome-wide analyses, RNA from two independent cultures of each cell line (DAUDI, RAJI, RCH-ACV and 697) was processed for use on Affymetrix (Santa Clara, CA, USA) GeneChip HuGene 1.0 ST Arrays, according to the manufacturer's Whole Transcript Sense Target Labeling Assay.

Microarray data analysis

All the microarray data analysis was performed with R and several packages available from CRAN[17] and Bioconductor.[18] The raw data (CEL files) were normalized and summarized with the Robust MultiArray Average method from the "affy" package.[19] Unsupervised clustering analysis of the gene expression profiles for entire probe set data was assessed through hierarchical clustering (Euclidean distance and complete agglomeration method) and principal component analysis (prcomp function which calls a sin-

gular value decomposition method for non-symmetric matrices) as implemented in the statistical computing package.[17] Differentially expressed genes for each comparison were selected using linear models and empirical Bayes methods[20] as implemented in the Limma package,[21] verifying the *P* values corresponding to moderated F-statistics, and selecting as differentially expressed genes those that had adjusted *P* values adjusted using the Benjamini and Hochberg method[22] lower than 0.05.

The enrichment of biological functions and pathways was analyzed using Ingenuity Pathway Analysis software (Ingenuity Systems, Mountain View, CA, USA) and all genes present in the Affymetrix Human Gene 1.0 ST as control.

Results

Highly variable susceptibility of acute leukemias and non-Hodgkin's lymphomas to γδ-PBL cytotoxicity

In our laboratory we have studied a collection of 23 samples of acute lymphoblastic leukemias and non-Hodgkin's lymphomas, and a panel of 20 tumor cell lines of hematopoietic origin. The latter included acute lymphoblastic leukemia (ALL) (JURKAT, MOLT4, RCH-ACV, 697, CEM, TOM-1, RS4-11, B15, REH, Bv173) and acute myelogenous leukemia (AML) (HL-60, HEL, THP-1) cell lines; and non-Hodgkin Burkitt's (DAUDI, RAJI, RAMOS), follicular (DOHH2) and lymphoblastic (Oz) lymphoma cell lines (for detailed description of these cell lines see *Online Supplementary Table S2*). Although the capacity of peripheral blood γδ T cells to target multiple tumor cell lines of hematopoietic origin is well documented,[7-9] we observed that a substantial fraction of cell lines (Figure 1A and B) and patient samples (Figure 1A and *data not shown*) were strikingly resistant to γδ-PBL (obtained from healthy donors) pre-activated (as illustrated by high CD69 levels) with HMB-PP, the most potent natural Vγ9Vδ2 T-cell activator known to date (6, 7) (*Online Supplementary Figure S1*). For example, the B-ALL cell lines Bv173, REH and 697 (Figure 1 A and B), and six primary samples obtained from B-ALL patients (Figure 1A and *data not shown*) remained mostly alive (Annexin V-) in co-cultures with fully-activated (100% $CD69^+$; data not shown) γδ-PBL. Similar data were obtained with primary T-ALL samples and the cell line CEM (Figure 1A). This resistance to γδ-PBL cytotoxicity contrasted sharply with the extensive killing observed for the B-ALL line RCH-ACV and the T-ALL line MOLT-4 (Figure 1A), among various other hematopoietic tumors (Figure 1B).

For systematic analysis of our killing assay data, we considered tumor samples with over 70% lysis as susceptible to γδ-PBL-mediated lysis ("γδ-susceptible"), and those under 30% lysed as "γδ-resistant". Importantly, susceptibility was independent of the γδ-PBL donor, as the pattern of susceptible/resistant lines was equivalent for 3 independent healthy donors (*Online Supplementary Figure S2A*). Moreover, the differences in susceptibility to γδ T cells were maintained when tumor cell lines were incubated with γδ T cells activated for a shorter time (12h) (*Online Supplementary Figure S2B*), further supporting the segregation between susceptible and resistant cell

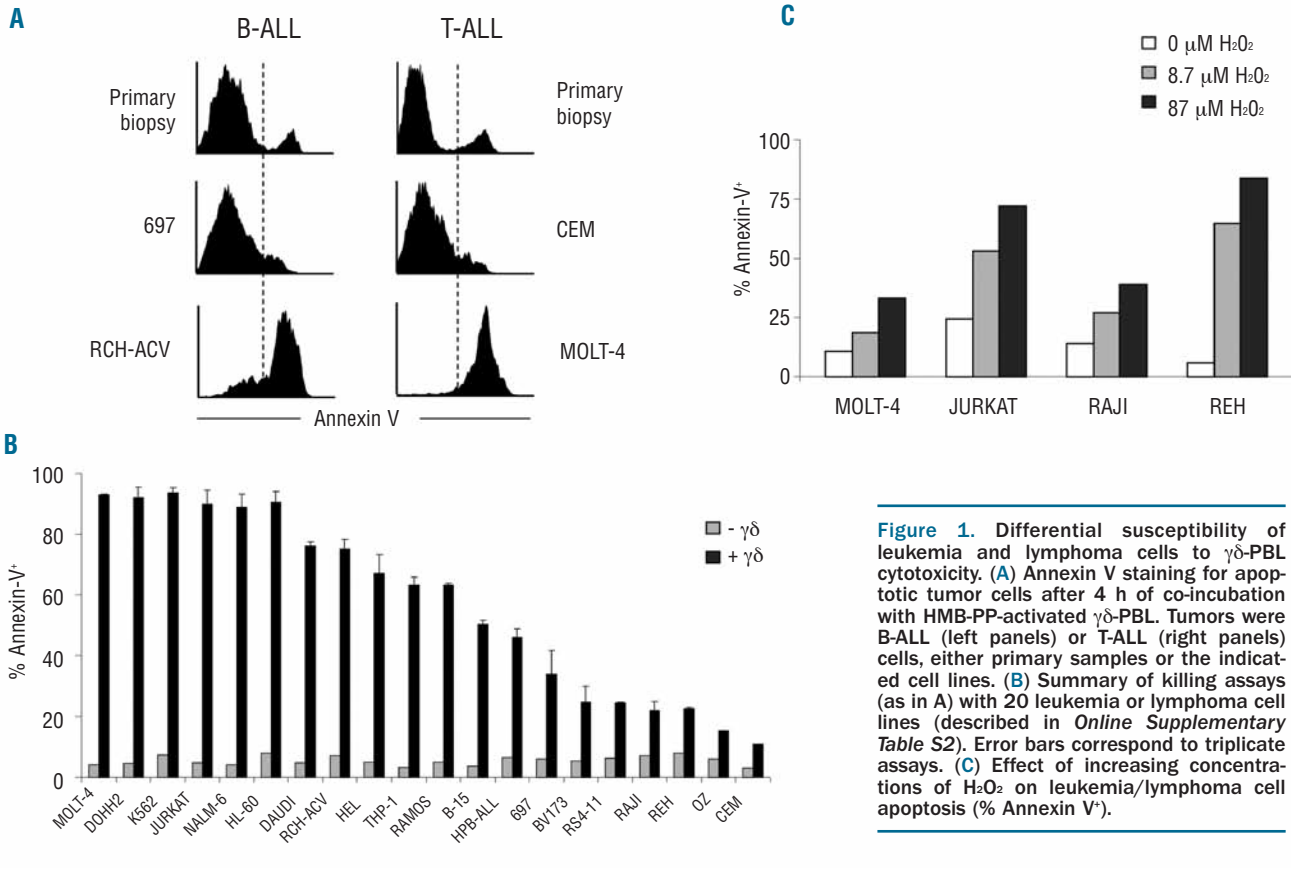

Figure 1. Differential susceptibility of leukemia and lymphoma cells to γδ-PBL cytotoxicity. (A) Annexin V staining for apoptotic tumor cells after 4 h of co-incubation with HMB-PP-activated γδ-PBL. Tumors were B-ALL (left panels) or T-ALL (right panels) cells, either primary samples or the indicated cell lines. (B) Summary of killing assays (as in A) with 20 leukemia or lymphoma cell lines (described in *Online Supplementary Table S2*). Error bars correspond to triplicate assays. (C) Effect of increasing concentrations of H_2O_2 on leukemia/lymphoma cell apoptosis (% Annexin V^+).

lines. As primary samples to reproduce and expand experiments aimed at dissecting the molecular mechanisms of tumor susceptibility to γδ-PBL cytotoxicity are difficult to obtain, we focused on our well-established panel of cell lines for the initial candidate searches and later extended our findings to patient samples.

We first considered that tumor resistance to γδ-PBL cytotoxicity could stem from intrinsic anti-apoptotic mechanisms developed by some leukemia/lymphoma cell lines. However, when we tested the effect of a pro-apoptotic stimulus (H_2O_2) we observed no association between resistance to apoptosis and to γδ-PBL cytotoxicity. Namely, the cell lines Jurkat (γδ-susceptible) and REH (γδ-resistant) were more sensitive to non-saturating concentrations of H_2O_2 than the cell lines MOLT-4 (γδ-susceptible) and RAJI (γδ-resistant) (Figure 1C). This suggests that susceptibility to γδ-PBL cytotoxicity is not related to the response to other death stimuli and probably involves a specific protein expression program (involved in tumor/γδ-PBL interactions) that we set out to characterize.

Genome-wide comparisons between γδ-susceptible and γδ-resistant hematopoietic tumors

The observed differences in susceptibility γδ-PBL cytotoxicity among hematopoietic tumors emphasize the importance of defining gene signatures that may predict the effectiveness of γδ T-cell based immunotherapies in the clinic. We performed a genome-wide analysis aimed at comparing the mRNA expression profiles of γδ-susceptible and γδ-resistant tumors. We employed cDNA microarrays to examine two pairs of hematopoietic tumor cell lines sharing the same cytogenetic alterations and cellular phenotypes (*Online Supplementary Table S2*): the Burkitt's lymphomas DAUDI (susceptible) and RAJI (resistant), and the B-ALL lines RCH-ACV (susceptible) and 697 (resistant).

First, samples were grouped according to the similarity of gene expression patterns using unsupervised clustering analysis (no group specification *a priori*). Based on the entire probe set data, two main groups could be defined which corresponded to the original cell type (Figure 2A): pre-B (697 and RCH-ACV) and mature B cells (DAUDI and RAJI). We next applied principal component analysis (PCA), which identifies new variables, to the principal components, which are linear combinations of the original variables (gene expression levels) and represent the largest variation found between samples.[23] Although the original cell type was the major source of variation between all samples (53.3% of total variation), PCA showed that component 3 was responsible for the segregation (16.4% of total variation) according to the susceptibility to γδ-PBL cytotoxicity (Figure 2B): susceptible (DAUDI and RCH-ACV) versus resistant (RAJI and 697).

To identify gene expression variations associated with susceptibility to γδ-PBL cytotoxicity, and to suppress the variations due to the transformed cell type (pre-B or mature B cells), we first compared tumors with identical origin, i.e. DAUDI *versus* RAJI, and RCH-ACV *versus* 697 (*Online Supplementary Tables S3 and S4*). We then used Bayesian linear models[20] and selected the common genes between both analyses: 340 genes (155 up- and 185 down-regulated in γδ-susceptible tumors) presented similar gene expression variations and were considered for further analysis (*Online Supplementary Table S5*). Bioinformatics analysis revealed an enrichment for functions related to cell-to-cell signaling and interaction, hematologic system development and function, immune

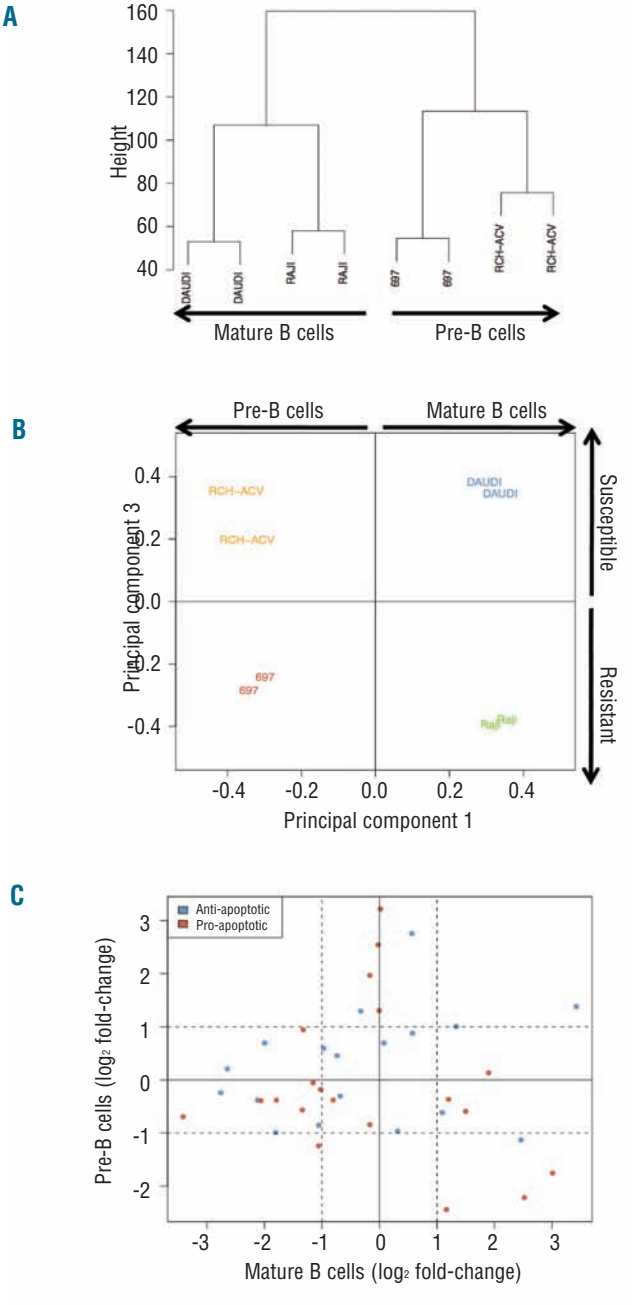

Figure 2. Comparison of gene expression in tumor cell lines susceptible or resistant to γδ-PBL cytotoxicity. Bioinformatics analyses of cDNA microarray comparisons between the Burkitt's lymphomas DAUDI and RAJI; and the B-ALL lines RCH-ACV and 697. (A) Unsupervised hierarchical clustering analysis. Samples with similar gene expression patterns are grouped together and connected with branches, producing a clustering tree (or dendrogram) on which the branch length inversely reflects the degree of similarity between samples. (B) Principal Component Analysis. The samples are plotted according to the first and third principal components (corresponding to the largest variation found between samples). (C) Variations in expression levels of anti- or pro-apoptotic genes in susceptible *versus* resistant tumor cell lines. Dashed lines indicate 2 fold-changes (in logarithmic scale) in the expression ratio susceptible/resistant.

cell trafficking (*P* value < 0.05; *Online Supplementary Table S6*). Some of the top pathways affected were interferon signaling, crosstalk between dendritic cells and natural killer cells, and molecular mechanisms of cancer (*P* value < 0.05; *Online Supplementary Table S7*).

The gene expression variations observed also suggested that, consistent with our previous experimental data (Figure 1C), the segregation between susceptible and resistant tumors is not associated with expression of anti- or pro-apoptotic genes (Figure 2C and *Online Supplementary Table S8*). Thus, up-/down-regulation of pro-/anti-apoptotic genes did not correlate with susceptibility to γδ-PBL cytotoxicity. Moreover, apoptotic related functions and pathways were not enriched in the panel of 340 genes (*Online Supplementary Table S7*). Based on these results, we favored the hypothesis that susceptibility or resistance to γδ-PBL cytotoxicity is conferred by signals presented at the tumor/γδ-PBL interface, i.e. on the surface of leukemia/lymphoma cells.

A set of cell surface proteins segregates between γδ-susceptible and γδ-resistant leukemia/lymphoma cell lines

T cells recognize their targets through cell surface antigens. We, therefore, focused our analysis of the panel of 340 genes on those encoding plasma membrane proteins (with extracellular domains), using a fold change threshold of 2 (log FC >1). These consisted of 8 genes up-regulated and 19 genes down-regulated in γδ-susceptible tumors when compared to resistant tumors (*Online Supplementary Table S9*). The mRNA expression levels of the 27 candidates were assessed by RT-qPCR (in independent samples) to validate the microarray results. Upon statistical analysis of the data, 22 out of the 27 genes were confirmed as differentially expressed in the two pairs of cell lines used for microarray comparisons: of these, 6 genes were up-regulated and 16 genes were down-regulated in γδ-susceptible tumors (Figure 3A). In order to have more stringent selection criteria, we extended our expression studies to a broader panel of cell lines, including 6 susceptible and 4 resistant cell lines (*Online Supplementary Figure S3*). This showed 10 genes with significant expression variation between susceptible and resistant tumors (*P* value < 0.05, Mann-Whitney test) (Figure 3B). Thus, our final panel of candidate markers of susceptibility to γδ-PBL cytotoxicity consisted of 3 genes enriched in γδ-suceptible tumors (*ULBP1, TFR2* and *IFITM1*), and 7 genes enriched in γδ-resistant leukemias/lymphomas (*CLEC2D, NRP2, SELL, PKD2, KCNK12, ITGA6* and *SLAMF1*) (Table 1).

Heterogeneity of expression of candidate markers in primary leukemia and lymphoma samples

We next determined the expression levels of each candidate marker in primary samples obtained from T-cell acute lymphoblastic leukemia (T-ALL) and non-Hodgkin's lymphoma (NHL) patients. Within the latter group, we sampled patients with common indolent (follicular) or aggressive (diffuse large B-cell - DLBCL) lymphomas. Gene expression levels in samples were compared with healthy PBMCs (for ALL) and reactive follicles (for NHL), taken as references (0 on log scale) in Figure 4A and B. Hence, a positive or negative (log scale) variation indicates higher or lower expression in tumors than in the control samples, respectively. Overall, the tumors exhibited very variable gene expression profiles. For example, among susceptibility-associated genes, *ULBP1* was over-expressed in a large number of primary samples, while *TFR2* was only enriched in three FL samples (FL 1, FL 2 and FL 8), and *IFITM1* was strongly depleted in various tumors (Figure 4A and B). On the other hand, all resistance-associated genes were over-expressed in FL sample 3, in contrast to the majority of primary samples analyzed. Moreover, there was no essential difference in some markers, such as ITGA6 or SELL, between the various patients (Figure 4A and B). Collectively, these data revealed a striking heterogeneity in the expression of particular candidate genes in primary tumors. When compared to our results with tumor cell lines (*Online Supplementary Figure S3*), these clinical data possibly reflect distinct selective pressures on the

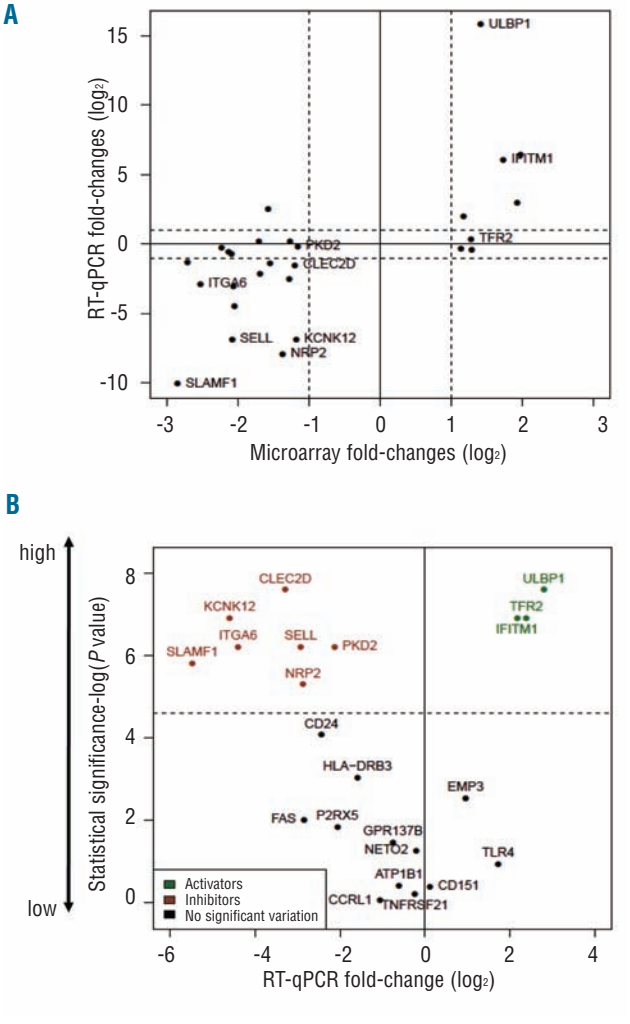

Figure 3. Variations in expression of genes encoding cell surface proteins that segregate between γδ-susceptible and γδ-resistant leukemia/lymphoma cell lines. (A) RT-qPCR validation of microarray results for the comparisons of Figure 2. The mRNA expression levels were normalized to GUSB and PSMB6 for each cell line. Plotted are the averages of relative expression levels in DAUDI versus RAJI (DAUDI/ RAJI) and RCH-ACV versus 697 (RCH-ACV/ 697). Dashed lines indicate 2 fold-change values (in logarithmic scale). (B) Statistical analysis of RT-qPCR results (detailed in Figure 4) in 6 susceptible and 4 resistant cell lines. Statistical significance was assessed by Mann-Whitney test (-log *P* value). Dashed line represents the statistical threshold *P*=0.01.

Table 1. Panel of cell surface proteins associated with the susceptibility or resistance of lymphomas/leukemias to γδ T-cell cytotoxicity. The statistical difference between the average gene expression in the 6 susceptible versus the 4 resistant tumors of Figure 4 was assessed by Mann-Whitney test ($P<0.05$).

Symbol	Description	Biological function	P value
Enriched in γδ-susceptible tumors			
ULBP1	UL16 binding protein	Ligand for NKG2D on NK and T cells; induces cytotoxicity, cytokine secretion	0
IFITM1 (CD225)	Interferon-induced transmembrane protein 1	Involved in cell proliferation and malignancy	0
TFR2	Transferrin receptor 2	Cellular uptake of transferrin-bound iron	0.004
Enriched in γδ-resistant tumors			
CLEC2D	C-type lectin 2, D	Ligand for the NK inhibitory receptor CD161	0.002
SELL	Selectin L	Adhesion of T cells to endothelial cells	0.001
SLAMF1	Signaling lymphocytic activation molecule 1	Bidirectional T cell to B cell stimulation	0
KCNK12	Potassium channel K, 12	Potassium channel	0
ITGA6	Integrin alpha 6	Integrin; receptor for laminin	0.014
PKD2	Polycystic kidney disease 2	Calcium channel	0.017
NRP2	Neuropilin 2	Co-receptor for VEGF; implicated in tumor growth and vascularization	0.018

expression of the genes that compose the candidate panel, the consequence of which should now be evaluated in clinical trials.

Discussion

The success of immunotherapy to tackle tumors, in particular those that prevail after chemo- or radiotherapy, critically depends on two factors: the specific activation of effector anti-tumor lymphocytes and the molecular recognition of tumor cells by activated lymphocytes. Concerning γδ T cells, research over the last 15 years has identified very potent and specific phosphoantigens, most notably HMB-PP,[6,7] that seem to fulfill the first requirement. There have been suggestions that phosphoantigens themselves,[6,24,25] or an F1-ATPase-related structure complexed with delipidated apolipoprotein A-I,[26] or the non-classical MHC protein ULBP4[27] could be responsible for tumor cell recognition by Vγ9Vδ2 PBL. However, despite this, the issue is still highly controversial. This naturally impacts on our ability to design effective therapeutic protocols based on γδ-PBL immunotargeting of tumors. Thus, only 33% of patients with prostate carcinoma[11] or non-Hodgkin's lymphoma[14] showed objective responses despite large activation and expansion of their Vγ9Vδ2 T cells *in vivo*. These considerations stress the importance of identifying tumor molecular signatures that may predict the response to activated γδ-PBL.

In this study, we set out to identify cell surface proteins involved in interactions between leukemia/lymphoma cells and γδ-PBL. Taking *in vitro* tumor cytolysis as functional readout, we screened a panel of 20 leukemia and lymphoma cell lines that faithfully reproduced the susceptibility/resistance of primary tumors (Figure 1A). The use of cell lines permitted experimental reproducibility and hence statistical robustness for the gene expression undertaken. Upon the identification of candidate markers, we analyzed their expression in 23 samples derived from T-ALL and NHL (FL and DLBCL) patients.

The choice of cDNA microarrays as screening tools was based on a multiplicity of previous studies that demonstrated how powerful and reliable they are in defining cancer molecular signatures.[28] Our analyses led to the identification of a large panel of genes differentially expressed between "γδ-susceptible" and "γδ-resistant" tumors. Importantly, we verified that there was no correlation between intrinsic anti-apoptotic properties and resistance to γδ-PBL cytotoxicity, both in terms of gene expression and response to a death stimulus. Thus, susceptibility or resistance to γδ-mediated lysis is more likely to be related to tumor recognition and immune evasion strategies, the molecular basis of which remains to be clarified. Of note, MHC class Ia expression did not consistently segregate between γδ-susceptible and γδ-resistant tumor cell lines (*Online Supplementary Figure S4*). For example, among susceptible lines, DAUDI and MOLT-4 expressed very low or undetectable levels, whereas JURKAT and RCH-ACV displayed high levels of surface MHC class I (*Online Supplementary Figure S4*). These data exclude a mechanism of "missing self" as the basis for γδ T-cell recognition of hematopoietic tumors.

Building upon stringent biological and statistical selection criteria, we narrowed our microarray data down to 10 genes encoding cell surface proteins (with extracellular domains), whose expression segregated with susceptibility *versus* resistance to γδ-PBL cytotoxicity. We believe it is important to make this gene profile available to the biomedical community. Thus, we propose the expression of each candidate gene to be evaluated during upcoming γδ T-cell based clinical trials. The genes with highest predictive value will constitute novel leukemia/lymphoma biomarkers, for which standardized quantification essays should be developed. This will provide clinicians with a key tool for the indication and monitoring of γδ T-cell based immunotherapies.

Furthermore, within the panel of 10 candidate markers, some are likely to play non-redundant roles in leukemia/lymphoma cell recognition by γδ-PBL. Thus, proteins that are enriched in γδ-susceptible tumors may provide activation signals, whereas markers of resistance may convey inhibitory signals to γδ-PBL. Provocatively, 7

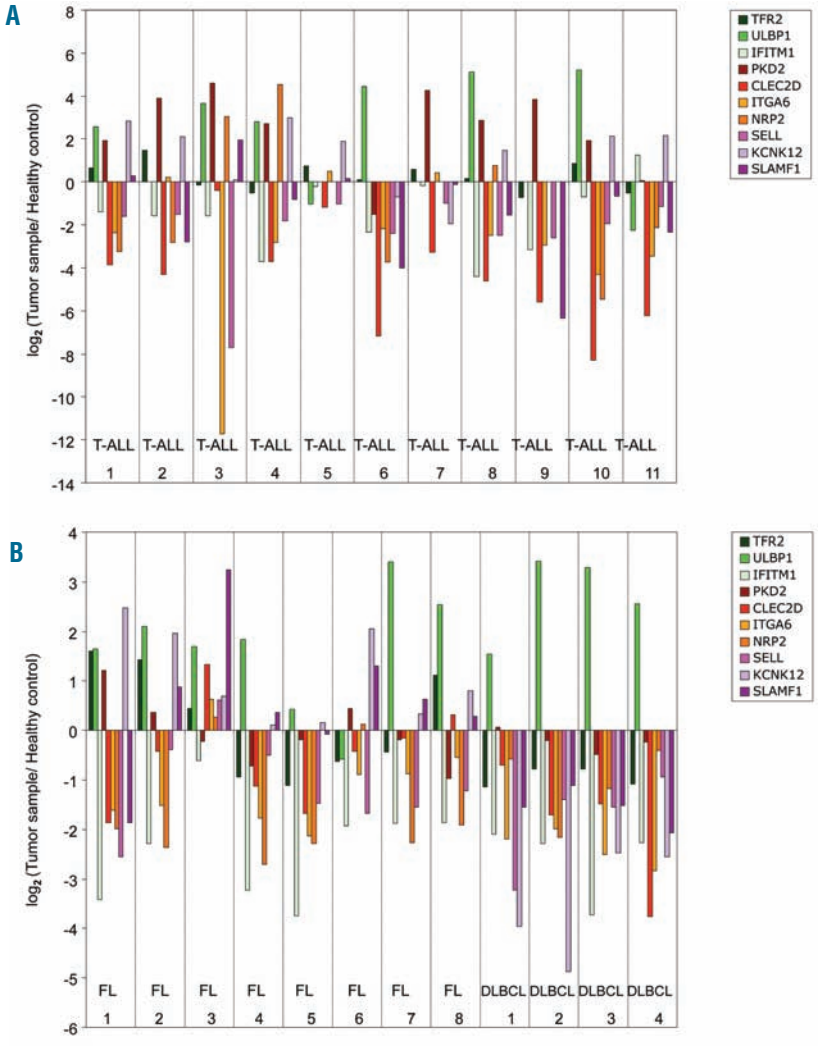

Figure 4. Quantification of mRNA expression levels of γδ-susceptibility markers in acute lymphoblastic leukemia and non-Hodgkin's lymphoma patients. (A) RT-qPCR analysis of mRNA expression in 11 T-cell acute lymphoblastic leukemia (T-ALL) samples, normalized to housekeeping genes (*GUSB* and PSMB6) and to reference PBMCs from healthy individuals. Values were converted to logarithmic scale. (B) RT-qPCR analysis of mRNA expression in 8 follicular lymphoma (FL) and 4 diffuse large B cell lymphoma (DLBCL) samples, normalized to housekeeping genes (*GUSB* and *PSMB6*) and to a reference sample - reactive follicles – obtained through the same procedure. Values were converted to logarithmic scale.

of the candidates are known to intervene in immune responses: 4 of them (ULBP1, IFITM1, CLEC2D and SLAMF1) provide stimulatory (or inhibitory) signals through receptors expressed on lymphocytes, while 3 (NRP2, SELL and ITGA6) control lymphocyte adhesion. ULBP1 is a ligand for the NKG2D receptor expressed on all cytotoxic lymphocyte lineages, including 100% of Vγ9Vδ2 T cells, which has been clearly implicated in anti-tumor responses.[29-32] IFITM1 was shown to modulate NK cell responses and its expression correlated with improved survival of gastric cancer patients.[33] By contrast, the expression of CLEC2D, a ligand for the inhibitory receptor CD161, inhibits NK cell responses and was associated with increased malignancy grade of glioblastoma.[34] NRP2 is another protein that can favor cancer progression by acting as a coreceptor for vascular endothelial growth factor (VEGF) and stimulating tumor growth (35). We will now proceed with individual knock-down (RNA interference) experiments in a functional (tumor killing) bioassay to dissect the role of each of the candidates in γδ-PBL targeting of leukemias and lymphomas. Given that some of these molecules can also provide costimulatory or inhibitory signals to NK cells, we also plan to address their role in NK cell targeting of hematopoietic malignancies.

In summary, this report establishes a panel of 10 putative markers of leukemia/lymphoma susceptibility to γδ-PBL cytotoxicity. The expression data collected from primary samples showed a striking heterogeneity for particular candidate genes, most notably *ULBP1*, whereas other genes, such as *IFITM1*, *ITGA6* or *SELL*, essentially did not vary among patients. It is, therefore, predictable that different components of the proposed panel will behave in very distinct ways when associated to therapeutic outcome in clinical trials. It will also be interesting to evaluate to what extent immunoselection may have conditioned the expression of these markers in tumors evolving in a dynamic interaction with γδ T lymphocytes. This will significantly add to our understanding of anti-tumor immunity and to our capacity to modulate it for cancer immunotherapy.

Authorship and Disclosures

BSS was the principal investigator and takes primary responsibility for the paper. AQG, DVC and TL performed the laboratory work for this study. ARG performed the bioinformatics analysis of the data. CF, JFL, JTB and MGS provided clinical samples and suggestions. AQG, DVC and BSS wrote the manuscript.

The authors reported no potential conflicts of interest.

References

1. Kabelitz D, Wesch D, He W. Perspectives of gammadelta T cells in tumor immunology. Cancer Res. 2007;67(1):5-8.
2. Girardi M, Oppenheim DE, Steele CR, Lewis JM, Glusac E, Filler R, et al. Regulation of cutaneous malignancy by gammadelta T cells. Science. 2001;294 (5542):605-9.
3. Gao Y, Yang W, Pan M, Scully E, Girardi M, Augenlicht LH, et al. Gamma delta T cells provide an early source of interferon gamma in tumor immunity. J Exp Med. 2003;198(3):433-42.
4. Liu Z, Eltoum IE, Guo B, Beck BH, Cloud GA, Lopez RD. Protective immunosurveillance and therapeutic antitumor activity of gammadelta T cells demonstrated in a mouse model of prostate cancer. J Immunol. 2008;180(9):6044-53.
5. Street SE, Hayakawa Y, Zhan Y, Lew AM, MacGregor D, Jamieson AM, et al. Innate immune surveillance of spontaneous B cell lymphomas by natural killer cells and gammadelta T cells. J Exp Med. 2004;199(6): 879-84.
6. Morita CT, Jin C, Sarikonda G, Wang H. Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vgamma2Vdelta2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens. Immunol Rev. 2007;215:59-76.
7. Correia DV, d'Orey F, Cardoso BA, Lanca T, Grosso AR, deBarros A, et al. Highly active microbial phosphoantigen induces rapid yet sustained MEK/Erk- and PI-3K/Akt-mediated signal transduction in anti-tumor human gammadelta T-cells. PLoS One. 2009;4(5):e5657.
8. Sicard H, Al Saati T, Delsol G, Fournie JJ. Synthetic phosphoantigens enhance human Vgamma9Vdelta2 T lymphocytes killing of non-Hodgkin's B lymphoma. Mol Med. 2001;7(10):711-22.
9. Kunzmann V, Wilhelm M. Anti-lymphoma effect of gammadelta T cells. Leuk Lymphoma. 2005;46(5):671-80.
10. Tokuyama H, Hagi T, Mattarollo SR, Morley J, Wang Q, Fai-So H, et al. V gamma 9 V delta 2 T cell cytotoxicity against tumor cells is enhanced by monoclonal antibody drugs--rituximab and trastuzumab. Int J Cancer. 2008;122(11): 2526-34.
11. Dieli F, Vermijlen D, Fulfaro F, Caccamo N, Meraviglia S, Cicero G, et al. Targeting human {gamma}delta} T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. Cancer Res. 2007;67(15):7450-7.
12. Kobayashi H, Tanaka Y, Yagi J, Osaka Y, Nakazawa H, Uchiyama T, et al. Safety profile and anti-tumor effects of adoptive immunotherapy using gamma-delta T cells against advanced renal cell carcinoma: a pilot study. Cancer Immunol Immunother. 2007;56(4):469-76.
13. Bennouna J, Bompas E, Neidhardt EM, Rolland F, Philip I, Galea C, et al. Phase-I study of Innacell gammadelta, an autologous cell-therapy product highly enriched in gamma9delta2 T lymphocytes, in combination with IL-2, in patients with metastatic renal cell carcinoma. Cancer Immunol Immunother. 2008;57(11):1599-609.
14. Wilhelm M, Kunzmann V, Eckstein S, Reimer P, Weissinger F, Ruediger T, et al. Gammadelta T cells for immune therapy of patients with lymphoid malignancies. Blood. 2003;102(1):200-6.
15. Gertner-Dardenne J, Bonnafous C, Bezombes C, Capietto AH, Scaglione V, Ingoure S, et al. Bromohydrin pyrophosphate enhances antibody-dependent cell-mediated cytotoxicity induced by therapeutic antibodies. Blood. 2009;113(20): 4875-84.
16. Abe Y, Muto M, Nieda M, Nakagawa Y, Nicol A, Kaneko T, et al. Clinical and immunological evaluation of zoledronate-activated Vgamma9gammadelta T-cell-based immunotherapy for patients with multiple myeloma. Exp Hematol. 2009; 37(8):956-68.
17. Team RDC. R: A Language and Environment for Statistical Computing. In: Computing RFfS, ed. Vienna, Austria, 2009.
18. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, et al. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. 2004;5(10):R80.
19. Gautier L, Cope L, Bolstad BM, Irizarry RA. affy--analysis of Affymetrix GeneChip data at the probe level. Bioinformatics. 2004;20(3):307-15.
20. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol. 2004;3:Article3.
21. Smyth GK. Limma: linear models for microarray data. In: R. Gentleman and V. Carey and S. Dudoit and R. Irizarry WH, ed. Bioinformatics and Computational Biology Solutions using R and Bioconductor. New York: Springer, 2005: 397-420.
22. Benjamini YH, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. Journal of the Royal Statistical Society Series B. 1995;57:289-300.
23. Ringner M. What is principal component analysis? Nat Biotechnol. 2008;26(3):303-4.
24. Li J, Herold MJ, Kimmel B, Muller I, Rincon-Orozco B, Kunzmann V, et al. Reduced expression of the mevalonate pathway enzyme farnesyl pyrophosphate synthase unveils recognition of tumor cells by Vgamma9Vdelta2 T cells. J Immunol. 2009;182(12):8118-24.
25. Vantourout P, Mookerjee-Basu J, Rolland C, Pont F, Martin H, Davrinche C, et al. Specific requirements for Vgamma9Vdelta2 T cell stimulation by a natural adenylated phosphoantigen. J Immunol. 2009;183(6): 3848-57.
26. Scotet E, Martinez LO, Grant E, Barbaras R, Jeno P, Guiraud M, et al. Tumor recognition following Vgamma9Vdelta2 T cell receptor interactions with a surface F1-ATPase-related structure and apolipoprotein A-I. Immunity. 2005;22(1):71-80.
27. Kong Y, Cao W, Xi X, Ma C, Cui L, He W. The NKG2D ligand ULBP4 binds to TCRgamma9/delta2 and induces cytotoxicity to tumor cells through both TCRgammadelta and NKG2D. Blood. 2009;114(2):310-7.
28. Sole X, Bonifaci N, Lopez-Bigas N, Berenguer A, Hernandez P, Reina O, et al. Biological convergence of cancer signatures. PLoS One. 2009;4(2):e4544.
29. Raulet DH. Roles of the NKG2D immunoreceptor and its ligands. Nat Rev Immunol. 2003;3(10):781-90.
30. Gomes AQ, Correia DV, Silva-Santos B. Non-classical major histocompatibility complex proteins as determinants of tumour immunosurveillance. EMBO Rep. 2007;8(11):1024-30.
31. Guerra N, Tan YX, Joncker NT, Choy A, Gallardo F, Xiong N, et al. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. Immunity. 2008;28(4):571-80.
32. Strid J, Roberts SJ, Filler RB, Lewis JM, Kwong BY, Schpero W, et al. Acute upregulation of an NKG2D ligand promotes rapid reorganization of a local immune compartment with pleiotropic effects on carcinogenesis. Nat Immunol. 2008;9(2):146-54.
33. Yang Y, Lee JH, Kim KY, Song HK, Kim JK, Yoon SR, et al. The interferon-inducible 9-27 gene modulates the susceptibility to natural killer cells and the invasiveness of gastric cancer cells. Cancer Lett. 2005; 221(2): 191-200.
34. Roth P, Mittelbronn M, Wick W, Meyermann R, Tatagiba M, Weller M. Malignant glioma cells counteract antitumor immune responses through expression of lectin-like transcript-1. Cancer Res. 2007;67(8):3540-4.
35. Pellet-Many C, Frankel P, Jia H, Zachary I. Neuropilins: structure, function and role in disease. Biochem J. 2008;411(2):211-26.

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Differentiation of human peripheral blood V$\delta 1^+$ T cells expressing the natural cytotoxicity receptor NKp30 for recognition of lymphoid leukemia cells

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Differentiation of human peripheral blood Vδ1$^+$ T cells expressing the natural cytotoxicity receptor NKp30 for recognition of lymphoid leukemia cells

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The success of cancer immunotherapy depends on productive tumor cell recognition by killer lymphocytes. γδ T cells are a population of innate-like lymphocytes endowed with strong, MHC-unrestricted cytotoxicity against tumor cells. This notwithstanding, we recently showed that a large proportion of human hematologic tumors is resistant to γδ peripheral blood lymphocytes (PBLs) activated with specific agonists to the highly prevalent Vγ9Vδ2 TCR. Although this probably constitutes an important limitation to current γδ T cell–mediated immunotherapy strategies, we describe here the differentiation of a novel subset of Vδ2$^-$ Vδ1$^+$ PBLs expressing natural cytotoxicity receptors (NCRs) that directly mediate killing of leukemia cell lines and chronic lymphocytic leukemia patient neoplastic cells. We show that Vδ1$^+$ T cells can be selectively induced to express NKp30, NKp44 and NKp46, through a process that requires functional phosphatidylinositol 3-kinase (PI-3K)/AKT signaling on stimulation with γ_c cytokines and TCR agonists. The stable expression of NCRs is associated with high levels of granzyme B and enhanced cytotoxicity against lymphoid leukemia cells. Specific gain-of-function and loss-of-function experiments demonstrated that NKp30 makes the most important contribution to TCR-independent leukemia cell recognition. Thus, NKp30$^+$ Vδ1$^+$ T cells constitute a novel, inducible and specialized killer lymphocyte population with high potential for immunotherapy of human cancer. (*Blood*. 2011;118(4):992-1001)

Introduction

Tumors develop in hosts endowed with a highly complex immune system that includes various lymphocyte subsets capable of recognizing and destroying transformed cells. It is now widely accepted that, although lymphocytes may constantly patrol tumor formation, cancer cells develop molecular strategies to evade immune surveillance, which are competitively selected under the pressure of the host immune system.[1] This dynamic process, termed "cancer immunoediting," is thought to constitute a major obstacle to cancer immunotherapy.[1]

Among multiple immune evasion mechanisms, we have recently shown that leukemia and lymphoma primary cells often down-regulate the nonclassical MHC protein, ULBP1, which is critical for recognition of hematologic tumors by γδ T cells expressing the counter-receptor NKG2D.[2] γδ T cells are innate-like lymphocytes that account for 1%-10% of peripheral blood lymphocytes (PBL) of healthy people and are capable of targeting a significant fraction of hematologic tumor cell lines tested in the laboratory.[3] However, we have demonstrated that many lymphoid leukemia cells are resistant to fully activated Vγ9Vδ2 T cells,[2,3] the dominant subset of γδ PBLs. Furthermore, clinical trials involving the in vivo administration of activators of Vγ9Vδ2 T cells have shown limited success, with objective responses restricted to 10%-33% of patients with either hematologic or solid tumors.[4-6] Even more modest has been the outcome of trials involving the adoptive transfer of activated and expanded Vδ2$^+$ cells, because no objective responses have been reported.[6] In fact, the simple ex vivo expansion of autologous Vδ2$^+$ T cells, whose surveillance the tumor managed to escape in vivo, may be condemned to little therapeutic effect on reinjection into the patient. Therefore, we believe it is critical to invest in strategies that endow γδ T cells with additional recognition machinery to detect tumors that have resisted the natural components present in vivo.

Besides Vγ9Vδ2 T cells, Vδ1$^+$ T cells are also endowed with potent antitumor cytolytic function, particularly as tissue-associated or tumor-infiltrating lymphocytes.[7-10] Moreover, Vδ1$^+$ T cells can constitute up to 30% of all γδ PBLs and may thus represent an important alternative population for adoptive cell therapy. However, this possibility remains poorly explored.

In this study we identified and characterized a novel Vδ1$^+$ PBL subset capable of targeting hematologic tumors highly resistant to fully activated Vγ9Vδ2 PBLs. We show that this Vδ1$^+$ population owes its specialized killer function to induced expression of natural cytotoxicity receptors (NCRs), which have been mostly regarded as NK-specific markers. Instead, we show that, although neither Vδ1$^+$ nor Vδ2$^+$ cells express NCRs constitutively, these can be selectively up-regulated in Vδ1$^+$ cells by AKT-dependent signals provided synergistically by γ_c cytokines (IL-2 or IL-15) and TCR stimulation. We further show that NKp30 and NKp44 are both functional in NCR$^+$ Vδ1$^+$ PBLs, and synergistically contribute to enhanced targeting of lymphocytic leukemia cells, with NKp30 playing the major role in this process. Thus, NKp30$^+$ Vδ1$^+$ PBL

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constitute a novel promising population for adoptive cell immunotherapy of hematologic malignancies.

Methods

Ethics statement

Research involving clinical samples was conducted according to the principles expressed in the Helsinki Declaration. All procedures were approved by the review board of Instituto Português de Oncologia de Lisboa (Portugal).

Isolation of human peripheral blood γδ T cells

Peripheral blood was collected from anonymous healthy volunteers, diluted in a 1:1 ratio (volume-to-volume) with PBS (Invitrogen Gibco), and centrifuged in Ficol-Paque (Histopaque-1077; Sigma-Aldrich) in a volume ratio of 1:3 (1 part ficol to 3 parts diluted blood) for 30 minutes at 1 500 rpm and 25°C. The interfase containing mononuclear cells was collected and washed (in PBS), and γδ T cells were isolated (to above 95% purity) by magnetic cell sorting via positive selection (with a FITC-labeled anti-TCRγδ antibody) or via negative selection (with a cocktail of Biotin-labeled antibodies; Miltenyi Biotec). When noted, Vδ1$^+$ cells were further purified by magnetic cell sorting via positive selection with a FITC-labeled anti-Vδ1 TCR antibody (Fisher Scientific) and anti-FITC microbeads (Miltenyi Biotec).

Cell culture

Isolated γδ PBLs were cultured at 10^6 cells/mL at 37°C, 5% CO^2 in round-bottom 96 well plates with RPMI 1640 and 2 mM l-glutamine (Invitrogen Gibco) supplemented with 10% FBS (Invitrogen Gibco), 1 mM sodium pyruvate (Invitrogen Gibco), and 50 mg/mL of penicillin and streptomycin (Invitrogen Gibco). The cells were expanded in the presence of 100 U/mL of rhIL-2 (Roche Applied Science), with or without 10 nM of HMB-PP (4-hydroxy-3-methyl-but-2-enyl pyrophosphate; Echelon Biosciences) and 1μg/mL of phytohemagglutinin (PHA; Sigma-Aldrich). Cells were washed and the culture medium was replaced every 5-6 days. To study the induction of NKp30 expression, γδ PBLs were cultured in the presence or absence of 100 U/mL of rhIL-2 (Roche Applied Science), 1μg/mL of soluble anti-CD3 antibody (eBioscience; clone OKT3), and 20ng/mL of rhIL-15 (Biolegend). For TCR blockade, freshly isolated γδ PBL were CFSE-labeled and then incubated for 7 days with anti-TCRγδ (Beckman Coulter; clone IMMU510) diluted 1:20 in complete medium supplemented with 1μg/mL PHA and 100 U/mL rhIL-2. To study the effects of chemical inhibitors of signal transduction, the MEK inhibitor UO126 and the PI-3K inhibitor LY294002 (both from Calbiochem) were added at 10 mM for a 2-hour incubation period and then maintained in culture with 100 U/mL rhIL-2 and 1μg/mL PHA for 7 days.

Flow cytometric cell sorting

For sorting of γδ PBL based on the expression of NKp30 and Vδ1$^+$ TCR, cells from PHA and IL-2–activated cultures were stained with anti-NKp30 (Biolegend; clone P30-15), anti-Vδ1 (Thermo Fisher Scientific; clone TS8.2), and sorted on a FACSAria cell sorter (BD Biosciences).

Leukemia patient samples

B-cell chronic lymphocytic leukemia cells were obtained from the peripheral blood of patients at presentation, after informed consent and institutional review board approval (Instituto Português de Oncologia de Lisboa, Portugal). Samples were enriched by density centrifugation over Ficol-Paque and then washed twice in 10% RPMI 1640.

In vitro tumor-killing assays

All tumor cell lines (details provided in supplemental Table 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article) were cultured in complete 10% RPMI 1640, maintained at 10^5 up to 10^6 cells/mL by dilution and splitting in a 1:3 ratio every 3-4 days. For cytotoxicity assays, magnetically purified γδ PBL were preactivated for 7-19 days in the presence of IL-2 (100 U/mL) and either 1μg/mL PHA or 10 nM HMB-PP. For receptor blocking, γδ PBLs were incubated for 2 hours with the blocking antibodies anti-NKp30 (clone F252), anti-NKp44 (clone KS38), anti-NKp46 (clone KL247), anti-TCRγδ (Beckman Coulter, clones IMMU510 or B1.1), or anti-Vδ1 TCR (Fisher Scientific, clones TCS1 or TS8.2). The blocking antibodies were maintained in the culture medium during the killing assays. Tumor cell lines or leukemia primary samples were stained with CellTrace Far Red DDAO-SE (1 μM; Molecular Probes, Invitrogen) and each batch of 3×10^4 tumor cells was incubated with 1.5×10^5 to 3×10^5 γδ T cells in RPMI for 3 hours at 37°C and 5% CO^2 on a round-bottom plate with 96 wells. Cells were then stained with annexin V–FITC (BD Biosciences) and analyzed by flow cytometry. For the redirected killing assays, PHA and IL-2–activated γδ PBL were incubated for 4 hours with the NCR agonists anti-NKp30 (clone AZ20), anti-Nkp44 (clone Z231) or anti-NKp46 (clone Bab281) during a standard ^{51}Cr release assay.

Flow cytometry analysis

Cells were labeled with the following fluorescent monoclonal antibodies: anti-CD3–PerCP-Cy5.5 (eBioscience; clone OKT3); anti-TCRγδ–FITC (eBioscience; clone B1.1); anti-CD69–PE (BD Pharmingen; clone FN50); anti-NKG2D–PE/Cy7 (Biolegend; clone 1D11); anti-2B4–APC (Biolegend, clone C1.7); anti–DNAM-1–Alexa-Fluor647 (Biolegend; clone DX11); anti-NKp30–APC (Biolegend; clone P30-15); anti-Vδ2 TCR-PE (Biolegend; clone B6); anti-NKp44–APC (Biolegend; clone P44-8); anti-NKp46–AlexaFluor647 (Biolegend; clone 9E2); anti-Vδ1 TCR-FITC (Thermo Fisher Scientific; clone TS8.2); anti-NKp30–PE (Biolegend; clone P30-15); anti–Mouse IgG1κ-APC Isotype Ctrl (Biolegend; clone MOPC-21); anti–Mouse IgG1κ-PE Isotype Ctrl (Biolegend, clone MOPC-21); anti-CD27–APC/Cy7 (Biolegend, clone O323); and anti-CD56–APC (Biolegend, clone HCD56). Cell proliferation was measured by following a standard CFSE staining protocol (CellTrace CFSE Cell Proliferation Kit, Invitrogen; final concentration 0.5 mM), while apoptosis was assessed by annexin V–FITC (BD Pharmingen) staining. Cells were analyzed on a FACSCanto flow cytometer (BD Biosciences).

RNA isolation and cDNA production

Total RNA was extracted using the RNeasy Mini Kit according to the manufacturer's protocol (QIAGEN). Concentration and purity was determined by spectrophotometry and integrity was confirmed using an Agilent 2100 Bioanalyzer with a RNA 6000 Nano Assay (Agilent Technologies). Total RNA was reverse-transcribed into cDNA using random hexamers and Superscript II first strand synthesis reagents (Invitrogen).

Real-time quantitative PCR

Real-time quantitative PCR (qPCR) was performed on ABI Prism 7500 FAST Sequence Detection System using SYBR Green detection system (both from Applied Biosystems). Primers were designed using Primer3 v.0.4.0 online program (http://primer3.sourceforge.net). For each transcript, quantification was done using the calibration curve method. β_2-microglobulin (*B2M*), Glucoronidase β (*GUSB*) and proteasome subunit β type 6 (*PSMB6*) were used as housekeeping controls for normalization of gene expression. The following primers were used: *B2M*, forward CTAT CCAG CGTA CTCC AAAG ATTC, reverse CTTG CTGA AAGA CAAG TCTG AATG; *PSMB6*, forward GGCG GCTA CCTT ACTA GCTG, reverse AAAC TGCA CGGC CATG ATA; *GUSB*, forward TGCA GCGG TGTA CTTC TG, reverse CCTT GACA GAGA TCTG GTAA TCA; *B7H6*, forward TCAC CAAG AGGC ATTC CGAC CT, reverse ACCA CCTC ACAT CGGT ACTC TC; *NKP44*, forward CCGT CAGA TTCT ATCT GGTG GT, reverse CACA CAGC TCTG GGTC TGG; *NKP46*, forward AAGA CCCC ACCT TTCC TGA, reverse TGCT GGCT CGCT CTCT AGT; *GZMB*, forward GGGG GACC CAGA GATT AAAA, reverse CCAT TGTT TCGT CCAT AGGA G. All samples were run in triplicate and repeated 3 times. Analysis of the qPCR results was performed using the ABI SDS v1.1 sequence analysis software (Applied Biosystems).

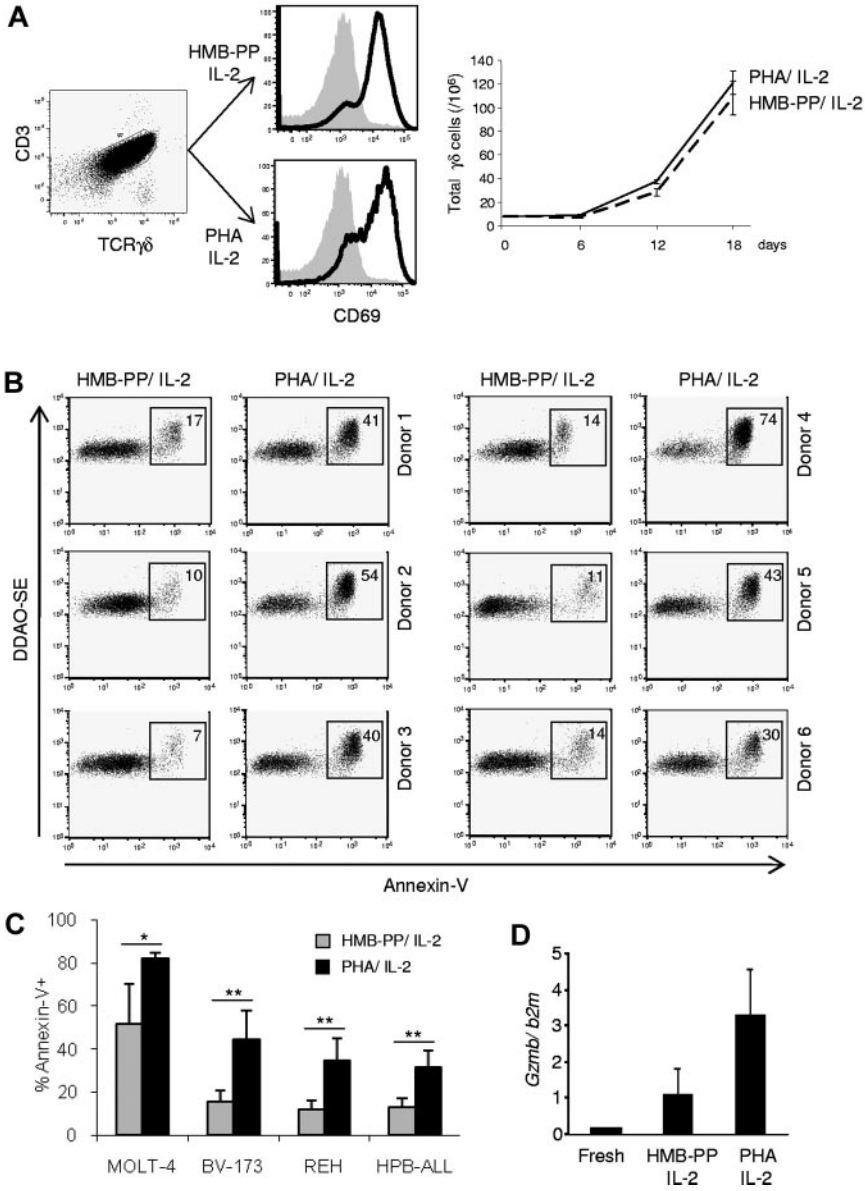

Figure 1. Enhanced antileukemia cytotoxicity of γδ PBL cultures activated with pan–T-cell mitogen. (A) γδ peripheral blood lymphocytes (γδ PBLs) were MACS-sorted from the peripheral blood of healthy volunteers (left panel), and stimulated with either HMB-PP and IL-2 or PHA and IL-2 for 4 to 19 days. Activation was evaluated by flow cytometry for CD69 up-regulation (middle panels; levels in freshly isolated control cells are shaded), and total cell numbers are shown on the right panel. (B-C) Preactivated (for 14 days, as in panel A) γδ PBLs were coincubated with DDAOse-labeled leukemia cells for 3 hours. Tumor cell lysis was evaluated by annexin-V staining using flow cytometry. (B) Representative results of 6 different donors for the Bv173 leukemia cell line. Percentages refer to annexin-V+ tumor cells. Basal tumor cell apoptosis (in the absence of γδ PBL) was < 5%. (C) Summary of the results of 6 different donors with 4 leukemia target cell lines. Error bars represent SD (n = 6, $*P < .05$; $**P < .01$). (D) Real-time PCR quantification of *GzmB* mRNA levels in freshly isolated, HMB-PP and IL-2–activated and PHA and IL-2–activated γδ PBL. Data in this figure are representative of 2 to 3 independent experiments with similar results.

Statistical analysis.

Differences between subpopulations were assessed using the Student *t* test and are indicated when significant as $*P < .05$; $**P < .01$; and $***P < .001$ in the figures.

Results

Enhanced cytotoxicity of γδ PBL cultures activated with pan–T-cell mitogen

We compared the antitumor killing capacity of γδ PBL cultures (always maintained in the presence of IL-2) activated either with PHA, a plant lectin that acts as a potent T-cell mitogen,[11] or the specific Vγ9Vδ2 TCR agonist HMB-PP.[12,13] Although both regimens were similarly efficient at activating γδ PBLs, as evaluated by CD69 up-regulation and cell proliferation (Figure 1A), we noted that samples activated with PHA were consistently better killers of hematopoietic tumor cell lines than samples (of the same donor origin) stimulated with HMB-PP (Figure 1B-C). This was valid across all donors tested (Figure 1B, supplemental Table 2, and data not shown) and was associated with higher expression of *GZMB* (Figure 1D), a key component of the lymphocyte cytolytic machinery. Of note, freshly isolated γδ PBLs, which lack *GZMB* expression (Figure 1D), displayed very poor antileukemia cytotoxicity (< 10% killing; not shown), as previously reported.[13]

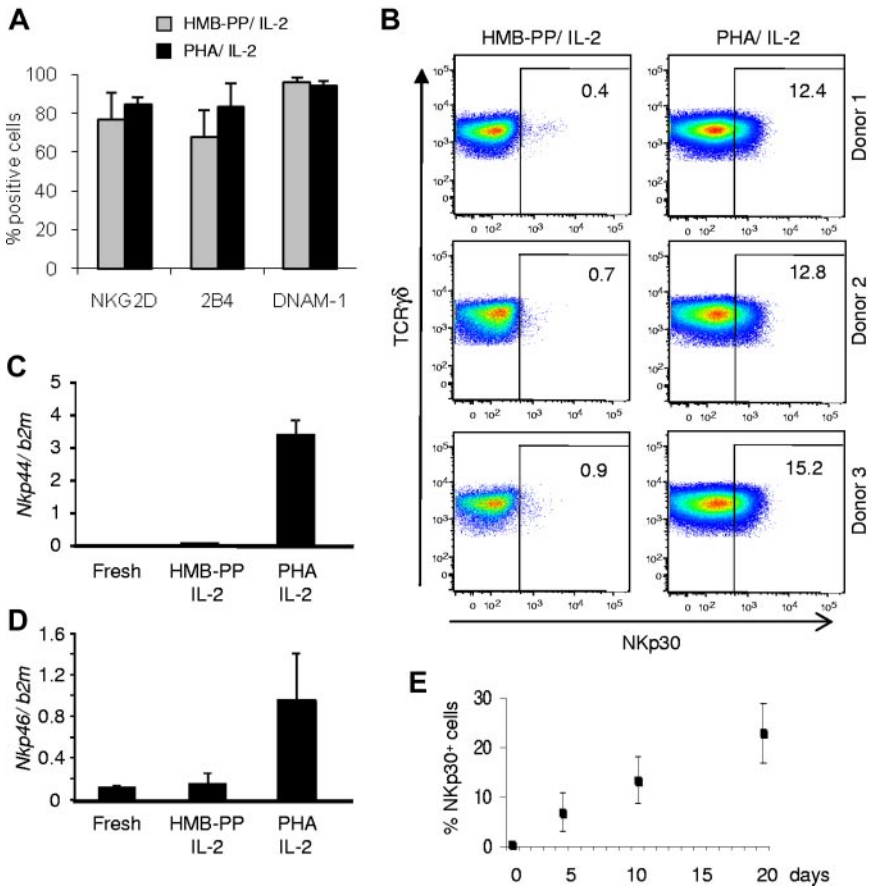

Figure 2. Induction of natural cytotoxicity receptor expression in γδ PBLs activated with pan–T-cell mitogen. γδ PBLs were cultured as described in Figure 1 for 4-19 days and analyzed by flow cytometry for surface expression of various NK receptors. (A) Results for NKG2D, 2B4 and DNAM-1 in 10-day cultures activated either with HMB-PP and IL-2 (gray) or PHA and IL-2 (black), derived from 6 independent healthy donors. Error bars represent SD (n = 6; $P > .05$). (B) Expression of NKp30 in the same cultures of (A). FACS plots correspond to cultures derived from 3 individual donors. Percentages refer to NKp30+ γδ PBLs. Isotype control staining is presented in supplemental Figure 2A. (C-D) Real-time PCR quantification of *Nkp44* (C) and *Nkp46* (D) mRNA levels in freshly isolated, HMB-PP and IL-2–activated and PHA and IL-2–activated γδ PBL. (E) Evolution of the percentage of NKp30+ cells in the cultures described in (A), analyzed up to day 19. Error bars represent SD (n = 5). Data in this figure are representative of 2 to 4 independent experiments with similar results.

The superior cytotoxic function of PHA-stimulated γδ PBL cultures was a surprising finding, because we and others have shown that HMB-PP is a very potent activator of the highly dominant Vγ9Vδ2 PBL subset.[13] We were particularly interested that, compared with HMB-PP–activated γδ PBL, PHA-stimulated cultures displayed improved cytotoxicity against various resistant leukemia cell lines, such as Bv-173, REH or HPB-ALL (Figure 1B-C), which we had shown to lack expression of the critical NKG2D ligand ULBP1.[2,3] Of note, PHA-stimulated γδ T cells did not target normal (healthy) PBMC (supplemental Figure 1). These data demonstrate that the pan–T-cell mitogen PHA is capable of increasing the cytolytic potential of medium-term (1-3 weeks) γδ PBL cultures against leukemia cells, which could be of great value for adoptive cell immunotherapy.

Induction of NCR expression on γδ PBLs activated with pan–T-cell mitogen

We next investigated the mechanism(s) underlying the enhanced cytotoxicity of PHA-activated γδ PBL cultures. We considered that this could be explained by differential expression of receptors such as NKG2D,[2,14,15] DNAM-1,[16,17] or 2B4,[18] all previously shown to participate in tumor cell recognition by killer lymphocytes. However, none of these candidates was differentially expressed between PHA-activated and HMB-PP–activated γδ PBL cultures (Figure 2A). By contrast, and unexpectedly, the natural cytotoxicity receptor NKp30, an important trigger of NK cell cytotoxicity,[19] was specifically found on PHA-stimulated γδ PBLs (Figure 2B; supplemental Figure 2A-B). Furthermore, the other NCR family members, NKp44 and NKp46, were also selectively expressed in these samples (Figure 2C-D; see next paragraph).

The proportion of NKp30+ cells increased steadily with culture time (Figure 2E), suggesting an association of NKp30 induction with cell proliferation. Although unlikely because of the very low background in fresh samples (Figure 2E), it was possible that a minute subset constitutively expressing NKp30 could be preferentially expanded in PHA-stimulated γδ PBL cultures. However, experiments with highly (> 99%) FACS-purified NKp30−cells demonstrated that NKp30− cells were able to acquire NKp30 expression as efficiently as unsorted cells on PHA and IL-2 stimulation (supplemental Figure 3). Moreover, under such conditions, NKp30− and NKp30+ cells proliferated to similar extent (not shown), further arguing against preferential expansion of NKp30+ cells under such conditions. These results suggest that NKp30 expression is induced de novo on γδ PBL activation by PHA and IL-2 treatment, which is coupled to cell proliferation.

NCRs are selectively expressed by proliferating Vδ1+ T cells

Considering that HMB-PP had been shown to be an optimal agonist of Vγ9Vδ2 cells,[12,13] we hypothesized that our findings derived from PHA-mediated activation of a distinct γδ PBL subset. Consistent with this, we observed that, by contrast with HMB-PP, treatment with PHA preferentially expanded Vδ2− cells among γδ PBL (Figure 3A). We verified that this was not because of differences in Vδ2+ cell apoptosis in the 2 experimental conditions (supplemental Figure 2C). The most likely Vδ2− population to expand so markedly (Figure 3A) were Vδ1+ cells, because other subsets are very rare in the peripheral blood of healthy adults.[20] When Vδ1 versus Vδ2 TCR usage was assessed, a dramatic Vδ1+ cell enrichment was found in PHA-activated cultures (> 80% of all γδ T cells after 19 days; Figure 3B; supplemental Figure 4).

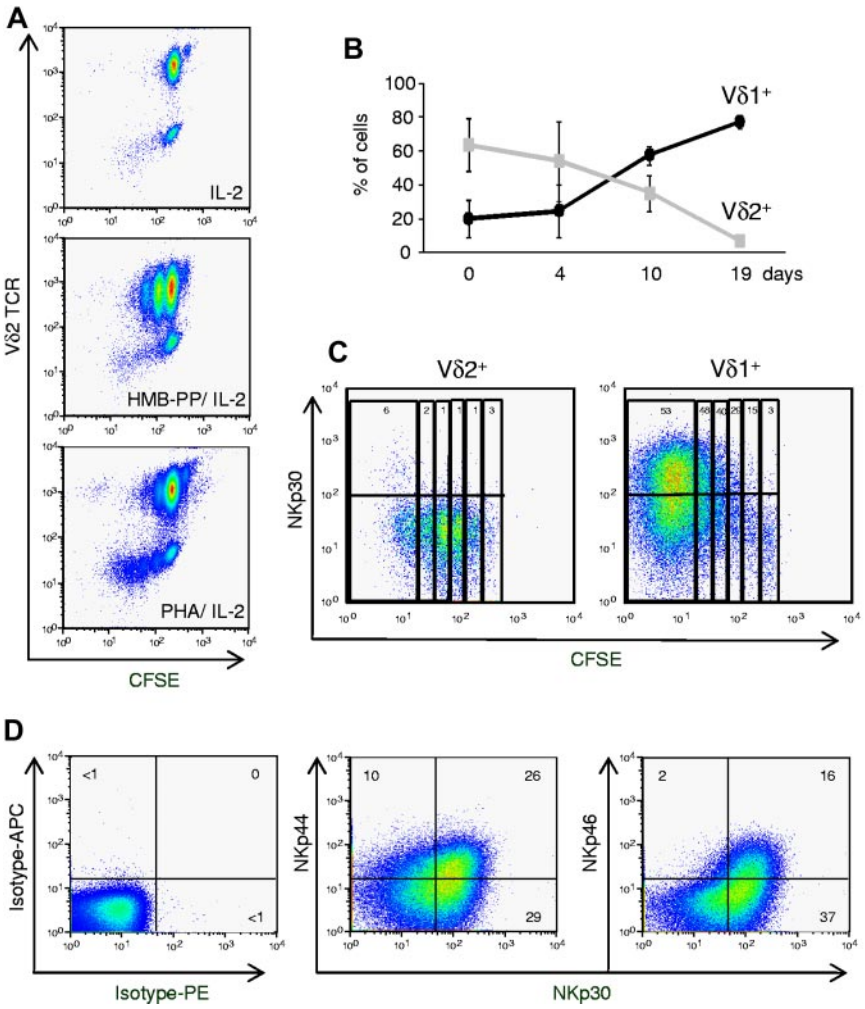

Figure 3. Natural cytotoxicity receptors are selectively expressed on proliferating Vδ1$^+$ T cells. (A) γδ PBLs were labeled with CFSE and cultured as described in Figure 1, or in the absence of T cell mitogens (ie, IL-2 alone). Flow cytometry analysis of CFSE dilution and Vδ2 TCR expression after 7 days in culture. (B) Percentage of Vδ1$^+$ or Vδ2$^+$ cells among total γδ PBLs cultured up to 19 days with PHA and IL-2. Error bars represent SD (n = 3). (C) NKp30 expression in PHA and IL-2–activated γδ PBL subsets. Vδ1$^+$ or Vδ2$^+$ cells were FACS-sorted from peripheral blood, labeled with CFSE and cultured with PHA and IL-2 for 7 days. Percentages refer to NKp30$^+$ cells within each cell division (according to CFSE levels and indicated by vertical rectangles). (D) Expression of NKp30, NKp44 and NKp46 in Vδ1$^+$ T cells after 19 days of PHA and IL-2 stimulation. Isotype mAb control stainings are also shown. Data in this figure are representative of 2-3 independent experiments with similar results.

Conversely, and as described,[13] HMB-PP–activated cultures were progressively dominated by Vδ2$^+$ cells (Figure 3A; supplemental Figure 4).

The induction of NKp30 expression was examined in parallel cultures of isolated Vδ1$^+$ or Vδ2$^+$ cells, which were stimulated with PHA and IL-2. Although neither freshly isolated Vδ1$^+$ nor Vδ2$^+$ cells expressed NKp30 (supplemental Figure 2B), this NCR was strongly induced (on PHA and IL-2 treatment) in Vδ1$^+$ but not Vδ2$^+$ cells (Figure 3C). Moreover, by following CFSE dilution, we demonstrated a striking accumulation of NKp30$^+$ cells with progressive division of Vδ1$^+$ cells (Figure 3C). These data suggest that activation of Vδ1$^+$ cells in PHA and IL-2 cultures induces NKp30 expression concomitantly with cell proliferation.

Whereas high percentages (> 50%) of NKp30$^+$ cells were usually detected after 2 to 3 weeks in culture, NKp44 (~ 30%) and NKp46 (< 20%) were expressed in lower proportions of Vδ1$^+$ cells (Figure 3D). Furthermore, most of NKp44$^+$ or NKp46$^+$ Vδ1$^+$ cells also expressed NKp30 (Figure 3D). We therefore considered NKp30 as the most informative marker of the inducible NCR$^+$ Vδ1$^+$ subset, and we set out to further characterize its differentiation.

NKp30 induction requires AKT-dependent γ_c cytokine and TCR signals

We next dissected the specific signals required for the differentiation of NCR$^+$ Vδ1$^+$ T cells. First, the 2 components of the activation protocol, IL-2 and PHA, were dissociated. IL-2, or its related γ_c cytokine, IL-15, alone were sufficient to induce some NKp30 expression, but the effect was modest compared with PHA and IL-2 (or PHA and IL-15) combinations (Figure 4A and not shown). On the other hand, PHA alone was not able to keep the cultures viable (data not shown), consistent with the critical role of γ_c cytokines in the survival of γδ T cells, particularly on activation and proliferation.[13,21]

Although PHA has been a widely used T cell mitogen, it is also a nonphysiologic compound capable of cross-linking a series of surface receptors, including the TCR.[22] We hypothesized that the molecular mediator of PHA stimulation could be the Vδ1$^+$ TCR complex. We therefore compared the ability of PHA and the OKT3 mAb, which specifically cross-links CD3ε chains of the TCR complex, to induce NKp30 expression (when combined with IL-2 or IL-15) in Vδ1$^+$ T cells. OKT3 was fully capable of mimicking PHA in these assays (Figure 4A-B), thus inducing NKp30 in proliferating Vδ1$^+$ T cells (supplemental Figure 5). Moreover, TCRγδ blockade in PHA and IL-2 cultures prevented NKp30 induction (Figure 4C). These data suggest that PHA treatment provides TCR signals to induce NCR expression on Vδ1$^+$ PBL. Moreover, the differences between cytokine alone or combination treatments with OKT3 (or PHA) highlight a marked synergy between γ_c cytokine and TCR signals in this process (Figure 4A-B).

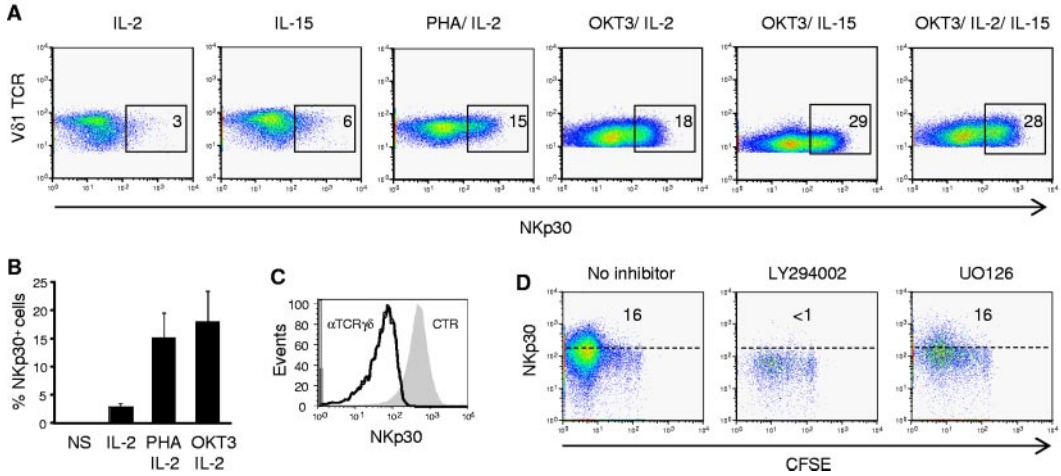

Figure 4. AKT-dependent γ_c cytokine and TCR signals induce NKp30 expression in Vδ1+ T cells. (A-B) Flow cytometry analysis of NKp30 expression on pregated Vδ1+ T cells from γδ PBL cultures after 7 days in the presence of IL-2 or IL-15, alone or in combination with PHA or OKT3 (anti-CD3ε mAb). (C) Effect of blocking anti-TCRγδ mAb on NKp30 induction in PHA and IL-2–activated γδ PBLs. The shaded gray area is pregated NKp30+ cells in 7-day control cultures. (D) Effect of chemical inhibitors LY294002 and UO126 on NKp30 induction in PHA and IL-2–activated γδ PBLs, prelabeled with CFSE. Data in this figure are representative of 2 to 3 independent experiments with similar results.

To further explore the molecular mechanisms of NCR induction, we used chemical inhibitors of key signal transduction pathways downstream of γ_c cytokine receptors and/or TCR signaling. Although blocking JAK signaling triggered extensive cell death before any NCR induction (not shown), coincubation with the PI-3K/ AKT inhibitor LY294002 specifically prevented NKp30 induction in proliferating Vδ1+ T cells (Figure 4D). AKT is involved in transducing both γ_c cytokine and TCR signals,[23] including TCRγδ signals.[13] By contrast, the MAPK/Erk inhibitor UO126 had no detectable effect on NKp30 induction in proliferating Vδ1+ T cells (Figure 4D). Importantly, the selective effect of LY294002 dissociated NCR induction from cell proliferation, thus demonstrating that Vδ1+ T-cell proliferation is necessary (Figure 3C; supplemental Figure 5) but not sufficient (Figure 4D) to induce NKp30 expression. Collectively, these data demonstrate that AKT-dependent γ_c cytokine and TCR signals synergize to induce NKp30 expression in Vδ1+ T cells.

Functional NKp30 and NKp44 trigger tumor cell killing by Vδ1+ PBLs

Although the previous data established clear associations between NKp30 expression and increased cytotoxicity of γδ (Vδ1+) PBL cultures, the functional role of NCRs in this system remained to be formally demonstrated. We therefore undertook gain-of function and loss-of-function experiments to evaluate the effect of NCR modulation on Vδ1+ enriched (> 80%; supplemental Figure 3A) PBL cultures, which expressed NCRs at levels similar to those in Figure 3D (not shown). First, using a reverse Ab-dependent cytotoxicity assay, we showed that cross-linking of NKp30 or NKp44, but not NKp46, produced significant increases in lysis of the P815 tumor cell targets (Figure 5A). These data demonstrate that induced NKp30 and NKp44 are functional and mediate tumor cell killing. To assess if they played nonredundant roles in targeting leukemia cells, we performed receptor blockade experiments using NCR-specific mAbs (kindly provided by Dr A. Moretta, University of Genova, Italy). We observed significant reductions in tumor cell lysis on NKp30 and NKp44 blockade (Figure 5B). As expected from the results in Figure 5A, NKp46 blockade did not affect tumor cell killing. Interestingly, a synergistic effect between NKp30 and NKp44 was also clearly observed. Of note, TCRγδ blockade in any setting (alone or in combination with anti-NCR mAbs) was a neutral event during the killing assay (Figure 5B). To further establish the TCR-independence of NCR+ Vδ1+ PBL cytotoxicity, we isolated PHA-activated Vδ1+ cells to very high purity (supplemental Figure 6) and used 3 different well-described anti-TCR blocking antibodies, including one (TCS1) specific for the Vδ1+ TCR. Again, we observed no effect on leukemia cell killing (Figure 5C). By contrast, inhibition of NKG2D had a significant (Figure 5C) and dose-dependent (supplemental Figure 6C) impact on tumor lysis. In fact, when we combined NCR and NKG2D inhibition, NCR+ Vδ1+ PBLs could not kill above background levels (Figure 5C). These data suggest that leukemia cell targeting by NCR+ Vδ1+ PBLs is a TCR-independent event mostly mediated by the synergistic function of NKp30, NKp44 and NKG2D.

NKp30+ Vδ1+ PBL are specialized killers that target resistant primary lymphocytic leukemias

To fully characterize the antitumor potential of NCR+ Vδ1+ PBL, we used FACS to sort NKp30+ cells to a high degree of purity (> 99%; Figure 6A) and performed a series of functional assays. As expected (Figure 3D), sorted NKp30+ cells also expressed NKp44 and NKp46 (Figure 6B), and the 3 NCRs were largely stable on the surface of the purified cells when cultured for 2 weeks with IL-2 alone (Figure 6C). These data demonstrate the feasible expansion of a stable NCR+ Vδ1+ T cell subset.

When the cytotoxic function of NKp30+ cells was assessed, an increased targeting of the resistant leukemia cell line Bv173 (among others; not shown) was observed (in comparison with NKp30− counterparts; Figure 6D). This correlated with higher expression of granzyme B (Figure 6E). Moreover, NKp30 expression also associated with higher degree of CD56 expression (supplemental Figure 7), which has been previously linked to cytotoxicity of human lymphocytes, including Vδ2+ T cells[24]).

Finally, we performed functional killing assays with primary samples obtained from B-cell chronic lymphocytic leukemia patients. We previously showed that such specimens are considerably resistant to γδ PBL activated and expanded with the specific Vγ9Vδ2 TCR agonist HMB-PP,[3] a finding confirmed in this study (Figure 6F-G). Importantly, HMB-PP and IL-2–activated γδ PBLs do not express NCRs (Figure 2B). We therefore compared their antitumor cytolytic activity with that of NKp30+ cells isolated from γδ PBL cultures activated with PHA and IL-2. We observed

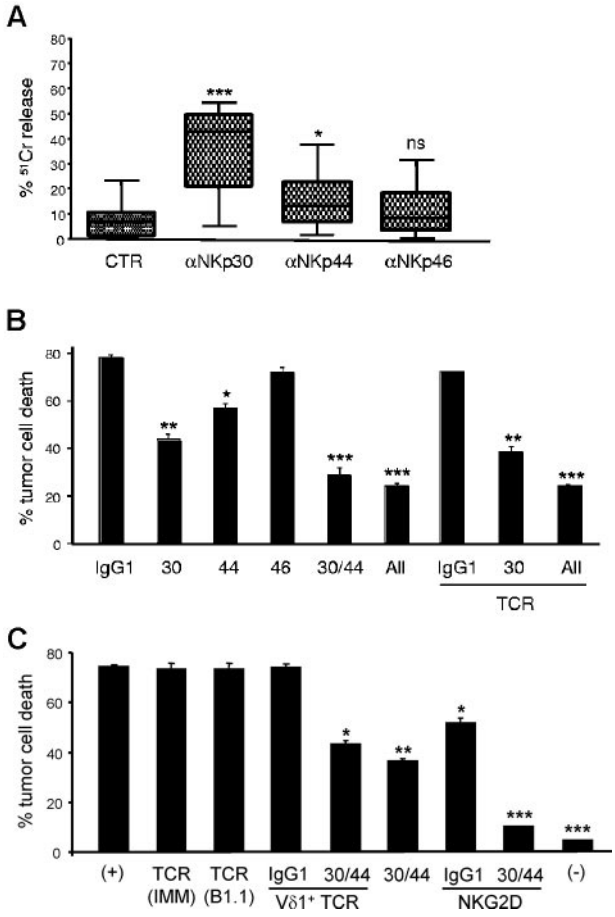

Figure 5. NKp30 and NKp44 mediate tumor cell killing by NCR$^+$ γδ PBLs. (A) Functional evaluation of NKp30, NKp44 and NKp46 using specific monoclonal antibodies in a 4-hour ^{51}Cr release redirected killing assay (at 2:1 effector:target ratio) of the FcγR$^+$ P815 target cell line by γδ PBLs activated and expanded with PHA and IL-2. Data are presented as mean and SD of 8 independent experiments performed in triplicate (*$P < .05$, ***$P < .001$; ns = not statistically significant). (B) γδ PBLs activated and expanded for 18 days in PHA and IL-2 were incubated (at 5:1 effector:target ratio) for 3 hours with the leukemia cell line MOLT-4 (as in Figure 1). Effect of blocking antibodies to NKp30, NKp44, NKp46 and TCRγδ (IMMU510) on tumor cell killing. (C) Vδ1$^+$ PBLs were MACS-sorted after 20 days in PHA and IL-2 cultures for the assay described in (B), but using blocking antibodies to pan-TCRγδ (IMMU510 and B1.1), Vδ1$^+$ TCR (TCS1), NKG2D or NKp30 and NKp44, or the depicted combinations. (+) refers to control cultures without inhibitory antibodies; (-) refers to tumor cell cultures without Vδ1$^+$ PBLs. Error bars represent SD (n = 3, *$P < .05$; **$P < .01$; ***$P < .001$).

that NKp30$^+$ γδ PBLs, obtained from 6 different donors, were consistently more efficient at eliminating primary B-CLL cells (Figure 6F-G). These data collectively suggest that highly cytotoxic NKp30$^+$ Vδ1$^+$ PBL are promising new candidates for adoptive cell immunotherapy of hematologic malignancies.

Discussion

Natural cytotoxicity receptors were identified by A. Moretta and colleagues over a decade ago, and were shown to play critical synergistic roles in the antitumor functions of NK cells.[19,25-27] In fact, NKp30 and NKp46 are widely considered to be 2 of the most specific NK markers[19,25-27] We now show that the combination of cytokine (IL-2 or IL-15) and mitogenic (PHA or OKT3) stimuli induces NCR expression in a sizeable Vδ1$^+$ PBL subset that is endowed with increased cytolytic activity against hematologic tumors. Although PHA is a nonphysiologic T-cell mitogen, we demonstrated that its effect on NCR induction was fully mimicked by crosslinking the TCR-CD3 complex on Vδ1$^+$ PBL. Thus, NCR induction is coupled to TCR-mediated proliferation of Vδ1$^+$ cells, while also requiring γ_c cytokine signals. This is consistent with previous reports demonstrating that the in vitro acquisition of NK receptors by liver[28] or umbilical cord[29] T cells depends on IL-15.

Among inducible NCRs, NKp30 is clearly the most important for the antitumor activity of Vδ1$^+$ T cells, based on the proportion of cells that express it (Figure 3D), the higher enhancement in Vδ1$^+$ T-cell cytotoxicity on NKp30 triggering (Figure 5A), and the significant reduction in leukemia cell killing on NKp30 blockade (Figure 5B). This notwithstanding, NKp44 (but not NKp46) is also functional in NCR$^+$ Vδ1$^+$ cells (Figure 5A), and appears to synergize with NKp30 for enhanced tumor targeting (Figure 5B). Of note, NKp30 engagement also augments the production of the key antitumor cytokine, interferon-γ, by NCR$^+$ Vδ1$^+$ cells (supplemental Figure 8).

Both NKp30[30] and NKp44[31] have been implicated in human NK cell recognition of virus-infected cells. Regarding tumors, antibody-mediated blocking experiments demonstrated important roles for these receptors in myeloma[32] and melanoma[33] cell targeting. Moreover, lack of NCR expression has been clinically correlated with poor survival in AML patients.[34]

Interestingly, NKp30 and NKp44 are not encoded in the genome of murine strains (such as C57Bl/6 or Balb/c) widely used for laboratory experimentation.[35] On the other hand, the major Vγ9Vδ2 subset of human PBL, and its reactivity toward phosphoantigens, are also primate-specific.[6] These observations highlight the special functional characteristics of primate γδ T cells; however, they also preclude the direct in vivo study of Vγ9Vδ2 or NKp30$^+$ Vδ1$^+$ T cells in the mouse.

Another aspect that currently limits our understanding of NCR function in immunity is the poor definition of their physiologic ligands, most notably in the context of tumors. In fact, viral hemagglutinin was until recently the only well-established ligand for NKp44[36] and NKp46.[37] This notwithstanding, a novel B7 family member, B7-H6, was lately described to bind NKp30.[38] B7-H6 was not detected in normal human tissues but was expressed on human tumor cells,[38] in line with the recognition of "stressed self" by innate and innate-like lymphocytes.[14,39] However, we obtained no evidence for a role of B7-H6 in NCR$^+$ Vδ1$^+$ T-cell recognition of lymphoid leukemias; in fact, many of the NCR$^+$ Vδ1$^+$ T-cell targets expressed lower levels of B7-H6 than healthy control cells (supplemental Figure 9). Brandt et al had also previously noted that only 24 of 119 tumor cell lines tested expressed B7-H6.[38] Thus, future lines of research should clarify the repertoire of relevant ligands expressed by tumors susceptible to NKp30-mediated cytotoxicity. Moreover, although we have thus far concentrated on hematologic malignancies, upcoming work will address whether NKp30$^+$ Vδ1$^+$ lymphocytes also possess enhanced cytotoxicity against solid tumors. Of note, Vδ1$^+$ T cells have previously been shown to be cytolytic against melanoma and various carcinomas.[10]

Vδ1$^+$ T cells are the predominant γδ T-cell subset during the fetal stage and early life,[40] when they are already able to respond to viral infection.[41] In adults, Vδ1$^+$ T-cell expansions have been associated with CMV infection,[42] HIV-1 infection,[43] and tumors of either epithelial[7-9] or hematopoietic[44,45] origin. An attractive prospect for adoptive transfer of activated Vδ1$^+$ T cells is that they may display particularly good capacity for homing to tissues because, contrary to their circulating Vδ2$^+$ counterparts, Vδ1$^+$ cells are preferentially tissue-associated lymphocytes.[9] Interestingly, the

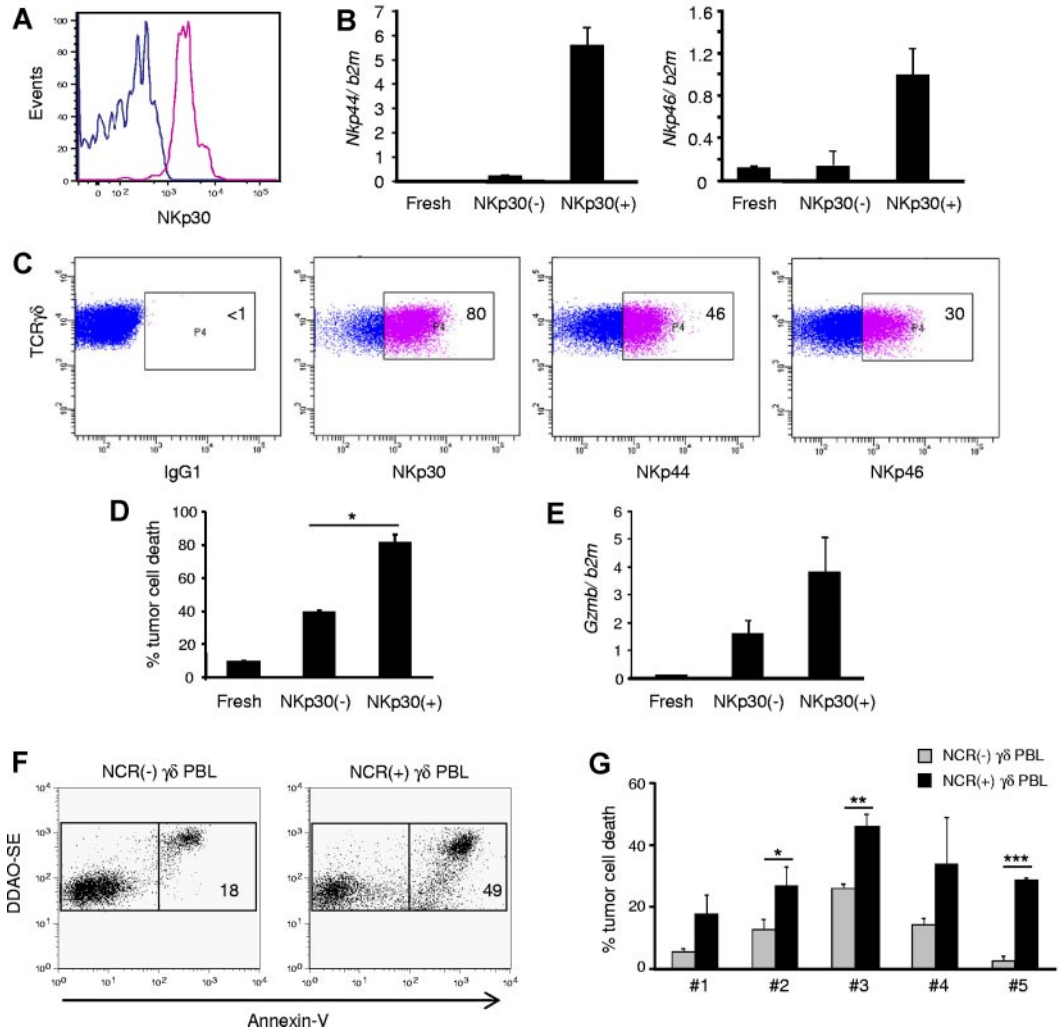

Figure 6. NKp30+ γδ PBLs are a stable subset endowed with enhanced cytotoxicity against chronic lymphocytic leukemia cells. NKp30+ and NKp30(−) γδ PBLs were FACS-sorted from 14-day PHA and IL-2–activated cultures. (A) Reanalysis of NKp30 expression in the purified populations. (B) Real-time PCR quantification of *Nkp44* (left) and *Nkp46* (right) mRNA levels in NKp30− or NKp30+ γδ T cells, compared with freshly isolated γδ PBLs. Error bars represent SD (n = 3). (C) Sorted NKp30+ γδ PBLs were cultured in the presence of IL-2. Analysis of NKp30, NKp44 and NKp46 expression after 14 days. (D) NKp30− or NKp30+ γδ T cells, or freshly isolated γδ PBLs, were used in killing assays with the leukemia cell line Bv173 (as in Figure 1). Tumor cell death was evaluated by annexin-V staining (n = 3, $*P < .05$). (E) Real-time PCR quantification of *GzmB* mRNA levels in freshly isolated, NKp30(−) or NKp30+ γδ T cells. Error bars represent SD (n = 3). (F-G) Representative plots (F) and data summary (G) for 5 primary B-cell chronic lymphocytic leukemia samples that were used in killing assays (as in Figure 1) with γδ PBLs obtained from 6 distinct donors and activated with either HMB-PP and IL-2 or PHA and IL-2. NCR(-) γδ PBL from HMB-PP and IL-2–activated cultures (gray bars) were compared with NCR(+) γδ PBL from PHA and IL-2–activated cultures (black bars). Error bars represent SD (n = 6, $*P < .05$; $**P < .01$; $***P < .001$).

abundance of Vδ1+ T cells at mucosal surfaces has been attributed to IL-15, which induces chromatin modifications that control TCR gene rearrangement.[46]

Our key demonstration that NKp30+ Vδ1+ cells are capable of targeting primary lymphoid leukemic cells is particularly relevant when taking into account that Vδ1+ T cells have been previously reported to be inefficient killers of primary leukemia or lymphoma cells,[44,45] which has been attributed to their lack of expression of NKG2D ligands.[44] Interestingly, a recent study in which γδ PBLs were activated with concanavalin A demonstrated higher killing ability of expanded Vδ1+ cells against B-CLL–derived cell lines.[47] It will be important to investigate whether this protocol also induces NKp30 expression on Vδ1+ cells. Thus, NKp30+ Vδ1+ cells may provide a valuable layer of intervention against lymphoid malignancies.

A surprising finding that deserves further investigation is the preferential expansion of Vδ1+ T cells (among γδ PBL) on PHA treatment in vitro (Figure 3B). Because this is not due to selective apoptosis of the dominant Vδ2+ counterparts (supplemental Figure 2), it must derive from a proliferative advantage of Vδ1+ cells when receiving PHA-dependent TCR signals (Figure 3A-B). Provocatively, we have previously observed that Vδ1+ T cells express significantly higher levels of the CD27 receptor (compared with Vδ2+ cells)[48]; CD27 costimulation enhances *Bcl2a1* and *Cyclin D2* expression and promotes γδ T-cell survival and proliferation.[48]

This study draws important novel (NCR-mediated) insight into the modus operandis of human γδ T cells, and supports the emerging paradigm of γδ T cells recognizing tumors via innate NK receptors rather than using the somatically rearranged TCRγδ (Figure 5B-C).[6] This notwithstanding, the TCR has a major, albeit indirect, contribution to the antitumor function of NKp30+ Vδ1+ cells. We showed that efficient induction of NKp30 expression on Vδ1+ cells depends on TCR stimulation; in its absence, γ_c cytokines can only effect a very modest up-regulation of NKp30 expression (Figure 4A-B). Thus, TCR signals are upstream of

NKp30-mediated tumor cell recognition by NKp30$^+$ Vδ1$^+$ lymphocytes. Interestingly, we have previously showed that, for Vδ2$^+$ cells, TCR signals are essential for cell activation and cytotoxic differentiation, "upstream" of tumor cell recognition via NKG2D.[2] We therefore propose a "2-step" model for human γδ T cells, in which they differentiate and are activated like prototypic T cells (ie, using the TCR), but rely essentially on NK receptors (such as NKG2D, NKp30 or DNAM-1) for tumor cell recognition. This is consistent with the critical role described by the Hayday and Girardi groups for NKG2D ligands in tumor surveillance by mouse γδ T cells,[49,50] and fits the general concept of NK receptors being the key molecular recognition determinants of "oncogenic stress."[39]

From a clinical perspective, this study describes a protocol to induce NKp30 ex vivo, which should make it very feasible to expand and inject large numbers of cells into patients. Importantly, we were able to efficiently expand NKp30$^+$ Vδ1$^+$ cells from B-CLL patients (supplemental Figure 10). On reinfusion, the activation status of the cells could potentially be maintained via administration of low doses of IL-2, which appears to be sufficient to sustain NKp30 expression. Thus, after this first report describing the differentiation of NKp30$^+$ Vδ1$^+$ lymphocytes, future work should evaluate their potential for adoptive cell immunotherapy of human cancer.

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Authorship

Contribution: D.V.C., M.F., and K.H. performed the experiments and analyzed the data; M.G.S. provided vital materials and technical help; D.M. and B.S.-S. designed the study and analyzed the data; and B.S.-S. wrote the manuscript.

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References

1. Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ. Natural innate and adaptive immunity to cancer. *Annu Rev Immunol.* 2011;29:235-71.
2. Lança T, Correia DV, Moita CF, et al. The MHC class Ib protein ULBP1 is a nonredundant determinant of leukemia/lymphoma susceptibility to gammadelta T-cell cytotoxicity. *Blood.* 2010; 115(12):2407-2411.
3. Gomes AQ, Correia DV, Grosso AR, et al. Identification of a panel of ten cell surface protein antigens associated with immunotargeting of leukemias and lymphomas by peripheral blood gammadelta T cells. *Haematologica.* 2010;95(8):1397-1404.
4. Wilhelm M, Kunzmann V, Eckstein S, et al. Gammadelta T cells for immune therapy of patients with lymphoid malignancies. *Blood.* 2003;102(1): 200-206.
5. Dieli F, Vermijlen D, Fulfaro F, et al. Targeting human gammadelta T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. *Cancer Res.* 2007; 67(15):7450-7457.
6. Gomes AQ, Martins DS, Silva-Santos B. Targeting gammadelta T lymphocytes for cancer immunotherapy: from novel mechanistic insight to clinical application. *Cancer Res.* 2010;70(24):10024-10027.
7. Maeurer MJ, Martin D, Walter W, et al. Human intestinal Vdelta1+ lymphocytes recognize tumor cells of epithelial origin. *J Exp Med.* 1996;183(4): 1681-1696.
8. Choudhary A, Davodeau F, Moreau A, Peyrat MA, Bonneville M, Jotereau F. Selective lysis of autologous tumor cells by recurrent gammadelta tumor-infiltrating lymphocytes from renal carcinoma. *J Immunol.* 1995;154(8):3932-3940.
9. Groh V, Steinle A, Bauer S, Spies T. Recognition of stress-induced MHC molecules by intestinal epithelial gammadelta T cells. *Science.* 1998; 279(5357):1737-1740.
10. Groh V, Rhinehart R, Secrist H, Bauer S, Grabstein KH, Spies T. Broad tumor-associated expression and recognition by tumor-derived gammadelta T cells of MICA and MICB. *Proc Natl Acad Sci U S A.* 1999;96(12):6879-6884.
11. Greaves M, Janossy G, Doenhoff M. Selective triggering of human T and B lymphocytes in vitro by polyclonal mitogens. *J Exp Med.* 1974;140(1): 1-18.
12. Chen Y, Shao L, Ali Z, Cai J, Chen ZW. NSOM/QD-based nanoscale immunofluorescence imaging of antigen-specific T-cell receptor responses during an in vivo clonal Vgamma2Vdelta2 T-cell expansion. *Blood.* 2008;111(8):4220-4232.
13. Correia DV, d'Orey F, Cardoso BA, et al. Highly active microbial phosphoantigen induces rapid yet sustained MEK/Erk- and PI-3K/Akt-mediated signal transduction in antitumor human gammadelta T cells. *PLoS ONE.* 2009;4(5):e5657.
14. Gomes AQ, Correia DV, Silva-Santos B. Non-classical major histocompatibility complex proteins as determinants of tumour immunosurveillance. *EMBO Rep.* 2007;8(11):1024-1030.
15. Nedellec S, Sabourin C, Bonneville M, Scotet E. NKG2D costimulates human Vgamma9Vdelta2 T cell antitumor cytotoxicity through protein kinase C theta-dependent modulation of early TCR-induced calcium and transduction signals. *J Immunol.* 2010;185(1):55-63.
16. Carlsten M, Bjorkstrom NK, Norell H, et al. DNAX accessory molecule-1 mediated recognition of freshly isolated ovarian carcinoma by resting natural killer cells. *Cancer Res.* 2007;67(3):1317-1325.
17. Toutirais O, Cabillic F, Le Friec G, et al. DNAX accessory molecule-1 (CD226) promotes human hepatocellular carcinoma cell lysis by Vgamma9Vdelta2 T cells. *Eur J Immunol.* 2009;39(5):1361-1368.
18. Lee KM, Forman JP, McNerney ME, et al. Requirement of homotypic NK-cell interactions through 2B4(CD244)/CD48 in the generation of NK effector functions. *Blood.* 2006;107(8):3181-3188.
19. Pende D, Parolini S, Pessino A, et al. Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells. *J Exp Med.* 1999;190(10):1505-1516.
20. Behr C, Couzi L, Taupin J, Déchanet-Merville J. Vdelta2neg gammadelta T cells, a multi-reactive tissue subset: from innate to adaptive altered-self surveillance. *Open Immunol.* 2009;2:106-118.
21. Casetti R, Perretta G, Taglioni A, et al. Drug-induced expansion and differentiation of Vgamma9Vdelta2 T cells in vivo: the role of exogenous IL-2. *J Immunol.* 2005;175(3):1593-1598.
22. Chilson OP, Kelly-Chilson AE. Mitogenic lectins bind to the antigen receptor on human lymphocytes. *Eur J Immunol.* 1989;19(2):389-396.
23. Cantrell D. Protein kinase B (Akt) regulation and function in T lymphocytes. *Semin Immunol.* 2002; 14(1):19-26.
24. Alexander AA, Maniar A, Cummings JS, et al. Isopentenyl pyrophosphate-activated CD56+ gammadelta T lymphocytes display potent antitumor activity toward human squamous cell carcinoma. *Clin Cancer Res.* 2008;14(13):4232-4240.
25. Sivori S, Vitale M, Morelli L, et al. p46, a novel natural killer cell-specific surface molecule that mediates cell activation. *J Exp Med.* 1997;186(7): 1129-1136.
26. Pessino A, Sivori S, Bottino C, et al. Molecular cloning of NKp46: a novel member of the immunoglobulin superfamily involved in triggering of natural cytotoxicity. *J Exp Med.* 1998;188(5):953-960.
27. Vitale M, Bottino C, Sivori S, et al. NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major

histocompatibility complex-restricted tumor cell lysis. *J Exp Med.* 1998;187(12):2065-2072.

28. Correia MP, Cardoso EM, Pereira CF, Neves R, Uhrberg M, Arosa FA. Hepatocytes and IL-15: a favorable microenvironment for T cell survival and CD8+ T cell differentiation. *J Immunol.* 2009; 182(10):6149-6159.
29. Tang Q, Grzywacz B, Wang H, et al. Umbilical cord blood T cells express multiple natural cytotoxicity receptors after IL-15 stimulation, but only NKp30 is functional. *J Immunol.* 2008;181(7): 4507-4515.
30. Arnon TI, Achdout H, Levi O, et al. Inhibition of the NKp30 activating receptor by pp65 of human cytomegalovirus. *Nat Immunol.* 2005;6(5):515-523.
31. Hershkovitz O, Rosental B, Rosenberg LA, et al. NKp44 receptor mediates interaction of the envelope glycoproteins from the West Nile and dengue viruses with NK cells. *J Immunol.* 2009; 183(4):2610-2621.
32. Carbone E, Neri P, Mesuraca M, et al. HLA class I, NKG2D, and natural cytotoxicity receptors regulate multiple myeloma cell recognition by natural killer cells. *Blood.* 2005;105(1):251-258.
33. Lakshmikanth T, Burke S, Ali TH, et al. NCRs and DNAM-1 mediate NK cell recognition and lysis of human and mouse melanoma cell lines in vitro and in vivo. *J Clin Invest.* 2009;119(5):1251-1263.
34. Fauriat C, Just-Landi S, Mallet F, et al. Deficient expression of NCR in NK cells from acute myeloid leukemia: Evolution during leukemia treatment and impact of leukemia cells in NCRdull phenotype induction. *Blood.* 2007;109(1):323-330.
35. Hollyoake M, Campbell RD, Aguado B. NKp30 (NCR3) is a pseudogene in 12 inbred and wild mouse strains, but an expressed gene in Mus caroli. *Mol Biol Evol.* 2005;22(8):1661-1672.
36. Arnon TI, Lev M, Katz G, Chernobrov Y, Porgador A, Mandelboim O. Recognition of viral hemagglutinins by NKp44 but not by NKp30. *Eur J Immunol.* 2001;31(9):2680-2689.
37. Mandelboim O, Lieberman N, Lev M, et al. Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells. *Nature.* 2001;409(6823):1055-1060.
38. Brandt CS, Baratin M, Yi EC, et al. The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. *J Exp Med.* 2009;206(7):1495-1503.
39. Raulet DH, Guerra N. Oncogenic stress sensed by the immune system: role of natural killer cell receptors. *Nat Rev Immunol.* 2009;9(8):568-580.
40. De Rosa SC, Andrus JP, Perfetto SP, et al. Ontogeny of gamma delta T cells in humans. *J Immunol.* 2004;172(3):1637-1645.
41. Vermijlen D, Brouwer M, Donner C, et al. Human cytomegalovirus elicits fetal gammadelta T cell responses in utero. *J Exp Med.* 2010;207(4):807-821.
42. Déchanet J, Merville P, Lim A, et al. Implication of gammadelta T cells in the human immune response to cytomegalovirus. *J Clin Invest.* 1999; 103(10):1437-1449.
43. Boullier S, Cochet M, Poccia F, Gougeon ML. CDR3-independent gamma delta V delta 1+ T cell expansion in the peripheral blood of HIV-infected persons. *J Immunol.* 1995;154(3):1418-1431.
44. Poggi A, Venturino C, Catellani S, et al. Vdelta1 T lymphocytes from B-CLL patients recognize ULBP3 expressed on leukemic B cells and upregulated by trans-retinoic acid. *Cancer Res.* 2004;64(24):9172-9179.
45. Catellani S, Poggi A, Bruzzone A, et al. Expansion of Vdelta1 T lymphocytes producing IL-4 in low-grade non-Hodgkin lymphomas expressing UL-16-binding proteins. *Blood.* 2007;109(5): 2078-2085.
46. Zhao H, Nguyen H, Kang J. Interleukin 15 controls the generation of the restricted T cell receptor repertoire of gamma delta intestinal intraepithelial lymphocytes. *Nat Immunol.* 2005;6(12): 1263-1271.
47. Siegers GM, Dhamko H, Wang XH, et al. Human Vdelta1 gammadelta T cells expanded from peripheral blood exhibit specific cytotoxicity against B-cell chronic lymphocytic leukemia-derived cells *Cytotherapy.* 2011;13:753-764.
48. DeBarros A, Chaves-Ferreira M, d'Orey F, Ribot JC, Silva-Santos B. CD70-CD27 interactions provide survival and proliferative signals that regulate T cell receptor-driven activation of human gammadelta peripheral blood lymphocytes. *Eur J Immunol.* 2011;41(1):195-201.
49. Girardi M, Oppenheim DE, Steele CR, et al. Regulation of cutaneous malignancy by gammadelta T cells. *Science.* 2001;294(5542):605-609.
50. Strid J, Roberts SJ, Filler RB, et al. Acute upregulation of an NKG2D ligand promotes rapid reorganization of a local immune compartment with pleiotropic effects on carcinogenesis. *Nat Immunol.* 2008;9(2):146-154.

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The MHC class Ib protein ULBP1 is a nonredundant determinant of leukemia/lymphoma susceptibility to {gamma}{delta} T-cell cytotoxicity

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Brief report

The MHC class Ib protein ULBP1 is a nonredundant determinant of leukemia/lymphoma susceptibility to γδ T-cell cytotoxicity

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On the path to successful immunotherapy of hematopoietic tumors, γδ T cells offer great promise because of their human leukocyte antigen (HLA)–unrestricted targeting of a wide variety of leukemias/lymphomas. However, the molecular mechanisms underlying lymphoma recognition by γδ T cells remain unclear. Here we show that the expression levels of UL16-binding protein 1 (ULBP1) determine lymphoma susceptibility to γδ T cell–mediated cytolysis. Consistent with this, blockade of NKG2D, the receptor for ULBP1 expressed on all Vγ9$^+$ T cells, significantly inhibits lymphoma cell killing. Specific loss-of-function studies demonstrate that the role of ULBP1 is nonredundant, highlighting a thus far unique physiologic relevance for tumor recognition by γδ T cells. Importantly, we observed a very wide spectrum of ULBP1 expression levels in primary biopsies obtained from lymphoma and leukemia patients. We suggest this will impact on the responsiveness to γδ T cell–based immunotherapy, and therefore propose ULBP1 to be used as a leukemia/lymphoma biomarker in upcoming clinical trials. (Blood. 2010;115:2407-2411)

Introduction

Cellular immunotherapy of hematopoietic malignancies is regarded as one of the most promising approaches to deal with the common relapse or resistance to conventional treatments. γδ T cells are innate-like lymphocytes capable of potent antitumor activity toward a variety of malignant cell types in both mice[1] and humans,[2] with special emphasis on lymphomas and leukemias.[3] Unlike their αβ counterparts, γδ T cells are not restricted by classic major histocompatibility complex (MHC) presentation but share many characteristics with NK cells, including the expression of "NK receptors," most notably NKG2D.[4,5] Most (60%-95%) human γδ peripheral blood lymphocytes (γδ-PBLs) express a Vγ9Vδ2 T-cell receptor (TCR)[6] and are specifically activated by nonpeptidic prenyl pyrophosphate intermediates of isoprenoid biosynthesis ("phosphoantigens"),[7,8] which constitutes the basis of current cancer immunotherapy strategies involving γδ T cells.[2,9,10]

Although several molecules have been proposed to play a role in tumor-Vγ9Vδ2 cell interactions, from phosphoantigens[7,11] to an F1-ATPase-related structure complexed with delipidated apolipoprotein A-I[12] and, more recently, DNAM-1 ligands[13] or the nonclassic MHC protein ULBP4,[14] a consensus about γδ T-cell recognition of tumors, particularly on physiologic (nonectopic) conditions, is yet to be reached.

Here we set out to determine the mechanism of leukemia/lymphoma cell recognition by γδ T cells, particularly relevant as previous γδ T cell–based clinical trials have shown a variable degree of success among patients.[9] The establishment of an in vitro model representative of this clinical scenario and the quantification and manipulation of candidate gene expression allowed us to demonstrate a nonredundant role for ULBP1 in determining the susceptibility of leukemia/lymphoma cells to γδ T cell–mediated cytolysis.

Methods

Cell culture

Peripheral blood mononuclear cell (PBMC) isolation, Vγ9Vδ2 PBL expansion, leukemia/lymphoma cell line cultures, and killing assays were performed as previously described.[8]

Leukemia/lymphoma biopsies

Pediatric B- or T-cell acute lymphoblastic leukemia cells were obtained from peripheral blood of patients after informed consent and institutional review board approval (Instituto Português de Oncologia, Lisbon, Portugal) in accordance with the Declaration of Helsinki. Lymphoma cells from lymph node biopsies were frozen in liquid nitrogen and used on diagnosis (Hospital de Santa Maria-CHLN, Lisbon, Portugal).

Quantitative RT-PCR

RNA extraction and quantitative reverse-transcribed polymerase chain reaction (RT-PCR) were performed as described,[8] using the primers listed in supplemental Table 1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article).

Antibodies

The following anti–human monoclonal antibodies were used: ULBP-1/clone IC1380F, MHC class I chain-related gene A (MICA)/clone 1300

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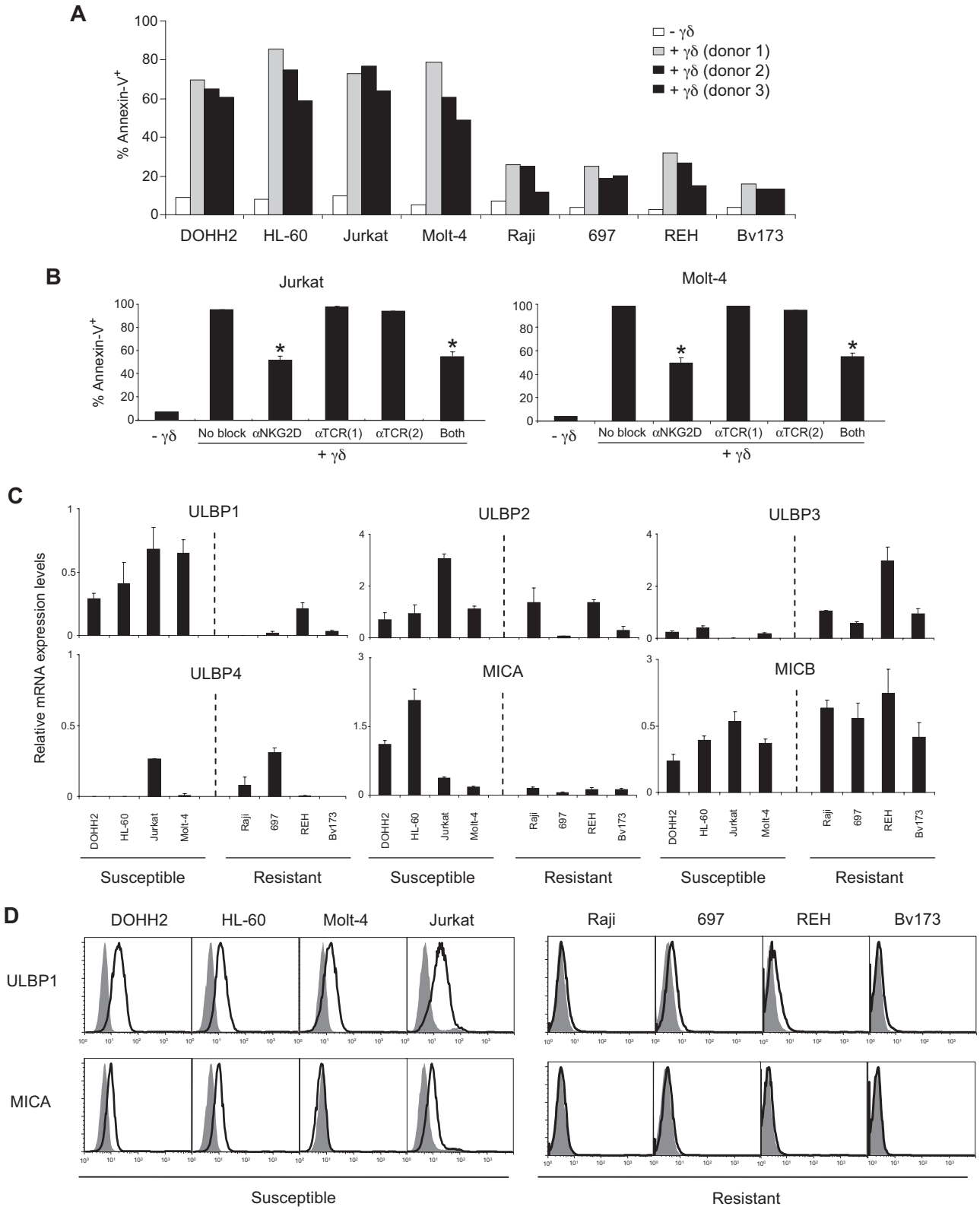

Figure 1. NKG2D mediates Vγ9Vδ2 T-cell recognition of hematopoietic tumors that endogenously overexpress ULBP1 and MICA. (A) Susceptibility of leukemia and lymphoma cell lines (described in supplemental Table 2) to HMB-PP–activated γδ-PBL (> 90% $V\gamma9^+$) cytotoxicity was assessed by coincubating 3×10^4 tumor cells (prelabeled with 1mM DDAO-SE) with 3×10^5 γδ-PBLs from 3 independent donors for 3 hours, then staining with annexin V–fluorescein isothiocyanate and analyzing by flow cytometry. (B) γδ-PBLs were incubated with saturating amounts of anti-NKG2D/clone 1D11 and anti-TCRγδ/clones B1.1[1] or IMMU510[2] blocking antibodies, or both anti-NKG2D and anti-TCRγδ,[1] for 1 hour at 4°C. γδ-PBLs were then cocultured either with Jurkat or Molt-4 leukemia lines, and tumor cell lysis was assessed as in panel A. Error bars represent SD (n = 3). *$P < .05$. (C) Quantitative RT-PCR quantification of mRNA levels of NKG2D ligands in cells lines of panel A, normalized to glucuronidase-β (GUSB) and proteasome subunit β type 6 (PSMB6) housekeeping genes. (D) Flow cytometric analysis of cell-surface expression of ULBP1 and MICA in the leukemia/lymphoma lines of panel A. Data presented in this figure (A-D) are representative of at least 3 independent experiments with consistent results.

(R&D Systems), NKG2D-PE/clone 1D11 (BioLegend), TCRγδ/clone B1.1 (eBioscience), and TCRγδ/clone IMMU510 (Beckman Coulter). Goat anti–mouse IgG-PE (Sigma-Aldrich) was used as secondary monoclonal antibody for ULBP1 and MICA staining.

RNA interference and overexpression

Lentiviral vectors expressing short hairpin RNA (shRNA) for the specific silencing of ULBP1 (CCTGGGAAGAACAAACTGAAA) and MICA (CTATGTCCGTTGTTGTAAGAA) were obtained from the RNAi Consortium and produced as previously described.[15] For overexpression of ULBP1, its coding sequence was amplified from a human EST clone (GenBank accession no. BC035416[16]) by PCR, cloned into pENTR-V5C2 vector, subcloned into pLenti6.2 (Invitrogen), and introduced into the Gateway System (Invitrogen). Lentiviruses were then pseudotyped as described.[15]

Results and discussion

γδ-PBLs, expanded and activated (~ 100% CD69$^+$; data not shown) with 4-hydroxy-3-methyl-but-2-enylpyrophosphate (HMB-PP), the most potent Vγ9Vδ2 TCR agonist yet known,[7,8,17] were able to mediate efficient killing of only a fraction of leukemia/lymphoma cell lines within a large panel established in our laboratory. Within the group selected for this study (supplemental Table 2), 4 lines were highly susceptible (60%-85% annexin V$^+$), whereas the other 4 lines were largely resistant (15%-30% annexin V$^+$) to γδ T-cell cytotoxicity (Figure 1A). Furthermore, we observed a consistent resistance of primary leukemia cells to γδ-PBL cytotoxicity (supplemental Figure 1), which stresses the importance of understanding the mechanisms of tumor cell recognition by γδ T cells.

Both TCRγδ and NKG2D have been implicated in Vγ9Vδ2 T cell–mediated killing of epithelial tumors.[18-20] To determine their importance in recognition of hematopoietic tumors, we performed specific antibody blockade experiments with HMB-PP–activated γδ-PBL (> 90% Vγ9$^+$) and 2 susceptible leukemia lines. We observed a significant reduction through NKG2D inhibition but not via TCRγδ blockade, and no additive effect (Figure 1B), suggesting that, although TCR-mediated activation greatly augments Vγ9Vδ2 T-cell cytolytic capacity,[8] the recognition of leukemia targets is essentially mediated by NKG2D. Moreover, we did not observe any Ca^{2+} influx in Vγ9Vδ2 T cells during the killing assay (data not shown), which is consistent with TCR-independent tumor cell recognition. Thus, γδ T cell–mediated surveillance of hematopoietic tumors appears to be a 2-step process where effector lymphocyte activation is achieved through TCR stimulation (presumably by endogenous phophoantigens[7,11]) but tumor cell recognition is predominantly mediated by NKG2D.

Based on these results, we hypothesized that the distinct leukemia/lymphoma susceptibilities were derived from differential expression of NKG2D-ligand(s) (NKG2DL), which was tested by quantitative RT-PCR. ULBP1 expression clearly segregated the best with susceptible versus resistant leukemias/lymphomas; on average, ULBP1 mRNA expression was 6-fold higher in susceptible than in resistant lines (Figure 1C). MICA was also highly expressed in some but not all susceptible lines, whereas ULBP3 was enriched in some resistant cell lines, and the expression of the other NKG2DL did not segregate with susceptibility to γδ-mediated killing (Figure 1C). Of note, ULBP4, recently suggested to be involved in γδ-PBL targeting of some epithelial tumors,[14] was very poorly expressed in leukemias/lymphomas (Figures 1C, 2D-E). We also confirmed by flow cytometry that ULBP1 and MICA were differentially expressed at the protein level and on the cell surface, although the correlation between mRNA and protein expression was not absolute (Figure 1D).

This prompted us to assess the physiologic role of these NKG2DL in leukemia targeting by γδ-PBL through loss-of-function studies using RNA interference. ULBP1 and MICA mRNA and protein expression levels were efficiently and specifically decreased on shRNA infection (Figure 2A; supplemental Figure 2). Loss of ULBP1 expression caused a very significant reduction (35%-50%) of γδ-PBL–mediated lysis of leukemia lines (Figure 2B), essentially "converting" these susceptible tumors into γδ-resistant lines. The residual cytotoxicity may be the result of other NK-like receptors, such as DNAM-1,[13] which we are currently investigating. Interestingly, MICA down-regulation did not impair γδ-PBL targeting of these tumors (Figure 2B). These data collectively suggest that ULBP1 plays a crucial and nonredundant role in γδ-PBL recognition of leukemias. Importantly, this constitutes the first physiologic evidence for lymphocyte requirement of NKG2DL expression on tumors because previous studies[14,19,21,22] concentrated on their ectopic expression. Along these lines, we have also overexpressed ULBP1 in a resistant lymphoma cell line and observed a marked increase in susceptibility to γδ-PBL cytotoxicity (Figure 2C).

These findings suggest that monitoring ULBP1 levels in leukemia/lymphoma could be of great value in the clinic. In considering this, we analyzed the expression of ULBP1, as well as the other NKG2DL, in 15 leukemia PBMC samples and 12 lymphoma biopsies, which were compared with healthy PBMC and reactive follicles, respectively. ULBP1 presented the highest degree of overexpression in leukemias and lymphomas (Figure 2D-E), as well as the broadest spectrum of expression levels, as translated by its dramatic variance across clinical samples (supplemental Table 3). Taking into account the impact of 2-fold reduction in ULBP1 levels on leukemia killing in vitro (Figure 2A-B), these results with primary biopsies strongly suggest a large variability in susceptibility to γδ-PBL cytotoxicity in the clinical population. We therefore propose ULBP1 to be tested as a biomarker in upcoming γδ T cell–based cancer clinical trials. Moreover, recent findings that proteasome inhibitor drugs specifically up-regulate ULBP1 expression in carcinoma cells[23] open new perspectives for cancer immunotherapy.

The expression of ULBP family members correlates with improved survival in cancer patients, and ectopic expression of ULBP1 in particular has been shown to elicit potent antitumor responses.[22,24] The role we attribute here to ULBP1 in the context of lymphomas and leukemias is probably not universal for γδ T-cell recognition of other tumor types. For example, susceptible epithelial tumors have been shown to express low or undetectable levels of ULBP1.[25] In this context, it is attractive to speculate that ULBP4, recently shown to ectopically trigger Vγ9Vδ2 T-cell cytotoxicity against ovarian and colon carcinomas,[14] may play, in epithelial tumors, the equivalent physiologic role of ULBP1 in hematopoietic tumors. This would constitute a novel paradigm for tumor recognition, by which stress-inducible, nonclassic MHC proteins that constitute ligands for NKG2D, would act as cellular reporters of transformation for both circulating Vδ2 and tissue-associated Vδ1 T lymphocytes, the latter known to recognize MICA/MICB and ULBP3.[2] Furthermore, NGK2D also plays critical roles in antitumor NK and CD8 T-cell responses[5,24] and has been shown to be an essential genetic factor for tumor surveillance in mice.[26] We therefore think that NKG2D/NKG2DL modulation entails great promise for cancer immunotherapy.

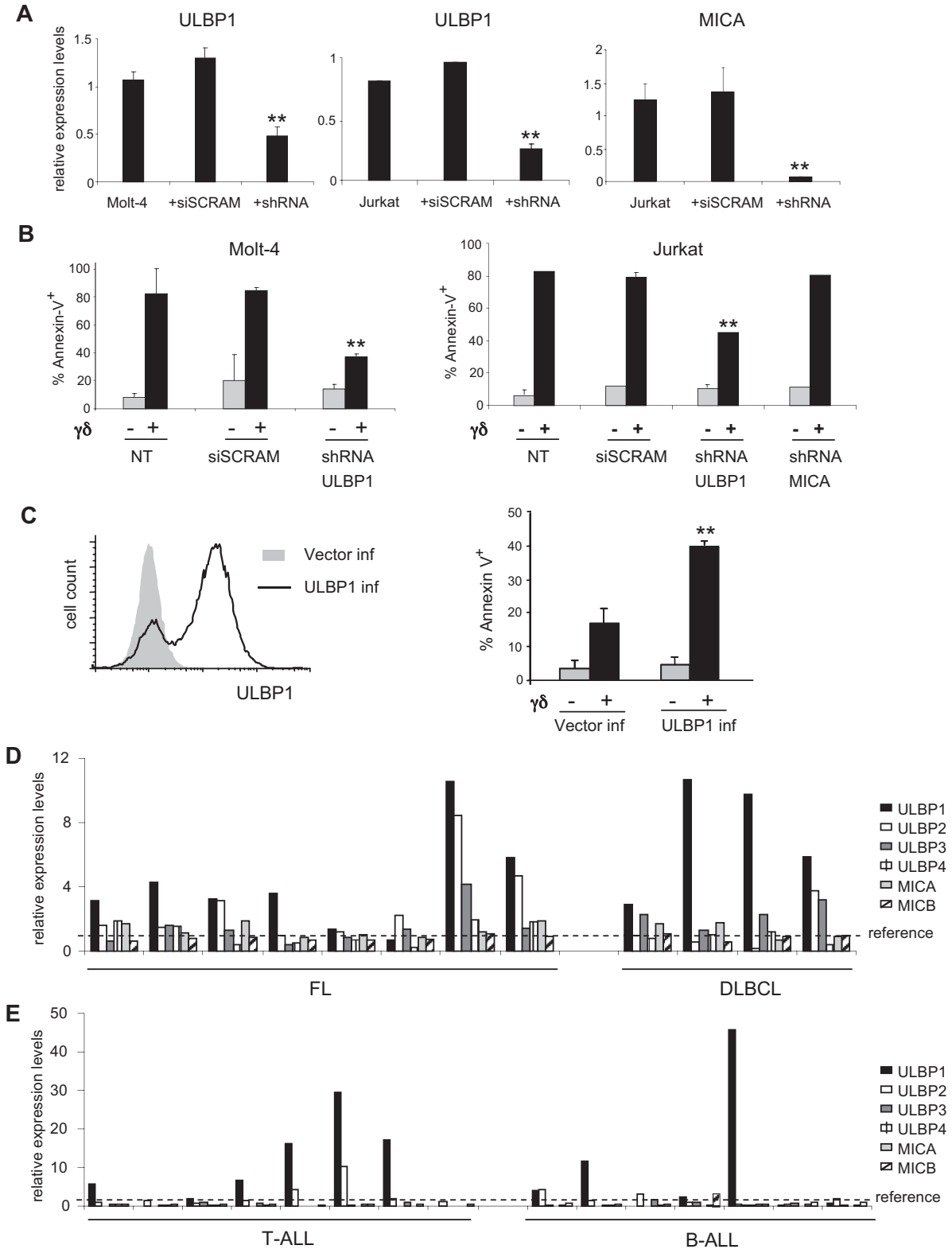

Figure 2 ULBP1 is required for Vγ9Vδ2 T-cell recognition of leukemia/lymphoma cells and displays a highly heterogeneous expression in cancer patients. (A) Lentiviral shRNA-mediated knockdown of ULBP1 and MICA in Molt-4 or Jurkat leukemia cells was confirmed by quantitative RT-PCR using GUSB and PSMB6 as endogenous references. Cells were infected with 10 μL of high-titer virus (10^7 CFU/mL) in media containing polybrene, submitted to selection 48 hours later, and collected for analysis 96 hours after infection. siSCRAM is an shRNA of scrambled (unspecific) sequence, used as an infection control. Error bars represent SD (n = 3). $^{**}P < .01$. (B) Molt-4 or Jurkat leukemia cells, subjected to ULBP1 or MICA shRNA knockdown (as in panel A), were used in in vitro killing assays either in the presence (+) or absence (−) of γδ-PBLs (as in Figure 1A). Nontransduced (NT) and siSCRAM-transduced cells were used as controls. (C) Raji lymphoma cells were lentivirally transduced with ULBP1 (or control vector), and surface expression of ULBP1 was assessed by flow cytometry (left). In vitro killing assays were then performed either in the presence (+) or absence (−) of γδ-PBLs (right). (D-E) Quantitative RT-PCR analysis of mRNA expression of NKG2DLs in 8 follicular lymphoma (FL) and 4 diffuse large B-cell lymphoma (DLBCL) biopsies, normalized to housekeeping genes (GUSB and PSMB6) and to a reference sample (reactive follicles) obtained through the same procedure and indicated by the dashed line (D); and in 8 T acute lymphoblastic leukemia (T-ALL) and 7 B acute lymphoblastic leukemia (B-ALL) PBMC samples, normalized to housekeeping genes (GUSB and PSMB6) and to reference PBMCs from healthy persons, indicated by the dashed line (E).

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Authorship

Contribution: T.L., D.V.C., and A.Q.G. performed the experiments; C.F.M., H.R., A.N.-C., C.F., J.S.R., J.T.B., and L.F.M. provided biologic materials and experimental assistance; and B.S.-S. and A.Q.G. designed the study and wrote the manuscript.

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References

1. Girardi M, Oppenheim DE, Steele CR, et al. Regulation of cutaneous malignancy by γδ T cells. *Science.* 2001;294(5542):605-609.
2. Kabelitz D, Wesch D, He W. Perspectives of γδ T cells in tumor immunology. *Cancer Res.* 2007; 67(1):5-8.
3. Kunzmann V, Wilhelm M. Anti-lymphoma effect of γδ T cells. *Leuk Lymphoma.* 2005;46(5):671-680.
4. Hayday AC. γδ cells: a right time and a right place for a conserved third way of protection. *Annu Rev Immunol.* 2000;18:975-1026.
5. Raulet DH. Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol.* 2003;3(10): 781-790.
6. Hinz T, Wesch D, Halary F, et al. Identification of the complete expressed human TCR V γ repertoire by flow cytometry. *Int Immunol.* 1997;9(8): 1065-1072.
7. Morita CT, Jin C, Sarikonda G, Wang H. Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vγ2Vδ2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens. *Immunol Rev.* 2007;215:59-76.
8. Correia DV, d'Orey F, Cardoso BA, et al. Highly active microbial phosphoantigen induces rapid yet sustained MEK/Erk- and PI-3K/Akt-mediated signal transduction in anti-tumor human γδ T cells. *PLoS ONE.* 2009;4(5):e5657.
9. Wilhelm M, Kunzmann V, Eckstein S, et al. γδ T cells for immune therapy of patients with lymphoid malignancies. *Blood.* 2003;102(1):200-206.
10. Dieli F, Vermijlen D, Fulfaro F, et al. Targeting human γδ T cells with zoledronate and interleukin-2 for immunotherapy of hormone-refractory prostate cancer. *Cancer Res.* 2007;67(15):7450-7457.
11. Li J, Herold MJ, Kimmel B, et al. Reduced expression of the mevalonate pathway enzyme farnesyl pyrophosphate synthase unveils recognition of tumor cells by Vγ9Vδ2 T cells. *J Immunol.* 2009; 182(12):8118-8124.
12. Scotet E, Martinez LO, Grant E, et al. Tumor recognition following Vγ9Vδ2 T cell receptor interactions with a surface F1-ATPase-related structure and apolipoprotein A-I. *Immunity.* 2005;22(1):71-80.
13. Toutirais O, Cabillic F, Le Friec G, et al. DNAX accessory molecule-1 (CD226) promotes human hepatocellular carcinoma cell lysis by Vγ9Vδ2 T cells. *Eur J Immunol.* 2009;39(5):1361-1368.
14. Kong Y, Cao W, Xi X, Ma C, Cui L, He W. The NKG2D ligand ULBP4 binds to TCRγ9/δ2 and induces cytotoxicity to tumor cells through both TCRγδ and NKG2D. *Blood.* 2009;114(2): 310-317.
15. Moffat J, Grueneberg DA, Yang X, et al. A lentiviral RNAi library for human and mouse genes applied to an arrayed viral high-content screen. *Cell.* 2006;124(6):1283-1298.
16. National Center for Biotechnology Information. Entrez Nucleotide (http://www.ncbi.nlm.nih.gov/nuccore). Accessed September 28, 2008.
17. Eberl M, Hintz M, Reichenberg A, Kollas AK, Wiesner J, Jomaa H. Microbial isoprenoid biosynthesis and human γδ T cell activation. *FEBS Lett.* 2003;544(1):4-10.
18. Corvaisier M, Moreau-Aubry A, Diez E, et al. Vγ9Vδ2 T cell response to colon carcinoma cells. *J Immunol.* 2005;175(8):5481-5488.
19. Bouet-Toussaint F, Cabillic F, Toutirais O, et al. Vγ9Vδ2 T cell-mediated recognition of human solid tumors: potential for immunotherapy of hepatocellular and colorectal carcinomas. *Cancer Immunol Immunother.* 2008;57(4):531-539.
20. Todaro M, D'Asaro M, Caccamo N, et al. Efficient killing of human colon cancer stem cells by γδ T lymphocytes. *J Immunol.* 2009;182(11):7287-7296.
21. Cerwenka A, Baron JL, Lanier LL. Ectopic expression of retinoic acid early inducible-1 gene (RAE-1) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor in vivo. *Proc Natl Acad Sci U S A.* 2001;98(20):11521-11526.
22. Sutherland CL, Rabinovich B, Chalupny NJ, Brawand P, Miller R, Cosman D. ULBPs, human ligands of the NKG2D receptor, stimulate tumor immunity with enhancement by IL-15. *Blood.* 2006;108(4):1313-1319.
23. Butler JE, Moore MB, Presnell SR, Chan HW, Chalupny NJ, Lutz CT. Proteasome regulation of ULBP1 transcription. *J Immunol.* 2009;182(10): 6600-6609.
24. Gomes AQ, Correia DV, Silva-Santos B. Non-classical major histocompatibility complex proteins as determinants of tumour immunosurveillance. *EMBO Rep.* 2007;8(11):1024-1030.
25. Wrobel P, Shojaei H, Schittek B, et al. Lysis of a broad range of epithelial tumour cells by human γδ T cells: involvement of NKG2D ligands and T-cell receptor- versus NKG2D-dependent recognition. *Scand J Immunol.* 2007;66(2):320-328.
26. Guerra N, Tan YX, Joncker NT, et al. NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. *Immunity.* 2008;28(4):571-580.

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Inhibition of murine γδ lymphocyte expansion and effector function by regulatory αβ T cells is cell-contact-dependent and sensitive to GITR modulation

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γδ T cells are highly cytolytic lymphocytes that produce large amounts of pro-inflammatory cytokines during immune responses to multiple pathogens. Furthermore, their ability to kill tumor cells has fueled the development of γδ-T-cell-based cancer therapies. Thus, the regulation of γδ-T-cell activity is of great biological and clinical relevance. Here, we show that murine $CD4^+CD25^+$ αβ T cells, the vast majority of which express the Treg marker, Foxp3, abolish key effector functions of γδ T cells, namely the production of the pro-inflammatory cytokines, IFN-γ and IL-17, cytotoxicity, and lymphocyte proliferation *in vitro* and *in vivo*. We further show that suppression is dependent on cellular contact between Treg and γδ T cells, results in the induction of an anergic state in γδ lymphocytes, and can be partially reversed by manipulating glucocorticoid-induced TNF receptor-related protein (GITR) signals. Our data collectively dissect a novel mechanism by which the expansion and pro-inflammatory functions of γδ T cells are regulated.

Key words: γδ T cells · IFN-γ · IL-17 · Treg · T-cell suppression

Supporting Information available online

Introduction

The integration of multiple effector and regulatory mechanisms dictates the course of immune responses to self and non-self antigens. γδ T cells are innate-like lymphocytes known to mount robust responses to infectious pathogens [1] and tumors [2], while also regulating tissue homeostasis and repair [3]. Importantly, several of these functions are non-redundant, as demonstrated by the immunopathology phenotypes of TCR-δ-deficient mice [4]. It is also well documented that upon pathogen challenge (for example, with *Plasmodium falciparum*, *Mycobacterium tuberculosis*, *Haemophilus influenzae*, or *Streptococcus pneumoniae*), γδ T cells are one of the immune populations that expands most dramatically in human peripheral blood [1, 5, 6].

In contrast with adaptive αβ T cells, activated γδ lymphocytes are capable of "immediate" cytotoxicity and cytokine secretion [5]. In fact, we have recently shown that murine γδ T cells become functionally competent in the thymus, particularly regarding the production of pro-inflammatory cytokines IFN-γ and IL-17 [7]. Furthermore, several reports have demonstrated the crucial contributions of γδ T cells to the reservoirs of such cytokines in the context of anti-tumor immunity [8], immune responses to infection [9], and autoimmunity [9, 10]. While these data highlight the importance of understanding how the

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pro-inflammatory and anti-tumor functions of γδ T cells are regulated [11, 12], this process remains poorly understood, particularly in murine models.

$CD4^+CD25^+$ αβ T cells are well known for their ability to control the activity of several immune cell populations *in vitro* and *in vivo*, including effector $CD4^+CD25^-$ and $CD8^+$ αβ T cells [13], NK [14] and NKT cells [15], B cells [16], DC [17], and monocytes/macrophages [18]. As a result, $CD4^+CD25^+$ αβ T cells, and particularly those that develop in the thymus through a Foxp3-dependent genetic program [19, 20], so-called "naturally occurring" Treg (nTreg), are pivotal to maintaining immune homeostasis and preventing inflammatory and autoimmune diseases [21]. On the other hand, Treg-suppressive functions are also known to diminish immunity to pathogens and to tumors [22, 23]. Provocatively, an inverse correlation between circulating human Treg frequencies and γδ-T-cell numbers in cancer patients has been recently reported [24].

Building on these foundations, we show here that Treg suppress γδ-T-cell proliferation, cytotoxicity, and cytokine production in co-cultures of lymphocytes isolated from both naïve and malaria-infected mice. Moreover, Treg also control γδ-T-cell expansion *in vivo*, upon adoptive transfer into lymphopenic mice. We further demonstrate that suppression is cell-contact-dependent and can be partially abrogated through manipulation of glucocorticoid-induced TNF receptor-related protein (GITR) receptor signaling. These data provide a novel mechanistic insight into the regulation of γδ T cells by suppressive αβ T cells.

Results

Treg suppress γδ cell cytotoxicity and cytokine production

γδ T cells are well known for their cytotoxic function in both mice [4] and humans [2], which constitutes a strong basis for γδ-T-cell-based cancer immunotherapies [2]. We tested whether this activity could be directly inhibited by Treg in 3-day co-cultures. $CD4^+CD25^+$ cells isolated from pooled spleen and lymph nodes of naïve C57BL/6 (B6) mice were >95% $Foxp3^+$ (Supporting Information Fig. 1A), and therefore largely represented nTreg. We used the P815 mastocytoma cell line as target for a redirected lysis assay, and observed a striking inhibition of γδ-T-cell-mediated apoptosis of P815, in a Treg dose-dependent manner that included complete suppression at a 1:1 ratio (Fig. 1A). In fact, even at a ratio that corresponds to a ninefold dilution of nTreg, these are still capable of considerable inhibition of γδ-cell cytotoxicity (Fig. 1A), thus demonstrating the extraordinary suppressive potency of nTreg.

In addition to cytotoxicity, cytokine secretion is another critical function of γδ T cells in immune responses. In particular, these lymphocytes have been shown to produce high amounts of IFN-γ [7, 8], IL-17 [10, 25], and TNF-α [7]. Interestingly, we observed an increased proportion of IFN-γ (and, to a lesser extent, IL-17) producers in γδ T cells isolated from TCR-α-deficient mice (Supporting Information Fig. 2), a widely used source of γδ T cells due to their increased numbers in this model [7]. As this cytokine phenotype could potentially reflect a suppressive role of endogenous αβ T cells in WT animals, we employed TCR-α-deficient mice as source of γδ T cells for the subsequent *in vitro* assays where the effect of Treg on γδ-T-cell function was studied. When we analyzed the supernatants of (24 h) co-cultures of γδ T cells with or without Treg, using a cytokine bead array (Supporting Information Fig. 1B), we detected a dramatic (20-fold) reduction in IFN-γ levels, accompanied by a 50% drop in TNF-α levels, in γδ:Treg samples (Fig. 1B). Of note, we verified that γδ-T-cell suppression is a property of Treg, but not of their $CD4^+CD25^-$ ("effector" T cells, Teff) counterparts, as these actually promoted the secretion of Th1-associated cytokines (Fig. 1B).

We have recently described two subsets of murine γδ T cells with distinct cytokine profiles: whereas $CD27^+$ γδ cells are essentially restricted to IFN-γ production, $CD27^-$ γδ cells are selectively capable of expressing IL-17, particularly upon TGF-β treatment *in vitro* [7]. When we investigated the effect of Treg on each of these particular γδ-T-cell subsets, we observed a complete loss of IFN-γ production by $CD27^+$ γδ cells (Fig. 1C), in parallel with a very significant inhibition of $CD27^-$ γδ cell cytokine production (Fig. 1D). Furthermore, both effects were dependent on the Treg:γδ ratio (Supporting Information Fig. 1C and D). These data demonstrate that Treg are very effective at inhibiting γδ-T-cell effector functions.

In vitro inhibition of γδ-T-cell proliferation by nTreg and iTreg

γδ-T-cell responses to TCR stimulation also involve extensive cellular proliferation. When we applied a conventional *in vitro* suppression assay [26], we observed that nTreg were clearly capable of inhibiting γδ-T-cell proliferation *in vitro*, in a dose-dependent manner, with a striking arrest observed at ratios of 1 Treg:3 γδ or above (Fig. 2A). Importantly, since each population represent *circa* 1% of all splenocytes or peripheral lymph node lymphocytes (Supporting Information Fig. 4A), this *in vitro* suppression (Fig. 2A) occurred at ratios that mimic the *in vivo* proportions of the two populations. Consistent with the proliferation blockade, we also observed a very significant decrease in cell size of activated γδ lymphocytes upon co-incubation with nTreg (Fig. 2B).

The peripheral generation of "adaptive" or "inducible" $Foxp3^+$ Treg (iTreg) through TGF-β-dependent "conversion" of $Foxp3^-$ $CD4^+$ T cells has been widely documented [27, 28]. We tested if iTreg could also suppress γδ-cell proliferation. Indeed, after *de novo* generation of over 70% $Foxp3^+$ $CD4^+$ T cells (data not shown), these significantly blocked γδ-cell expansion *in vitro* (Fig. 2C). Note that the inequivalent proportions of Foxp3-expressing cells within the iTreg and nTreg populations (70% *versus* 95%) may justify the difference in the extent of suppression scored in our assays (Fig. 2C).

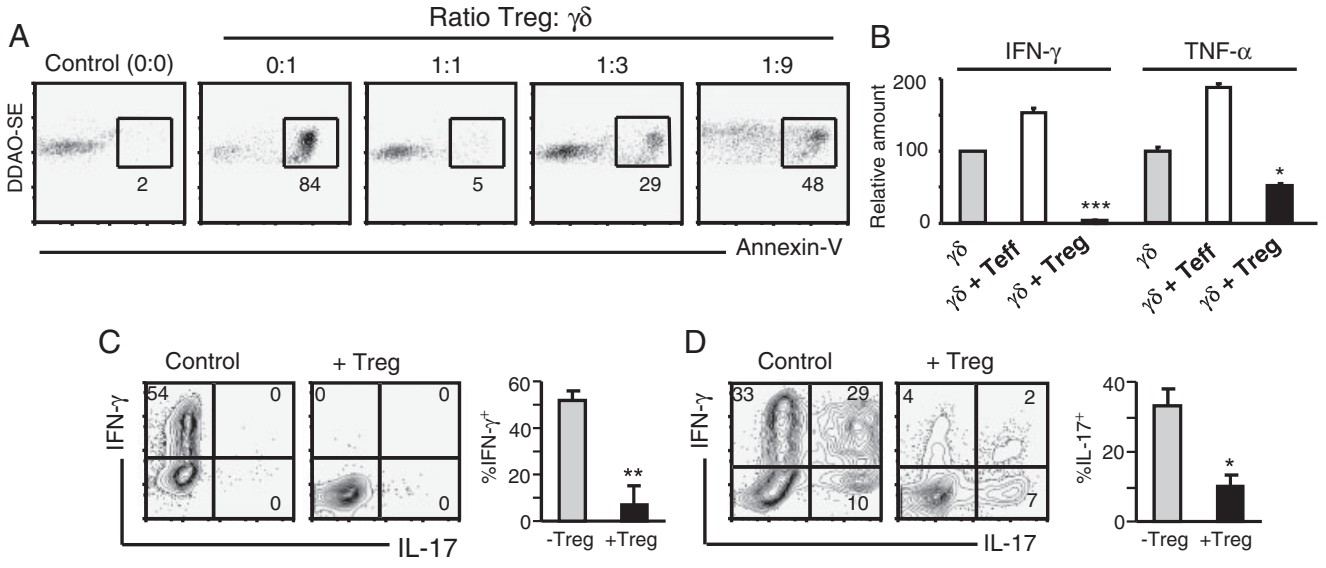

Figure 1. Suppression of γδ-cell cytotoxicity and cytokine production by αβ Treg. (A) Redirected lysis assay of (DDAO-SE-labeled) mastocytoma P815 target cell line by γδ-T cells in co-cultures with titrated amounts of Treg. γδ T cells and Treg were isolated from C57BL/6 (B6) mice. Tumor-cell apoptosis was evaluated by Annexin V staining 4 h after addition of P815 cells. (B) IFN-γ and TNF-α in the supernatants of 24 h B6 γδ cell cultures, alone or together (at 1:1 ratio) with either $CD4^+CD25^-$ (Teff) or $CD4^+CD25^+$ (Treg). Results of Cytokine Bead Array analysis were normalized to the amounts detected in cultures of γδ cells alone. Data show mean+SD ($n = 3$; $^*p<0.05$; $^{***}p<0.001$). (C and D) Intracellular staining for IFN-γ and IL-17 in TGF-β-supplemented cultures of $CD27^+$ (C) or $CD27^-$ (D) B6.*Tcra*$^{-/-}$ γδ T cells, alone (control) or in the presence of B6 Treg at 1:1 ratio. Bar graphs show IFN-γ^+ within $CD27^+$ (C) or IL-17^+ within $CD27^-$ (D) γδ cells. Data show mean+SD ($n = 3$, $^*p<0.05$; $^{**}p<0.01$). Data are representative of three independent experiments.

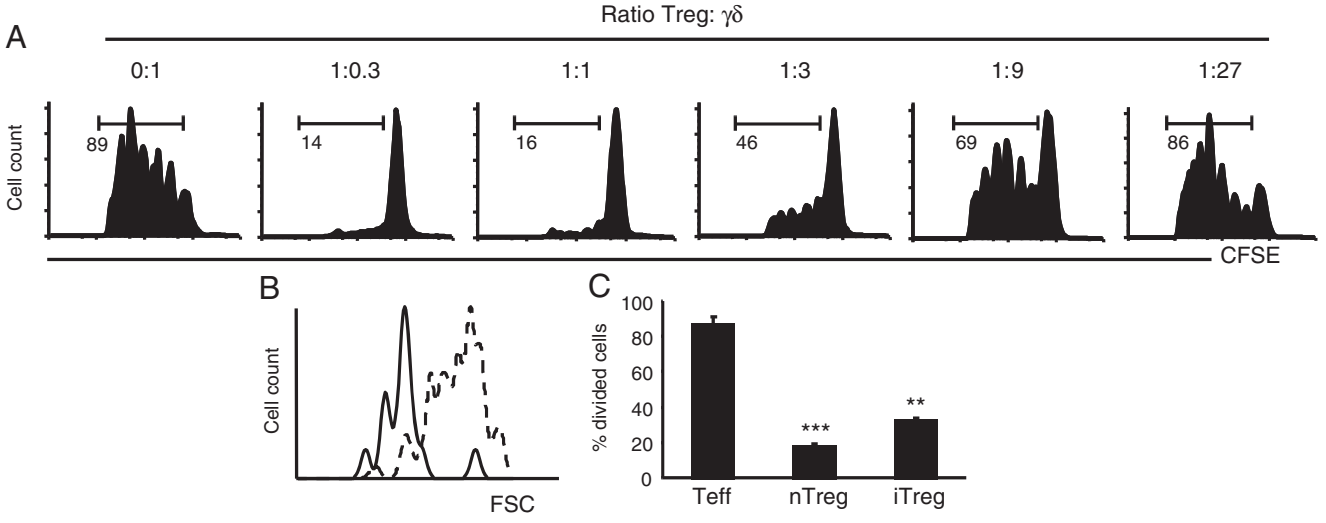

Figure 2. Inhibition of γδ-T-cell proliferation by nTreg and iTreg. (A) Proliferation of CFSE-labeled γδ cells in co-cultures with titrated amounts of nTreg. γδ T cells were isolated from B6.*Tcra*$^{-/-}$ mice, while Treg were obtained from B6 animals. CFSE-labeled γδ-cell responders were cultured with anti-CD3ε and IL-7 in the presence of APC and variable ratios of Treg or Teff. Indicated is percentage of divided γδ cells after 3 days of culture, as assessed by flow cytometry. Data are representative of five independent experiments. (B) Forward side scatter (FSC; cell size) of γδ cells cultured in the absence (dashed line) or presence (full line) of nTreg at 1:1 ratio. (C) Percentage of divided γδ cells, as evaluated by flow cytometry, after 3 days in co-culture (at 1:1 ratio) with nTreg or iTreg. Data show mean+SD ($n = 3$; $^{**}p<0.01$; $^{***}p<0.001$).

Treg abolish effector functions of malaria-activated γδ T cells

Having performed the previous experiments with naïve lymphocytes, we asked whether these findings could be extended to T cells that had been conditioned during the course of an infection. Recent reports have shown that human Treg can inhibit IFN-γ secretion by Vγ9Vδ2 PBL activated *in vitro* by *M. tuberculosis* antigens [11, 29]. Here, we used an *in vivo* model of malaria infection [30] to obtain γδ cells at the peak of their (rapid) response and test their susceptibility to Treg suppression *in vitro*. C57BL/6 mice infected with *Plasmodium berghei* ANKA showed a

marked expansion of γδ cells in peripheral lymphoid organs (spleen and lymph nodes) within the first 5 days of infection (Fig. 3A), which correlated with an increased expression of the activation marker CD69 (Fig. 3B). Moreover, the absolute number of IFN-γ- and IL-17-producing γδ cells increased by approximately five- and tenfold, respectively (Fig. 3C). When γδ cells isolated at day 3 post-infection were co-incubated with Treg obtained from the same mice, the proliferation of the former was completely inhibited (Fig. 3D). In contrast, $CD4^+CD25^-$ Teff facilitated γδ-cell proliferation (Fig. 3D), potentially through the provision of IL-2 (Fig. 3E). We also analyzed the expression of IFN-γ and IL-17 in these cultures, and observed that, in stark contrast with γδ:Teff samples, γδ:Treg co-cultures completely lacked both cytokines (Fig. 3F and G). These data collectively demonstrate that Treg block the effector functions of malaria-activated γδ T cells.

In vivo suppression of γδ-T-cell expansion by Treg

Murine models offer the possibility to test Treg functions *in vivo*. A widely used assay is the adoptive transfer of Treg and αβ Teff populations into lymphopenic mice, which has clearly shown that Treg prevent the homeostatic expansion of αβ Teff [31]. We adapted this system to test the effect of Treg on γδ-cell expansion in Rag2-deficient animals; we added *P. berghei* ANKA infection as an extra stimulus (1 day after the injection of lymphocytes) due to the relatively poor homeostatic expansion of γδ cells (data not shown) [32]. In this adapted model, Teff and Treg engrafted and expanded comparably after 4 days (Fig. 4A) and largely maintained their characteristics, in particular CD25 expression (Fig. 4B). However, the co-injected γδ cells expanded to very distinct levels in the two scenarios, both in terms of percentages (Fig. 4C) and absolute numbers (data not shown): approximately fivefold less γδ cells accumulated in the spleens of Treg:γδ when compared with Teff:γδ-injected animals. Of note, γδ cells expanded to a similar degree when injected alone or in the presence of Teff (data not shown). These data constitute important *in vivo* evidence for the relevance of the Treg-mediated effects previously observed *in vitro* (Fig. 2 and 3).

Moreover, endogenous γδ T cells expanded significantly more (upon malaria infection) in TCR-α-deficient mice than in WT controls (Fig. 4D), consistent with an aggregate inhibitory effect of αβ T cells on γδ-cell proliferation. It should be noted, however, that we cannot exclude a distinct homeostatic behavior of γδ cells due to the lack of competing αβ T cells.

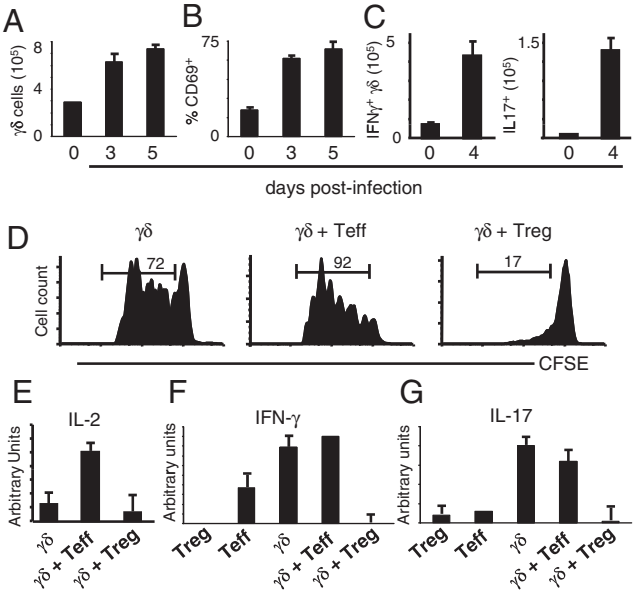

Figure 3. Treg-mediated suppression of proliferation and cytokine production of malaria-activated γδ T cells (A–C). γδ-T-cell absolute numbers (A), percentage of CD69-expressing cells (B) and numbers of IFN-γ- or IL-17-producing cells (C) per spleen of C57BL/6 mice during the course of a *P. berghei* ANKA infection. Data show mean+SD (n = four mice). (D) Proliferation of CFSE-labeled γδ cells obtained from day 3-infected animals, co-cultured alone or with either $CD4^+CD25^-$ (Teff) or $CD4^+CD25^+$ (Treg) at 1:1 ratio. Indicated is the percentage of divided γδ cells after 3 days, as evaluated by flow cytometry. (E) Real-time quantitative PCR for IL-2 in cells obtained from 24 h cultures of γδ T cells, alone or together with either $CD4^+CD25^-$ (Teff) or $CD4^+CD25^+$ (Treg) at 1:1 ratio. Gene expression was normalized to the housekeeping gene Efa1. (F and G) Real-time quantitative PCR for IFN-γ (E) or IL-17 (F) in cells obtained from cultures identical to (E). Gene expression was normalized to the housekeeping gene Efa1. Data are representative of two independent experiments (each involving four to five animals).

Cell contact/Treg-dependent "anergy" in γδ cells can be partially abrogated *via* GITR

We next sought to dissect the mechanisms underlying Treg-mediated suppression of γδ-T-cell activities. We first addressed the cell-contact-dependence of this phenomenon by employing a classical transwell system. We observed that the inhibition of γδ-cell proliferation by Treg was abolished when the two populations were separated by a cell-impermeable membrane (Fig. 5A). To gain further insight into this cell-contact-dependent mechanism, we considered that Treg could induce γδ-cell apoptosis through a Perforin [33]/Granzyme [34]-mediated mechanism. Although we could indeed detect significant expression of granzymes in γδ:Treg, but not in γδ:Teff, co-cultures (Fig. 5B), we did not observe an increase of apoptotic (Annexin V^+) γδ cells in γδ:Treg samples when compared with controls (Fig. 5C). In particular, most γδ T cells that did not proliferate in γδ:Treg co-cultures remained negative for Annexin V (Fig. 5D). We therefore propose that Treg-induced apoptosis is not a major factor in the suppression of γδ-cell responses, and that instead γδ cells are rendered "anergic" (unresponsive to TCR stimulation) through the delivery of inhibitory signals from Treg. However, when we used a specific mAb to GITR in γδ:Treg co-cultures, we could observe a significant and dose-dependent restoration of γδ-cell proliferation (Fig. 5E and F). In contrast, IL-10 (Fig. 5E), TGF-β or their combination (data not shown) blockade had no effect in these assays, which is consistent with suppression being cell-contact-dependent.

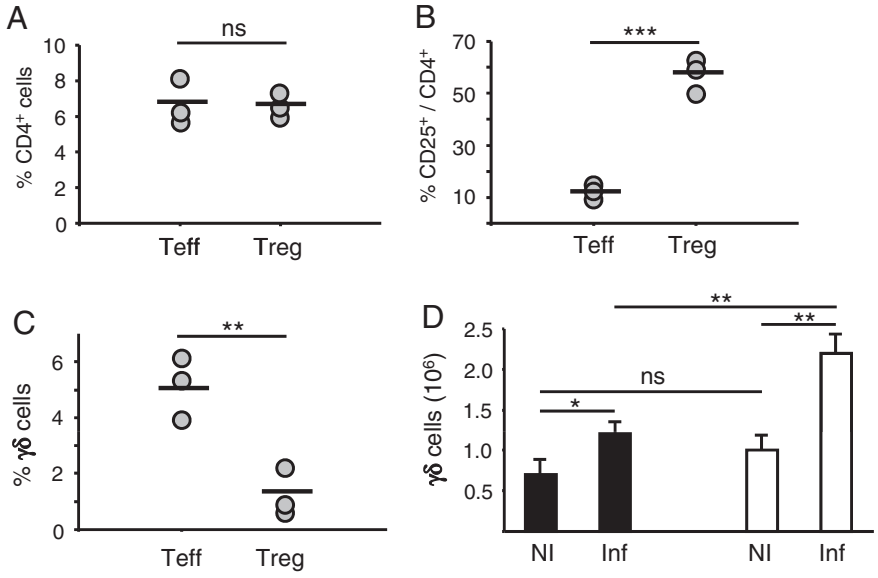

Figure 4. *In vivo* suppression of γδ-T-cell expansion. γδ cells (isolated from B6.*Tcra*$^{-/-}$ mice) were injected together with CD4$^+$ T cells (from B6 mice) into Rag2-deficient mice, and these were infected with *P. berghei* ANKA 1 day later. Each dot represents one animal. Expansion (A) and CD25 expression (B) of Teff or Treg populations analyzed in the spleen of the animals 4 days after co-injection with γδ cells. Percentages refer to electronically gated "live lymphocytes" according to flow cytometry forward and side scatter parameters. ($n = 3$; ns, non-significant; ***$p<0.001$). (C) Abundance of γδ cells in the spleens analyzed in (A and B) ($n = 3$; **$p<0.01$). (D) B6 (black bars) or B6.*Tcra*$^{-/-}$ (white bars) mice were infected with *P. berghei* ANKA and their spleens were collected after 3 days. Total γδ cell counts *per* spleen were estimated by flow cytometry ($n = 3$; ns, non-significant; *$p<0.05$; **$p<0.01$). Data are representative of two independent experiments (each involving three animals).

We next asked if Treg-mediated suppression of γδ-T-cell cytokine production was also sensitive to GITR modulation. We detected a partial recovery in IFN-γ and IL-17 production upon α-GITR mAb treatment of γδ:Treg co-cultures (Fig. 5G). Of note, α-GITR mAb treatment had no direct inhibitory effect on γδ-T-cell proliferation (Supporting Information Fig. 3) or cytokine production (Fig. 5G); in fact, α-GITR mAb treatment appeared to stimulate IFN-γ/IL-17 double producers (Fig. 5G).

Our data collectively demonstrate that murine γδ-T-cell effector functions and expansion upon activation are very effectively inhibited by αβ Treg through a contact-dependent mechanism that can be partially abrogated by manipulating GITR signals.

Discussion

The thymus produces functional αβ nTreg and γδ T cells from common early precursors that follow distinct developmental pathways [7, 26] but that end up sharing the unconventional property of being positively selected on high avidity signals [35–38], which presumably reflects their self-reactivity [21, 39, 40]. While the different homing properties of the two T-cell subsets may prevent their interaction in various tissues, γδ cells may often come into contact with Treg in peripheral lymphoid organs, namely the spleen and lymph nodes (Supporting Information Fig. 4). However, the outcome of such interactions is largely unknown, particularly in the mouse system, where Treg functions have been otherwise extensively dissected [21]. This study provides a first detailed characterization of the suppressive effects of murine Treg on γδ-cell activity.

The potent cytotoxicity and cytokine production properties of γδ T cells potentially justify their evolutionary conservation and have fueled the development of γδ-T-cell-based therapies. Our results clearly demonstrate that these effector functions, along with cell expansion upon activation, are severely impaired when γδ cells come into contact with Treg. Importantly, this happens at ratios (close to 1:1) that mimic their relative physiologic abundance (Supporting Information Fig. 4). Furthermore, both functional subsets of γδ T cells that we have recently described [7] are effectively suppressed by Treg. Thus, by showing that all major effector functions attributed to murine γδ cells are strongly inhibited by Treg, our data significantly add to the particular parameters scored in previous human studies [11, 24, 29]. Of note, the employment of naïve mice raised in specific pathogen-free housing conditions allowed us to assign these effects to either nTreg or iTreg (see Fig. 2), which would be essentially impossible to do in human volunteers.

Although multiple molecular events have been proposed to explain Treg suppression of immune cell (particularly αβ Teff) functions [13], none is able to account for all aspects of Treg activity, and it is therefore likely that combinations of various mechanisms operate at any given time. Treg can interact with other immune populations *via* soluble factors and/or *via* cell-contact-dependent mechanisms. Whereas *in vivo* studies have suggested a major role for soluble factors, such as IL-10 [41], TGF-β [42], or IL-35 [43], most *in vitro* experiments described a cell-contact-dependent mechanism, involving CTLA-4 [44, 45], GITR [46],

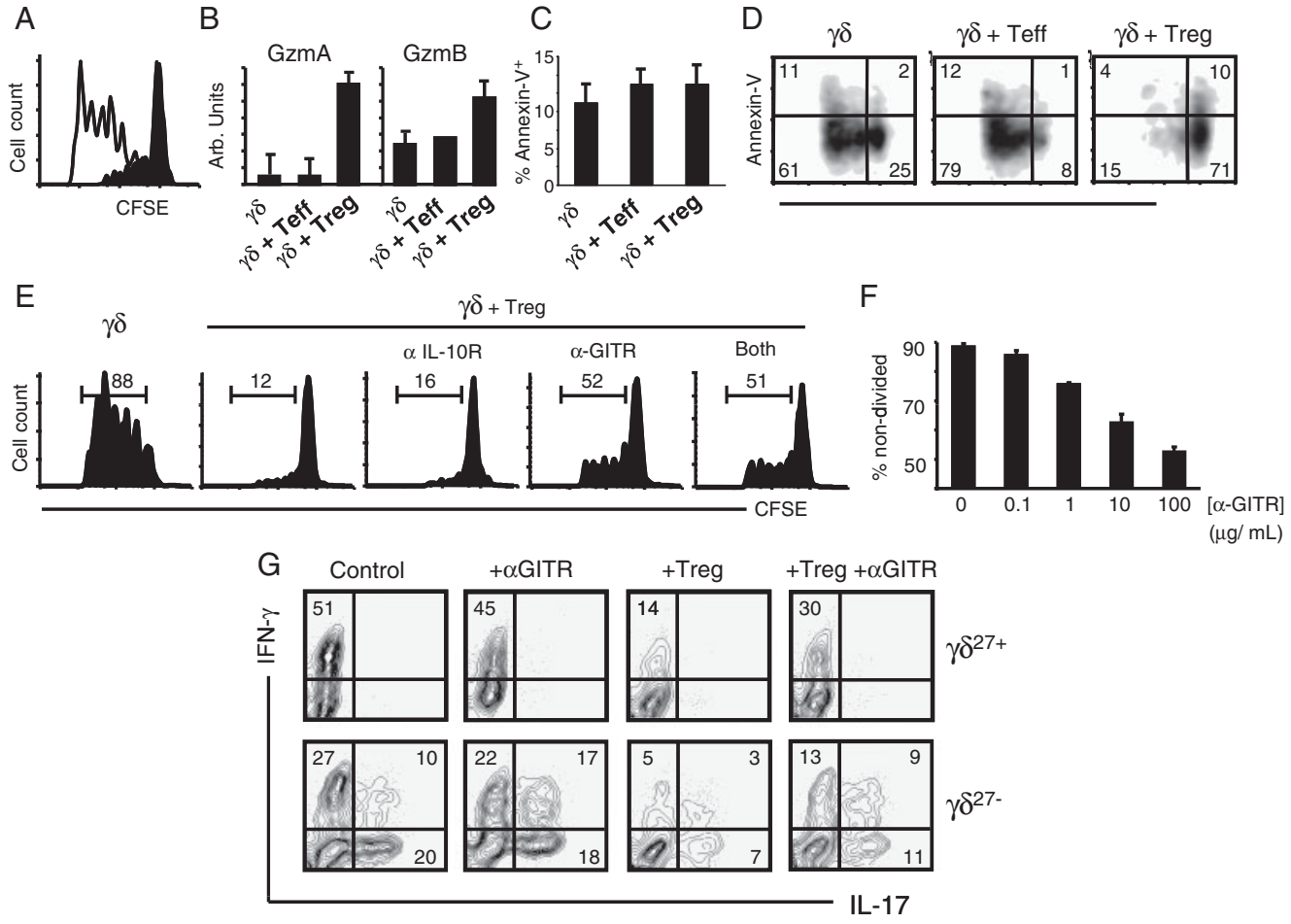

Figure 5. Cell-contact-dependent, GITR-mediated inhibition of γδ-T-cell proliferation by αβ Treg. (A) Proliferation of CFSE-labeled γδ cells (isolated from B6.*Tcra*$^{-/-}$ mice) in co-cultures of with Treg (from B6 mice) at 1:1 ratio, in the absence (black filling) or presence (white filling) of a transwell cell-impermeable membrane. (B) Real-time quantitative PCR for Granzyme A and Granzyme B in cells obtained from 24 h cultures of γδ T cells, alone or together with either Teff or Treg at 1:1 ratio. Gene expression was normalized to the housekeeping gene Efa1. (C) Percentage of Annexin-V$^+$ cells in 3-day co-cultures of γδ cells with either Teff or Treg at 1:1 ratio. (D) Percentage of Annexin-V$^+$ cells in 3-day co-cultures (identical to (B)) using CFSE-labeled γδ cells. (E) Proliferation of CFSE-labeled γδ cells alone or in co-cultures with Treg at 1:1 ratio. Where noted, specific mAb against GITR or IL-10R were added separately or together ("both") to the cultures. Indicated is percentage of divided γδ cells after 3 days, as evaluated by flow cytometry. (F) Effect of increasing amounts of anti-GITR mAb on γδ-cell proliferation (as in (E)). Indicated is the percentage of non-divided γδ cells. Data show mean+SD ($n = 3$). (G) Intracellular staining for IFN-γ and IL-17 expression in CD27$^+$ (upper panel) or CD27$^-$ (lower panel) γδ cells, alone or in co-culture with Treg at 1:1 ratio. Where noted, 100 μg/mL of anti-GITR mAb was added to the cultures. Data are representative of two to three independent experiments.

Perforin [33], or Granzyme B [34]. Our data suggest that Perforin/Granzyme-induced apoptosis does not play a major role in Treg-mediated suppression of mouse γδ-T-cell activity. Contrary to a previous report on human γδ T cells [11], we failed to observe an effect by blocking murine IL-10; this is consistent with the requirement for cell–cell interactions that transpires from our transwell experiments. Importantly, our results demonstrate that the modulation of GITR signals can partially reverse the suppressive effect of Treg on γδ-T-cell proliferation and cytokine production. GITR is a Treg-biased gene product [47, 48] that binds a ligand (GITR-L) expressed on endothelial and APC [49]. The administration of α-GITR mAb was previously shown to induce autoimmune manifestations in mice [48], and to abrogate Treg-mediated suppression of Teff activity *in vitro* [47, 48]. This was possibly due to GITR triggering rather than blocking, as the effect of the antibody was mimicked by GITR-L fusion proteins [46, 50]. However, cross-linking of GITR was later shown to co-stimulate both Treg and Teff [50–52]. Therefore, the precise mechanistic involvement of GITR in Treg-mediated suppression remains under scrutiny [49].

Future lines of research should address Treg suppression of γδ-T-cell functions in animal models of tumor rejection, where the potential of GITR modulation should also be assessed and the dynamics of these two lymphocyte populations in human pathology examined, particularly as circulating human Treg frequencies and γδ-T-cell numbers have been inversely correlated in cancer patients [24].

The unique responsiveness of the major γδ cell subset (Vγ9Vδ2) of human peripheral blood to non-peptidic phophory-

lated intermediates of isoprenoid biosynthesis pathways (phosphoantigens) [53] is the basis of current immunotherapy strategies involving γδ cells. Not only are phosphoantigen-activated Vγ9Vδ2 cells high producers of Th1 cytokines and very cytotoxic against a variety of tumors [54], but they can also induce DC maturation [55], and even perform as CD80/86-expressing APC [56]. While the recent characterization of (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate as a very potent agonist of Vγ9Vδ2 responses [57, 58] strengthens the principle of γδ-cell-based immunotherapy, the data we report here, by clarifying how γδ-cell activities can be negatively modulated, stress the importance of combining Treg inhibition with γδ-cell activation for future immunotherapeutic strategies.

Materials and methods

Mice

C57BL/6 (used as source of αβ and γδ-T-cell populations) and B6.TCR-α-deficient mice (source of γδ T cells), previously obtained from Jackson Laboratories, were bred and maintained in specific pathogen-free conditions, and used at 5–10 wk of age. All experiments involving mice were performed in compliance with the relevant laws and institutional guidelines and have been approved by the Instituto de Medicina Molecular animal ethics committee.

Cell isolation and flow cytometry

Single-cell suspensions were obtained from lymph nodes and spleen, after lympholyte M (Cerderlane Laboratories) gradient centrifugation. For APC, erythrocyte-depleted splenocytes were treated with mitomycin C (25 μg/mL; Sigma) at 37°C for 1 h. Cells were surface stained with anti-TCR-δ (GL3), anti-CD4 (RM4-5), anti-CD25 (PC61), and anti-CD27 (LG.7F9), all from BD Biosciences, for 15 min on ice. Cells were analyzed on FACS Calibur (data analyzed on FloJo) and sorted on FACSAria (BD Biosciences).

In vitro generation of "inducible" Foxp3$^+$ T cells

Sorted CD4$^+$CD25$^-$ cells were incubated for 3 days on plate-bound anti-CD3ε (10 μg/mL; 145.2C11; BD Phaewigen) with IL-2 (100 U/mL; Sigma) and TGF-β (5 ng/mL; R&D Systems). To confirm the "conversion", intracellular staining for Foxp3 protein was performed using a commercial kit (eBiosciences) and analyzed by flow cytometry.

In vitro proliferation assays

Sorted γδ-T-cell responders were labeled with 5 μM CFSE (Invitrogen) for 5 min at room temperature and washed with RPMI 1640 (Invitrogen) supplemented with 10% FBS. In total, 25×10^3 γδ T (or Teff) cells were co-cultured in a 96-well round bottom plates in the presence of APC (10^5) with variable ratios of Treg or Teff and 0.5 μg/mL anti-CD3ε (145.2C11; BD Pharmigen) and 20 ng/mL IL-7 (PeproTech) in RPMI 1640 with 10% FBS. Co-cultures of 25×10^3 Teff with or without Treg in the same conditions described above were used as controls. After 72 h, CFSE dilution was assessed by flow cytometry. To assess apoptosis, cells were stained with 1:50 dilution of APC-labeled Annexin V (BD Biosciences) in 1× Annexin binding buffer (BD Biosciences). Where noted, a 96-well transwell plate (Costar) with a 0.4 μm membrane support was used to separate cell populations in 3-day co-cultures. When noted, the following antibodies were added at saturating concentrations (100–200 μg/mL) to the cultures: anti-GITR (YGITR 765.4.2), anti-IL10R (1B1.2), and anti-TGF-β (clone 1D11), all kindly provided by Hermann Waldmann (Oxford, UK).

Cytokine production

For intracellular staining, cells were stimulated with PMA (50 ng/mL) and ionomycin (1 μg/mL) for 4 h at 37°C, 10 μg/mL BrefeldinA (Sigma) was added during the last 2 h, cells were fixed (15 min, 4°C) in 2% paraformaldehyde, permeabilized (30 min, room temperature) with 0.5% saponin, and finally stained with indicated antibodies. For evaluation of secretion, the Mouse Inflammation Cytometric Bead Array kit (BD Biosciences) was used according to the manufacturer's instructions. Cells were seeded at 2×10^5 cells/well, culture supernatants were labeled, and analyzed on a FACSCanto (BD Biosciences).

Cytotoxic assays

γδ T cells were co-cultured with APC and 1 μg/mL anti-CD3 antibody for 3 days and then mixed with 1 μM DDAOse-labeled (10 min at 10°C) P815 target cells at indicated ratios, in the presence of 1 μg/mL anti-CD3 antibody. Redirected lysis was performed for 4 h and target apoptosis was evaluated by Annexin V staining.

RNA extraction and real-time quantitative PCR

RNA from 5×10^5 cultured cells was extracted using TRIzol (Invitrogen) and treated with Rnase-free RQ1DNase (Promega). Production of cDNA was carried out using Superscript II Reverse transcriptase following the manufacturer's instructions; 145.2C11; BD Pharmingen. All PCR were preformed in the Rotor Gene 6000 (Corbett Research) in a conventional 50-cycle program.

Primers used in the quantitative PCR assays were:

IFN-γ, Fwd: 5′-TAGCTCTGAGACAATGAACGCTAC-3′; IFN-γ, Rev: 5′-TCCTCCAGATATCCAAGAAGAGAC-3′; IL-17, Fwd: 5′-TGTGAAGGTCAACCTCAAAGTC-3′; IL-17, Rev: 5′-AGGGATATCTATCAGGGTCTTCATT-3′; Granzyme A, Fwd: 5′-TGAGAGAAGTCAACATCACTGTCA-3′; Granzyme A, Rev: 5′-GCAAAATACCATCACATAGCAGAG-3′;

Granzyme B, Fwd: 5′-CTAAAGCTGAAGAGTAAGGCCAAG-3′; Granzyme B, Rev: 5′-GATCCTTCTGTACTGTCAGCTCAA-3′; IL-2, Fwd: 5′-GCTGTTGATGGACCTACAGGA-3′; IL-2, Rev: 5′-TTCAATTCTGTGGCCTGCTT-3′; Efa1, Fwd: 5′-ACACGTAGATTCCGGCAAGT-3′; Efa1, Rev: 5′-AGGAGCCCTTTCCCATCTC-3′.

Gene expression was quantified by the standard curve method, normalized to Efa1, and expressed in arbitrary units.

Malaria infection

Mice were infected intraperitonially with 10^6 GFP-transgenic *P. berghei* ANKA-infected red blood cells, and parasitemia was evaluated [30] by flow cytometry at day 3. Single-cell suspension was obtained from lymph nodes and spleen; cells were then stained, sorted, and cultured as described in the *cell isolation and flow cytometry* section.

Adoptive transfer and *in vivo* T-cell expansion

Sorted γδ (5×10^5) cells were stained with CFSE as described in *in vitro proliferation assays* section and were co-injected intravenously with either 10^6 Teff or Treg into Rag2-deficient mice. After 24 h, the mice were infected intraperitonially with 10^6 *P. berghei* ANKA-infected red blood cells. Splenocytes were collected at day 3 post-infection and proliferation of γδ cells was accessed by flow cytometry.

Confocal microscopy

Spleen tissue sections of 20 μm were fixed in acetone and then fixed and permeabilized using a Fixation/Permeabilization buffer (eBiosciences). The following antibodies were used for staining: anti-mouse γδ TCR-biotin (clone UC7-13D5) (eBiosciences), streptavidin-Alexa Fluor 568 (Molecular Probes, Invitrogen), rabbit anti-GFP (Abcam) and donkey anti-rabbit Alexa Fluor 647 (Molecular Probes, Invitrogen). Nuclear DNA content was detected with DAPI Fluoromount G (Southern Biotech). Immunofluorescence microscopy was performed with a LSM 510 META confocal microscope (Zeiss). Separate images were collected with a 63× objective for each fluorochrome and then overlaid to obtain a multicolor image.

Statistical analysis

The statistical signicance of differences between populations was assessed using Student's *t*-test; *p* values below 0.05 were considered signicant.

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References

1 **Chen, Z. W.,** Immune regulation of γδ T cell responses in mycobacterial infections. *Clin. Immunol.* 2005. **116**: 202–207.

2 **Kabelitz, D., Wesch, D. and He, W.,** Perspectives of γδ T cells in tumor immunology. *Cancer Res.* 2007. **67**: 5–8.

3 **Jameson, J. and Havran, W. L.,** Skin γδ T-cell functions in homeostasis and wound healing. *Immunol. Rev.* 2007. **215**: 114–122.

4 **Hayday, A. and Tigelaar, R.,** Immunoregulation in the tissues by γδ T cells. *Nat. Rev. Immunol.* 2003. **3**: 233–242.

5 **Beetz, S., Wesch, D., Marischen, L., Welte, S., Oberg, H. H. and Kabelitz, D.,** Innate immune functions of human γδ T cells. *Immunobiology* 2008. **213**: 173–182.

6 **Dieli, F., Troye-Blomberg, M., Farouk, S. E., Sireci, G. and Salerno, A.,** Biology of γδ T cells in tuberculosis and malaria. *Curr. Mol. Med.* 2001. **1**: 437–446.

7 **Ribot, J. C., deBarros, A., Pang, D. J., Neves, J. F., Peperzak, V., Roberts, S. J., Girardi, M. *et al.*,** CD27 is a thymic determinant of the balance between interferon-γ- and interleukin-17-producing γδ T cell subsets. *Nat. Immunol.* 2009. **10**: 427–436.

8 **Gao, Y., Yang, W., Pan, M., Scully, E., Girardi, M., Augenlicht, L. H., Craft, J. and Yin, Z.,** γδ T cells provide an early source of interferon-γ in tumor immunity. *J. Exp. Med.* 2003. **198**: 433–442.

9 **O'Brien, R. L., Roark, C. L. and Born, W. K.,** IL-17-producing γδ T cells. *Eur. J. Immunol.* 2009. **39**: 662–666.

10 **Jensen, K. D., Su, X., Shin, S., Li, L., Youssef, S., Yamasaki, S., Steinman, L. *et al.*,** Thymic selection determines γδ T cell effector fate: antigen-naive cells make interleukin-17 and antigen-experienced cells make interferon-γ. *Immunity* 2008. **29**: 90–100.

11 **Li, L. and Wu, C. Y.,** CD4+ CD25+ Treg cells inhibit human memory γδ T cells to produce IFN-γin response to *M. tuberculosis* antigen ESAT-6. *Blood* 2008. **111**: 5629–5636.

12 **Martinet, L., Fleury-Cappellesso, S., Gadelorge, M., Dietrich, G., Bourin, P., Fournie, J. J. and Poupot, R.,** A regulatory cross-talk between Vγ9Vδ2 T lymphocytes and mesenchymal stem cells. *Eur. J. Immunol.* 2009. **39**: 752–762.

13 **Tang, Q. and Bluestone, J. A.,** The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. *Nat. Immunol.* 2008. **9**: 239–244.

14 **Ghiringhelli, F., Menard, C., Terme, M., Flament, C., Taieb, J., Chaput, N., Puig, P. E. *et al.*,** CD4+CD25+ regulatory T cells inhibit natural killer cell functions in a transforming growth factor-beta-dependent manner. *J. Exp. Med.* 2005. **202**: 1075–1085.

15 **Azuma, T., Takahashi, T., Kunisato, A., Kitamura, T. and Hirai, H.,** Human CD4+ CD25+ regulatory T cells suppress NKT cell functions. *Cancer Res.* 2003. **63**: 4516–4520.

16 **Lim, H. W., Hillsamer, P., Banham, A. H. and Kim, C. H.,** Cutting edge: direct suppression of B cells by CD4+ CD25+ regulatory T cells. *J. Immunol.* 2005. **175**: 4180–4183.

17 **Misra, N., Bayry, J., Lacroix-Desmazes, S., Kazatchkine, M. D. and Kaveri, S. V.,** Cutting edge: human CD4+CD25+ T cells restrain the maturation and antigen-presenting function of dendritic cells. *J. Immunol.* 2004. **172**: 4676–4680.

18 **Taams, L. S., van Amelsfort, J. M., Tiemessen, M. M., Jacobs, K. M., de Jong, E. C., Akbar, A. N., Bijlsma, J. W. and Lafeber, F. P.,** Modulation of monocyte/macrophage function by human CD4+CD25+ regulatory T cells. *Hum. Immunol.* 2005. **66**: 222–230.

19 **Fontenot, J. D., Gavin, M. A. and Rudensky, A. Y.,** Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat. Immunol.* 2003. **4**: 330–336.

20 **Hori, S., Nomura, T. and Sakaguchi, S.,** Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003. **299**: 1057–1061.

21 **Sakaguchi, S., Yamaguchi, T., Nomura, T. and Ono, M.,** Regulatory T cells and immune tolerance. *Cell* 2008. **133**: 775–787.

22 **Mills, K. H.,** Regulatory T cells: friend or foe in immunity to infection? *Nat. Rev. Immunol.* 2004. **4**: 841–855.

23 **Zou, W.,** Regulatory T cells, tumour immunity and immunotherapy. *Nat. Rev. Immunol.* 2006. **6**: 295–307.

24 **Kunzmann, V., Kimmel, B., Herrmann, T., Einsele, H. and Wilhelm, M.,** Inhibition of phosphoantigen-mediated γδ T-cell proliferation by CD4+ CD25+ FoxP3+ regulatory T cells. *Immunology* 2009. **126**: 256–267.

25 **Roark, C. L., Simonian, P. L., Fontenot, A. P., Born, W. K. and O'Brien, R. L.,** γδ T cells: an important source of IL-17. *Curr. Opin. Immunol.* 2008. **20**: 353–357.

26 **Pennington, D. J., Silva-Santos, B., Silberzahn, T., Escorcio-Correia, M., Woodward, M. J., Roberts, S. J., Smith, A. L. *et al.*,** Early events in the thymus affect the balance of effector and regulatory T cells. *Nature* 2006. **444**: 1073–1077.

27 **Apostolou, I. and von Boehmer, H.,** In vivo instruction of suppressor commitment in naive T cells. *J. Exp. Med.* 2004. **199**: 1401–1408.

28 **Chen, W., Jin, W., Hardegen, N., Lei, K. J., Li, L., Marinos, N., McGrady, G. and Wahl, S. M.,** Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J. Exp. Med.* 2003. **198**: 1875–1886.

29 **Mahan, C. S., Thomas, J. J., Boom, W. H. and Rojas, R. E.,** CD4(+) CD25(high) Foxp3(3+) regulatory T cells downregulate human Vδ2(+) T-lymphocyte function triggered by anti-CD3 or phosphoantigen. *Immunology* 2009. **127**: 398–407.

30 **Pamplona, A., Ferreira, A., Balla, J., Jeney, V., Balla, G., Epiphanio, S., Chora, A. *et al.*,** Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. *Nat. Med.* 2007. **13**: 703–710.

31 **Zelenay, S., Lopes-Carvalho, T., Caramalho, I., Moraes-Fontes, M. F., Rebelo, M. and Demengeot, J.,** Foxp3+ CD25- CD4 T cells constitute a reservoir of committed regulatory cells that regain CD25 expression upon homeostatic expansion. *Proc. Natl. Acad. Sci. USA* 2005. **102**: 4091–4096.

32 **French, J. D., Roark, C. L., Born, W. K. and O'Brien R, L.,** γδ T cell homeostasis is established in competition with T cells and NK cells. *Proc. Natl. Acad. Sci. USA* 2005. **102**: 14741–14746.

33 **Grossman, W. J., Verbsky, J. W., Barchet, W., Colonna, M., Atkinson, J. P. and Ley, T. J.,** Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* 2004. **21**: 589–601.

34 **Gondek, D. C., Lu, L. F., Quezada, S. A., Sakaguchi, S. and Noelle, R. J.,** Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J. Immunol.* 2005. **174**: 1783–1786.

35 **Apostolou, I., Sarukhan, A., Klein, L. and von Boehmer, H.,** Origin of regulatory T cells with known specificity for antigen. *Nat. Immunol.* 2002. **3**: 756–763.

36 **Haks, M. C., Lefebvre, J. M., Lauritsen, J. P., Carleton, M., Rhodes, M., Miyazaki, T., Kappes, D. J. and Wiest, D. L.,** Attenuation of γδ TCR signaling efficiently diverts thymocytes to the alphabeta lineage. *Immunity* 2005. **22**: 595–606.

37 **Hayes, S. M., Li, L. and Love, P. E.,** TCR signal strength influences lineage fate. *Immunity* 2005. **22**: 583–593.

38 **Jordan, M. S., Boesteanu, A., Reed, A. J., Petrone, A. L., Holenbeck, A. E., Lerman, M. A., Naji, A. and Caton, A. J.,** Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide. *Nat. Immunol.* 2001. **2**: 301–306.

39 **Pennington, D. J., Silva-Santos, B. and Hayday, A. C.,** γδ T cell development--having the strength to get there. *Curr. Opin. Immunol.* 2005. **17**: 108–115.

40 **Romagnoli, P., Hudrisier, D. and van Meerwijk, J. P.,** Preferential recognition of self antigens despite normal thymic deletion of CD4(+)CD25(+) regulatory T cells. *J. Immunol.* 2002. **168**: 1644–1648.

41 **Hara, M., Kingsley, C. I., Niimi, M., Read, S., Turvey, S. E., Bushell, A. R., Morris, P. J. *et al.*,** IL-10 is required for regulatory T cells to mediate tolerance to alloantigens in vivo. *J. Immunol.* 2001. **166**: 3789–3796.

42 **Li, M. O., Wan, Y. Y. and Flavell, R. A.,** T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation. *Immunity* 2007. **26**: 579–591.

43 **Collison, L. W., Workman, C. J., Kuo, T. T., Boyd, K., Wang, Y., Vignali, K. M., Cross, R. *et al.*,** The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* 2007. **450**: 566–569.

44 **Wing, K., Onishi, Y., Prieto-Martin, P., Yamaguchi, T., Miyara, M., Fehervari, Z., Nomura, T. and Sakaguchi, S.,** CTLA-4 control over $Foxp^{3+}$ regulatory T cell function. *Science* 2008. **322**: 271–275.

45 **Read, S., Greenwald, R., Izcue, A., Robinson, N., Mandelbrot, D., Francisco, L., Sharpe, A. H. and Powrie, F.,** Blockade of CTLA-4 on CD4+ CD25+ regulatory T cells abrogates their function in vivo. *J. Immunol.* 2006. **177**: 4376–4383.

46 **Ji, H. B., Liao, G., Faubion, W. A., Abadia-Molina, A. C., Cozzo, C., Laroux, F. S., Caton, A. and Terhorst, C.,** Cutting edge: the natural ligand for glucocorticoid-induced TNF receptor-related protein abrogates regulatory T cell suppression. *J. Immunol.* 2004. **172**: 5823–5827.

47 **McHugh, R. S., Whitters, M. J., Piccirillo, C. A., Young, D. A., Shevach, E. M., Collins, M. and Byrne, M. C.,** CD4(+)CD25(+) immunoregulatoryT cells: gene expression analysis reveals a functional role for the glucocorticoid-induced TNF receptor. *Immunity* 2002. **16**: 311–323.

48 **Shimizu, J., Yamazaki, S., Takahashi, T., Ishida, Y. and Sakaguchi, S.,** Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance. *Nat. Immunol.* 2002. **3**: 135–142.

49 **Nocentini, G. and Riccardi, C.,** GITR: a multifaceted regulator of immunity belonging to the tumor necrosis factor receptor superfamily. *Eur. J. Immunol.* 2005. **35**: 1016–1022.

50 **Tone, M., Tone, Y., Adams, E., Yates, S. F., Frewin, M. R., Cobbold, S. P. and Waldmann, H.,** Mouse glucocorticoid-induced tumor necrosis factor

receptor ligand is costimulatory for T cells. *Proc. Natl. Acad. Sci. USA* 2003. **100**: 15059–15064.

51 **Kanamaru, F., Youngnak, P., Hashiguchi, M., Nishioka, T., Takahashi, T., Sakaguchi, S., Ishikawa, I. and Azuma, M.,** Costimulation via glucocorticoid-induced TNF receptor in both conventional and CD25+ regulatory CD4+ T cells. *J. Immunol.* 2004. **172**: 7306–7314.

52 **van Olffen, R. W., Koning, N., van Gisbergen, K. P., Wensveen, F. M., Hoek, R. M., Boon, L., Hamann, J. *et al.*,** GITR triggering induces expansion of both effector and regulatory CD4+ T cells in vivo. *J. Immunol.* 2009. **182**: 7490–7500.

53 **Morita, C. T., Jin, C., Sarikonda, G. and Wang, H.,** Nonpeptide antigens, presentation mechanisms, and immunological memory of human Vγ2Vδ2 T cells: discriminating friend from foe through the recognition of prenyl pyrophosphate antigens. *Immunol. Rev.* 2007. **215**: 59–76.

54 **Bonneville, M. and Scotet, E.,** Human Vγ9Vδ2 T cells: promising new leads for immunotherapy of infections and tumors. *Curr. Opin. Immunol.* 2006. **18**: 539–546.

55 **Devilder, M. C., Maillet, S., Bouyge-Moreau, I., Donnadieu, E., Bonneville, M. and Scotet, E.,** Potentiation of antigen-stimulated Vγ9Vδ2 T cell cytokine production by immature dendritic cells (DC) and reciprocal effect on DC maturation. *J. Immunol.* 2006. **176**: 1386–1393.

56 **Brandes, M., Willimann, K. and Moser, B.,** Professional antigen-presentation function by human γδ T Cells. *Science* 2005. **309**: 264–268.

57 **Correia, D. V., d'Orey, F., Cardoso, B. A., Lanca, T., Grosso, A. R., deBarros, A., Martins, L. R. *et al.*,** Highly active microbial phosphoantigen induces rapid yet sustained MEK/Erk- and PI-3K/Akt-mediated signal transduction in anti-tumor human γδ T-cells. *PLoS One* 2009. **4**: e5657.

58 **Ali, Z., Shao, L., Halliday, L., Reichenberg, A., Hintz, M., Jomaa, H., Chen, Z. W.,** Prolonged (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate-driven antimicrobial and cytotoxic responses of pulmonary and systemic Vγ2Vδ2 T cells in macaques. *J. Immunol.* 2007. **179**: 8287–8296.

Abbreviations: **B6:** C57BL/6 mice · **GITR:** glucocorticoid-induced TNF receptor-related protein · **iTreg:** "inducible" Foxp3$^+$ Treg · **nTreg:** naturally occurring Treg · **Teff:** effector T cell

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Non-classical major histocompatibility complex proteins as determinants of tumour immunosurveillance

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Tumours develop in vertebrate organisms endowed with immune systems that are potentially able to eradicate them. Nevertheless, our ever-increasing understanding of the complex interactions between lymphocytes and tumour cells fuels the long-standing hope of developing efficient immunotherapies against cancer. This review focuses on a versatile family of proteins, the major histocompatibility complex class Ib, which has been recently implicated in both the establishment of anti-tumour immune responses and in tumour immune response evasion. We focus on a subset of class Ib proteins, human leukocyte antigen (HLA)-G, Qa-2, CD1d and NKG2D ligands, which bind to either stimulatory or inhibitory receptors expressed on T, natural killer (NK) and NKT lymphocytes, and thereby modulate their anti-tumour activity.

Keywords: MHC; cancer; innate; lymphocyte; natural killer

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Introduction

Paul Ehrlich's discoveries 100 years ago seduced immunologists with the idea that cytotoxic cells are able to eliminate tumours, much like they destroy virus-infected cells. In the 1950s, MacFarlane Burnet proposed that the immune system surveys cellular transformation and prevents the development of tumours (Burnet, 1957). This 'tumour surveillance hypothesis' has prompted the identification of a panoply of tumour-specific or tumour-enriched peptides over the past 20 years. These peptides are presumably the antigens presented by classical major histocompatibility complex (MHC) molecules to the cytotoxic T lymphocytes (CTL) of the adaptive immune system, particularly T-cell receptor (TCR) $\alpha\beta^+$ $CD8^+$ cells. Although their identification allowed the development of tumour vaccines—which are being assayed in clinical trials—the overall success of such immunotherapeutic strategies has been limited by the common downregulation of such antigens and/or their MHC class Ia presentation elements in advanced tumours. However, cancer immune surveillance is likely to involve both the adaptive and the innate immune systems, as is true for host immunity to pathogens. Indeed, many reports have illustrated the capacity of innate lymphocytes, γδ T, natural killer (NK) and NKT cells, to detect and destroy tumour cells independently of classical MHC presentation (Girardi *et al*, 2001, 2003; Smyth *et al*, 2001; Street *et al*, 2004).

The molecular mechanisms responsible for the immune recognition of tumour cells are still the subject of much debate. However, recent data has highlighted the many roles played by members of a plastic protein family: the non-classical or class Ib MHC (MHC Ib). MHC Ib molecules are structurally related to class Ia proteins because they show a typical (α1–α2) MHC fold on a single polypeptide, which, in the case of Ib, does not pair up obligatorily with β2-microglobulin (Rodgers & Cook, 2005). Furthermore, although many MHC Ib genes are also located in the MHC locus, they tend to be oligomorphic—few alleles exist in the population—which is in marked contrast with the extensive polymorphism of class Ia. The oligomorphism of class Ib might therefore be an advantage for the design of cancer therapies with a wider application within the cancer patient population. Amino-acid sequence identity/homology is not a useful criterion to establish relationships between class Ib members, as functional equivalents across species—for example, proteins that bind to a given receptor—are often not orthologous. Some class Ib genes are in fact more closely related to class Ia genes in evolutionary terms than to other class Ib members (Rodgers & Cook, 2005). MHC Ib proteins have been largely shown to bind to stimulatory or inhibitory receptors expressed on T, NK and/or NKT lymphocytes (Fig 1); there are, however, a few exceptions such as the haemochromatosis antigen (HFE) or the neonatal Fc receptor (FcRn).

Here, we focus on the ability of some MHC Ib proteins to act as 'reporters' of cellular transformation and trigger anti-tumour immune responses. We have selected three examples to illustrate the various mechanisms by which MHC Ib can activate or inhibit anti-tumour lymphocytes.

Qa-2 and HLA-G present peptides to $CD8^+$ T cells

Murine Qa-2 and human leukocyte antigen (HLA)-G are 'young' MHC Ib proteins in that they diverged from MHC Ia less than 20

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million years ago, and therefore have many similarities with the classical Ia molecules: they present a repertoire of nonameric peptides, contain a CD8 binding loop, associate with β2-microglobulin (β2m), display similar exon–intron organization and even share a high degree of amino-acid sequence homology (Seliger *et al*, 2003).

The murine Qa-2 region, located in the H2-Q class Ib locus, encodes the proteins Q8 and Q9 in C57BL/6 (B6) mice, which differ from each other by 20 amino acids located in the α1 and α2 domains. These proteins have overlapping peptide-binding motifs and are recognized by cross-reactive anti-tumour CTLs, and therefore appear to be functionally equivalent (Chiang & Stroynowski, 2006). Surface expression of Qa-2 molecules requires a functional TAP (transporter associated with antigen processing) peptide transporter (Tabaczewski *et al*, 1994) and is dependent on ERAP1 (endoplasmic-reticulum-associated peptidase 1) processing, suggesting that peptides loaded onto Qa-2 molecules are generated through the conventional class Ia antigen-processing pathway (Yan *et al*, 2006). Qa-2 transcripts are widely expressed at low levels in healthy tissues, both in haematopoietic and in non-haematopoietic cell types (Ungchusri *et al*, 2001). However, Q9 expression was found to be lost or severely reduced in a large panel of *in vivo*-derived tumour cell lines, including cells derived from T- and B-cell lymphoma, mastocytoma, melanoma and hepatoma (Ungchusri *et al*, 2001). Serological and reverse-transcriptase-PCR (RT-PCR) analyses have also shown that the primary B16 melanoma tumour cell line and all its variants had no Q8 and Q9 expression, whereas the control melanocyte line was positive for these antigens (Chiang *et al*, 2003). These results suggest that Q8 and Q9 expression is silenced early during tumour progression.

Several *in vitro* and *in vivo* studies have nonetheless shown Q9 involvement in tumour rejection. Syngeneic B6 mice injected with the B78H1 melanoma cell line—selectively devoid of TAP2 and class Ia (Kb and Db) transcripts and genetically manipulated to re-express Q9 on its surface—were protected from melanoma outgrowth, unlike mice challenged with class Ia-negative B78H1 or with TAP2-transfected parental melanoma cells (Chiang *et al*, 2003). Subsequent studies have shown $CD8^+$ CTL involvement in Q9-mediated protection from melanoma development, as protection is lost in CD8 knockout and severe combined immunodeficiency (SCID) mice, but not in CD4 knockouts (Chiang & Stroynowski, 2004). Furthermore, it was demonstrated that mice surviving the original challenge became resistant to subsequent doses of Q9-positive melanoma, suggesting that Qa-2 acted as a restriction element for anti-tumour $CD8^+$ T cells, and that both effector and memory cells were generated in immunized mice (Chiang & Stroynowski, 2004). Q9-restricted CTL responses able to recognize lung carcinoma and T-cell lymphoma have also been observed (Chiang & Stroynowski, 2005). In addition, Q9 was shown to act as a restriction element for a tumour antigen common to these tumours and melanoma. It was observed that CTL clones raised in response to a challenge with Q9-expressing 3LLA9F1 lung carcinoma, RMA T-cell lymphoma or GMQ9TAP B78H1 melanoma efficiently killed all of these tumours in cytotoxicity assays in a Q9-restricted manner. This also suggests that CTLs generated in the primary response establish a pool of memory cells that exhibit cross-reactivity against various tumours (Chiang & Stroynowski, 2005). Qa-2 proteins are also recognized by NK lymphocytes, and these have been shown to be essential for the rejection of large doses of Q9-positive melanoma cells even in the presence of a CTL response (Chiang *et al*, 2003). Therefore, it will be important to analyse the interplay between $CD8^+$ T and NK cells in Qa-2-dependent anti-tumour responses.

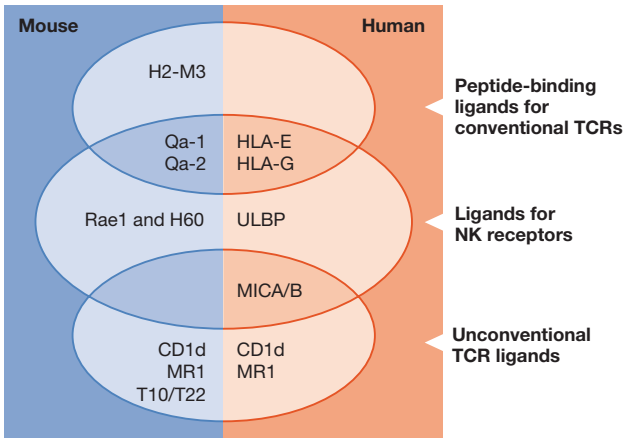

Fig 1 | Major histocompatibility complex class Ib proteins bind various immunoreceptors. Listed are major histocompatibility complex class Ib (MHC Ib) molecules for which receptor binding has been well characterized. Conventional T-cell receptors (TCRs) are those of polyclonal αβ T cells; unconventional TCRs correspond to oligoclonal T-cell subsets, such as natural killer (NK) T cells (for CD1d), γδ T cells (T10/T22, MICA/B) or gut-associated T cells (MR1). Proposed functional homologues between mice and humans are in the same row. H60, histocompatibility antigen 60; HLA, human leukocyte antigen; MICA/B, MHC class I chain-related peptides A/B; Rae1, retinoic acid early inducible gene 1; ULBP, UL16 binding protein.

Although there is no human orthologue of Qa-2, some have suggested HLA-G is its functional homologue as both proteins have immunoregulatory roles, are involved in embryonic development, and exist in membrane-bound and soluble forms that arise by alternative splicing (Comiskey *et al*, 2003). HLA-G expression is augmented in various tumours, including melanomas, breast, renal, ovarian and lung carcinomas, gliomas, B and T non-Hodgkin lymphomas, acute leukaemias and colorectal cancers (Rouas-Freiss *et al*, 2005). However, it is important to note that HLA-G expression is not detected in all tumours; for example, HLA-G expression has been reported in ocular tumours such as retinoblastoma, whereas it has not been detected in uveal melanomas, even after treatment with interferon-γ (IFN-γ; Hurks *et al*, 2001). *HLA-G* expression is highly inducible and cytokines, such as interleukin (IL)-10, leukaemia inhibitory factor (LIF), tumour necrosis factor-α (TNF-α) and IFN-γ, differentially regulate its transcription in several tumour cell lines (Carosella & Dausset, 2003). Stress-inducing factors, such as heat shock and arsenite treatment, also induce *HLA-G* transcription in tumour cells (Ibrahim *et al*, 2000). Furthermore, it was recently shown that *HLA-G* expression is regulated by hypoxia in a hypoxia-inducible factor 1 (HIF1)-dependent manner (Mouillot *et al*, 2007). HLA-G1 protein expression can also be controlled at the post-translational level because its levels on the cell surface of carcinoma and melanoma cells are reduced upon activation of the NF-κB signalling pathway (Zidi *et al*, 2006). Unlike HLA class I genes, *HLA-G* expression is controlled by *cis*-acting epigenetic

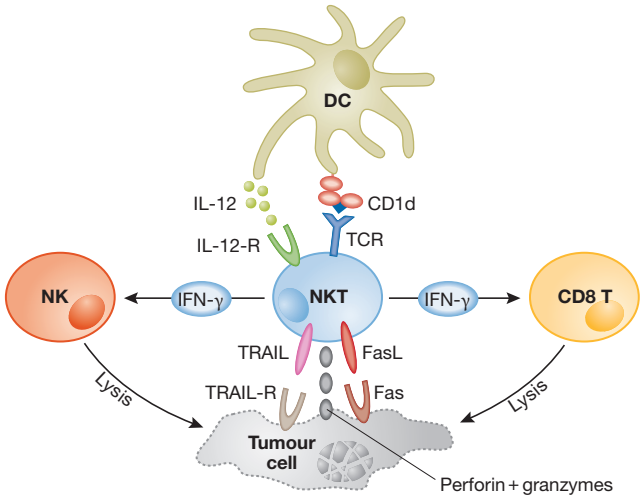

Fig 2 | Anti-tumour responses mediated by CD1d-activated type I natural killer T cells. After receiving activation signals from dendritic cells (DCs)—through CD1d and IL-12—NKT cells can either lyse tumour cells directly using the perforin/granzyme system or ligands for death receptors, or stimulate other cytotoxic cells such as NK and CD8$^+$ T cells through IFN-γ secretion. IFN-γ, interferon-γ; IL-12, interleukin 12; NK, natural killer; NKT, natural killer T cell; TCR, T-cell receptor; TRAIL, tumour necrosis factor-related apoptosis inducing ligand.

mechanisms, such as DNA methylation/demethylation and histone deacetylation/acetylation (Chang *et al*, 2003; Guillaudeux *et al*, 1995; Mouillot *et al*, 2005). For example, when cells of HLA-G positive melanoma tumours are subject to long-term *in vitro* culture, their HLA-G expression levels decrease over time, correlating with methylation of the *HLA-G* promoter. This suggests that lack of exposure to tumour microenvironmental conditions might lead to silencing of the gene (Chang *et al*, 2003).

Qa-2 expression in murine tumours makes them more susceptible to CTL-mediated lysis, whereas the aberrant expression of HLA-G antigens by human tumour cells seems to constitute an immune evasion mechanism. This would be analogous with their function in trophoblasts, which is to protect the fetus from maternal immune attack (Carosella *et al*, 2003). Indeed, several lines of evidence have shown that HLA-G can inhibit anti-tumour lymphocytes (Rouas-Freiss *et al*, 2005). First, HLA-G molecules were shown to interact directly with inhibitory receptors present on the surface of T (ILT-2) and NK cells (KIR2DL4, ILT-2), as well as monocytes/macrophages and dendritic cells (ILT-2 and ILT-4). Second, HLA-G molecules stabilize the cell surface expression of other non-classical HLA molecules such as HLA-E, which convey additional suppressive signals to lymphocytes through the widely expressed CD94/NKG2A killing inhibitory receptor. Third, HLA-G1 ligation decreases the secretion of IFN-γ and TNF-α, which are crucial for anti-tumour immunity (Rouas-Freiss *et al*, 2005). Fourth, antigen-presenting cells (APCs) that express HLA-G1 were shown to induce CD4$^+$ T-cell anergy and to trigger their differentiation into suppressor cells that block CTL function (LeMaoult *et al*, 2004). This effect could either be a consequence of the interaction of HLA-G with an unknown receptor expressed by CD4$^+$ T cells, or an inhibitory effect of HLA-G on the APCs themselves. The HLA-G$^+$ APCs present in cancer patients might thus be partly responsible for tumour immune escape owing to their suppressive properties. Furthermore, soluble HLA-G5 has been directly implicated in the inhibition of CD4$^+$ and CD8$^+$ T-cell proliferation. It causes cell cycle arrest by altering the balance between inhibitory molecules and cyclins, inducing the accumulation of $p27^{kip}$ and the reduction of cyclins D2, E, A and B (Bahri *et al*, 2006). Recently, it has also been shown that HLA-G molecules are transferred from tumour cells to activated NK cells by trogocytosis (Caumartin *et al*, 2007), which consists of the rapid transfer of intact cell-surface proteins between cells in contact with each other. The acquisition of HLA-G1 by NK cells blocks their proliferation and cytolytic function owing to its interaction with ILT2 on other NK cells and therefore induces a temporary state of immunosuppression in the NK-cell population (Caumartin *et al*, 2007). Consistent with its immunoevasive role, HLA-G expression in some malignancies, such as colorectal cancers, correlates significantly with increased depth of invasion, histological grade, lymph nodal metastasis and clinical stages of the disease, suggesting that its presence could act as an independent prognostic factor for cancer patients (Ye *et al*, 2007).

Despite the vast amount of data, the role of HLA-G as an inhibitory ligand for anti-tumour lymphocytes remains controversial. For example, the main NK cell receptor for HLA-G, KIR2DL4, can also function as an activating receptor. Indeed, KIR2DL4 engagement has been reported either to inhibit cytotoxicity (Ponte *et al*, 1999) or to promote cytotoxicity and IFN-γ secretion (Kikuchi-Maki *et al*, 2003; Rajagopalan *et al*, 2001). Therefore, the elucidation of the functional consequences of HLA-G ligation, as well as the specific roles of membrane-bound and soluble forms of the protein (Morandi *et al*, 2007), require further investigation.

CD1d presents lipids to NKT cells

CD1d, a conserved member of the small CD1 protein family, presents lipid/glycolipid—instead of peptidic—antigens to TCR αβ complexes expressed by anti-tumour NKT cells in mice and humans (Godfrey *et al*, 2004). The dependence on CD1d for development and activation is the defining characteristic of NKT cells, which also express NK-cell-associated surface markers. NKT cell subsets can be further characterized by their TCR repertoire: type I NKT cells express an invariant TCR (using the Vα14Jα18 gene segments in mice and the Vα24 segment in humans), whereas type II cells display a more diverse repertoire (Godfrey *et al*, 2004). Although NKT cells are endowed with cytolytic factors that eliminate tumour cells such as perforin, Fas-ligand and TRAIL (tumour necrosis factor-related apoptosis inducing ligand) (Kawano *et al*, 1998), they also secrete large amounts of IFN-γ, which, in turn, stimulates anti-tumour NK and CD8$^+$ CTLs (Smyth *et al*, 2002; Fig 2).

CD1d can present various ligands to NKT cells (Zhou *et al*, 2004; Kinjo *et al*, 2005; Mattner *et al*, 2005), including tumour-derived lipids and glycolipids (Brutkiewicz, 2006). For example, the disialoganglioside GD3 is strongly upregulated in some tumours such as melanomas and can be cross-presented by CD1d-expressing murine APCs to NKT cells (Wu *et al*, 2003). Furthermore, some glycolipid fractions of tumour cell membranes are presented by CD1d and activate NKT cells (Gumperz *et al*, 2000). However, it has also been shown that glycolipid shedding by tumour cells can inhibit CD1d-mediated recognition of the tumour target (Sriram *et al*, 2002). Although the repertoire of transformation-induced glycolipids

Table 1 | mRNA/protein expression patterns for NKG2D ligands

NKG2D ligand	Basal	Augmented*
MICA/B (human)	Protein in gastrointestinal epithelium, endothelial cells, fibroblasts; mRNA in keratinocytes Soluble protein in placenta	Protein in melanoma, myeloma, lymphoma, and colon, breast, lung, liver, ovary, kidney and cervical carcinomas Soluble protein in gastrointestinal and prostate cancers, lymphomas and neuroblastoma
ULBP (human)	ULBP1, 2, 3 mRNA in heart, brain, lung, liver, thymus, testis, lymph nodes, tonsils and bone marrow ULBP4 mRNA mainly in skin and small intestine	Both mRNA and surface protein in melanoma, various leukaemias and carcinomas (stomach, colon, cervical and ovarian carcinomas for ULBP2 and ULBP3); ULBP1 and ULBP2 in T-cell lymphomas
Rae1 (mouse)	mRNA in embryonic tissues mRNA absent (or very rare) in healthy adult tissues	mRNA and surface protein in lymphomas, lung, prostate and cutaneous carcinomas (variable)
H60 (BALB/c mouse)	mRNA in embryonic tissues, adult thymus and spleen, but generally absent in healthy adult tissues	Both mRNA and surface protein in lymphomas, prostate and cutaneous carcinomas (variable)
Mult1 (mouse)	mRNA in thymus, spleen, lymph nodes, liver, gut, heart and lung	mRNA in T-cell lymphomas and sarcomas

*'Augmented' refers to transcription induction or increased surface protein expression, relative to 'basal' levels present in healthy tissues (Raulet, 2003, and references therein).
H60, histocompatibility antigen 60; MICA/B, MHC class I chain-related peptide A/B; MULT1, murine UL16-binding protein-like transcript 1; Rae1, retinoic acid early inducible gene 1; ULBP, UL16-binding protein.

presented by CD1d remains to be characterized, α-galactosylceramide (α-GalCer), which is extracted from the marine sponge *Agelas mauritianus*, is the most potent stimulator of NKT cells known so far and has proven to be effective against a wide range of tumours. α-GalCer protects against the development of primary sarcomas induced chemically or owing to a lack of genetic tumour suppression (Hayakawa *et al*, 2003), as well as against metastasis of multiple tumour cell lines: B16 (melanoma), EL4 (thymoma), Colon-26 (colon adenocarcinoma), 3LL (lung carcinoma), RM-1 (prostate carcinoma) and DA3 (mammary carcinoma) (Swann *et al*, 2004). In the absence of CD1d—as in CD1d$^{-/-}$ mice—methylcholanthrene-induced sarcomas developed faster and at a higher frequency than in wild-type mice (Crowe *et al*, 2002). Similar results were obtained using Jα18$^{-/-}$ mice (Smyth *et al*, 2000; Crowe *et al*, 2002), which selectively lack type I NKT cells, suggesting that these invariant lymphocytes are the crucial subset responsible for tumour suppression. Not unexpectedly, the transfer of wild-type type I NKT cells into Jα18$^{-/-}$ mice resulted in protection from tumour development. Murine NKT lymphocytes have been recently characterized as strikingly heterogeneous in their response to tumours; type I NKT lymphocytes eliminate CD1d^{+} cells, whereas type II NKT actually suppress tumour immunity in several mouse tumour models (Terabe *et al*, 2005). It has been shown that downregulation of tumour immunity by NKT cells might be accomplished through an IL-13 and TGF-β-dependent mechanism that shuts down anti-tumour CD8^{+} T lymphocytes (Terabe *et al*, 2003). Another group has also reported differential anti-tumour effects by subdividing NKT cells according to their tissue origin—thymus or liver—and CD4 expression (Crowe *et al*, 2005). Future research will therefore attempt to manipulate the balance between distinct NKT cell subsets towards the promotion of anti-tumour immunity.

Several studies of cancer patients suffering from a wide range of tumours have shown decreased numbers of NKT cells in the peripheral blood, as compared with healthy volunteers (Swann *et al*, 2004). Furthermore, deficient NKT-cell production of IFN-γ has been associated with the progression from pre-malignant to malignant multiple myeloma, and the NKT dysfunction could be corrected *in vitro* by culturing the NKT cells with α-GalCer-pulsed dendritic cells (Dhodapkar *et al*, 2003). After the disappointing outcome of the first clinical trial with soluble α-GalCer in patients with solid tumours (Giaccone *et al*, 2002), the use of α-GalCer-loaded dendritic cells has produced promising pre-clinical data in mice (Fujii *et al*, 2002) and in phase I human clinical trials (Nieda *et al*, 2004; Ishikawa *et al*, 2005); phase II trials are currently under way.

NKG2D ligands directly activate anti-tumour lymphocytes

The immunoreceptor NKG2D provides important stimulatory signals to NK and T cells (Bauer *et al*, 1999). In humans, NKG2D is constitutively expressed on the cell surface of NK, CD8^{+} T and γδ T lymphocytes, but is absent from CD4^{+} T cells and monocytes (Raulet, 2003). Several ligands for both the human and mouse NKG2D have been identified and, surprisingly, there is low amino-acid sequence homology between them. Human NKG2D binds to MHC I chain-related (MIC) peptides A and B (MICA and MICB), and to UL16-binding proteins (ULBP, members 1–4). Mouse NKG2D binds to retinoic acid early inducible gene 1 (Rae1), histocompatibility antigen 60 (H60), and murine UL16-binding protein-like transcript 1 (MULT1). MICA/B, H60 and MULT1 are transmembrane proteins, whereas ULBP1–4 and Rae1 localize to the cell surface using glycosylphosphatidylinositol (GPI) linkages. None of the NKG2D ligands bind to additional—peptide or lipid—antigens but rather interact directly with the receptor. In addition, in contrast to the MHC Ib molecules described above, NKG2D ligands do not associate with β2-microglobulin (Raulet, 2003). For some authors, the unorthodoxy of NKG2D ligands prompts their formal designation as 'MHC class I-related' instead of MHC Ib (Sullivan *et al*, 2006).

The murine ligands Rae1 and H60 are rare in healthy adult tissues, but their transcription is strongly induced in keratinocytes after their *in vivo* exposure to carcinogens (Girardi *et al*, 2001), and they are overexpressed in the cutaneous papillomas and carcinomas that subsequently develop, as well as in other tumours (Table 1). The expression of Rae1 or H60 by target cells was shown

to enhance cytolysis and the production of IFN-γ by anti-tumour CTLs (Diefenbach *et al*, 2000) and γδ T lymphocytes (Girardi *et al*, 2001), leading to tumour rejection *in vivo*. Furthermore, transfection of *rae1* or *h60* or *mult1* into NK-cell-resistant target cells made them susceptible to NK-cell-mediated killing and stimulated IFN-γ secretion by NK cells (Carayannopoulos *et al*, 2002; Cerwenka *et al*, 2000; Diefenbach *et al*, 2001). However, recent data suggest that IFN-γ can paradoxically downregulate H60 expression in tumours (Bui *et al*, 2006). Future experiments should try to reconcile these findings and elucidate the dynamics of H60 during tumour development *in vivo*.

The human MICA and MICB proteins—which are 91% identical at the amino-acid level—show a restricted and low expression in healthy tissues, but are strongly induced by cellular stress (including heat shock) and transformation, and accumulate in various tumour cell lines, particularly those of epithelial origin (Table 1). Atypically for MHC Ib molecules, the *MIC* genes are highly polymorphic: there are 61 *MICA* and 30 *MICB* alleles (Raulet, 2003). Although MICA/B was suggested to bind not only to NKG2D, but also to the Vγ1$^+$ TCR expressed by human intraepithelial γδ T lymphocytes (Wu *et al*, 2002), this claim is highly controversial. In fact, the identity of the majority of γδ TCR ligands remains an unresolved biological issue in which MHC Ib proteins are expected to have a crucial role (Steele *et al*, 2000; Sullivan *et al*, 2006; Thedrez *et al*, 2007). The membrane-bound form of MICA provides stimulatory signals to killer lymphocytes, whereas a soluble version (sMICA), shed from the cell surface by matrix metalloproteinases, systemically downregulates surface NKG2D and impairs tumour cytolysis mediated by T and NK cells, therefore constituting an important immune evasion mechanism for tumours (Groh *et al*, 2002; Salih *et al*, 2002).

Distantly related to the MIC proteins are the members of the ULBP family (23–26% amino-acid identity). In contrast to Rae1 or MICA, ULBPs are expressed at significant levels in a wide range of healthy tissues and cell lines of both epithelial and nonepithelial origin (Table 1; Cosman *et al*, 2001). Ectopic expression of ULBP1 or ULBP2 on murine EL4 or RMA tumour cells elicits potent anti-tumour responses in syngeneic B6 and SCID mice, recruiting NK, NKT and T cells to the tumour (Sutherland *et al*, 2006). Similarly, tumour cells that are insensitive to NK cells can be lysed effectively when transfected with ULBPs (Kubin *et al*, 2001).

Conclusion

In summary, NKG2D recognition of target tumour cells constitutes a potent anti-tumour mechanism, but its clinical application depends largely on the control and manipulation of the expression of NKG2D ligands. Therefore, the mechanisms controlling their specific regulatory signals need to be elucidated.

The immune system 'sculpts' tumours by selecting those that, owing to their reduced immunogenicity, escape its recognition/destruction. This process of elimination versus escape creates a dynamic relationship that has been termed 'cancer immunoediting' (Dunn *et al*, 2002). Non-classical MHC proteins can function as indicators of cellular transformation to anti-tumour lymphocytes, either directly or through the presentation of endogenous antigens that are (over-)expressed in tumours. Therefore, silencing MHC Ib expression might be a major immune evasion mechanism used by tumours, as described for Qa-2 during melanoma progression (Chiang *et al*, 2003). Another immune evasion strategy that involves MHC Ib proteins consists on the generation of soluble protein versions that block or internalize the corresponding receptors on lymphocytes, as mentioned for MICA (Groh *et al*, 2002; Salih *et al*, 2002). Similarly, the sustained surface expression of murine Rae1 has also been shown to have an inhibitory effect on NKG2D-expressing anti-tumour lymphocytes (Ogasawara *et al*, 2003; Oppenheim *et al*, 2005). Furthermore, CD1d-restricted type II NKT cells downregulate anti-tumour immunity (Terabe *et al*, 2005), whereas type I NKT cells promote tumour elimination. Such behaviour highlights the importance of determining the specific signals delivered by MHC Ib proteins to manipulate them towards protection. We believe that a better understanding of the underlying regulatory mechanisms of MHC Ib will make it possible to engineer vaccination approaches against various cancers that take advantage of the oligomorphism of most MHC Ib molecules.

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REFERENCES

Bahri R, Hirsch F, Josse A, Rouas-Freiss N, Bidere N, Vasquez A, Carosella ED, Charpentier B, Durrbach A (2006) Soluble HLA-G inhibits cell cycle progression in human alloreactive T lymphocytes. *J Immunol* **176:** 1331–1339

Bauer S, Groh V, Wu J, Steinle A, Phillips JH, Lanier LL, Spies T (1999) Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. *Science* **285:** 727–729

Brutkiewicz RR (2006) CD1d ligands: the good, the bad, and the ugly. *J Immunol* **177:** 769–775

Bui JD, Carayannopoulos LN, Lanier LL, Yokoyama WM, Schreiber RD (2006) IFN-dependent down-regulation of the NKG2D ligand H60 on tumours. *J Immunol* **176:** 905–913

Burnet M (1957) Cancer: a biological approach. III. Viruses associated with neoplastic conditions. IV. Practical applications. *Br Med J* **1:** 841–847

Carayannopoulos LN, Naidenko OV, Fremont DH, Yokoyama WM (2002) Cutting edge: murine UL16-binding protein-like transcript 1: a newly described transcript encoding a high-affinity ligand for murine NKG2D. *J Immunol* **169:** 4079–4083

Carosella ED, Dausset J (2003) Progress of HLA-G in cancer. *Semin Cancer Biol* **13:** 315–316

Carosella ED, Moreau P, Le Maoult J, Le Discorde M, Dausset J, Rouas-Freiss N (2003) HLA-G molecules: from maternal–fetal tolerance to tissue acceptance. *Adv Immunol* **81:** 199–252

Caumartin J, Favier B, Daouya M, Guillard C, Moreau P, Carosella ED, LeMaoult J (2007) Trogocytosis-based generation of suppressive NK cells. *EMBO J* **26:** 1423–1433

Cerwenka A, Bakker AB, McClanahan T, Wagner J, Wu J, Phillips JH, Lanier LL (2000) Retinoic acid early inducible genes define a ligand family for the activating NKG2D receptor in mice. *Immunity* **12:** 721–727

Chang CC, Murphy SP, Ferrone S (2003) Differential *in vivo* and *in vitro* HLA-G expression in melanoma cells: potential mechanisms. *Hum Immunol* **64:** 1057–1063

Chiang EY, Stroynowski I (2004) A nonclassical MHC class I molecule restricts CTL-mediated rejection of a syngeneic melanoma tumour. *J Immunol* **173:** 4394–4401

Chiang EY, Stroynowski I (2005) Protective immunity against disparate tumours is mediated by a nonpolymorphic MHC class I molecule. *J Immunol* **174:** 5367–5374

Chiang EY, Stroynowski I (2006) The role of structurally conserved class I MHC in tumour rejection: contribution of the Q8 locus. *J Immunol* **177:** 2123–2130

Chiang EY, Henson M, Stroynowski I (2003) Correction of defects responsible for impaired Qa-2 class Ib MHC expression on melanoma cells protects mice from tumour growth. *J Immunol* **170:** 4515–4523

Comiskey M, Goldstein CY, De Fazio SR, Mammolenti M, Newmark JA, Warner CM (2003) Evidence that HLA-G is the functional homolog of mouse Qa-2, the *Ped* gene product. *Hum Immunol* **64:** 999–1004

Cosman D, Mullberg J, Sutherland CL, Chin W, Armitage R, Fanslow W, Kubin M, Chalupny NJ (2001) ULBPs, novel MHC class I-related molecules, bind to CMV glycoprotein UL16 and stimulate NK cytotoxicity through the NKG2D receptor. *Immunity* **14:** 123–133

Crowe NY, Smyth MJ, Godfrey DI (2002) A critical role for natural killer T cells in immunosurveillance of methylcholanthrene-induced sarcomas. *J Exp Med* **196:** 119–127

Crowe NY, Coquet JM, Berzins SP, Kyparissoudis K, Keating R, Pellicci DG, Hayakawa Y, Godfrey DI, Smyth MJ (2005) Differential antitumour immunity mediated by NKT cell subsets *in vivo*. *J Exp Med* **202:** 1279–1288

Dhodapkar MV, Krasovsky J, Osman K, Geller MD (2003) Vigorous premalignancy-specific effector T cell response in the bone marrow of patients with monoclonal gammopathy. *J Exp Med* **198:** 1753–1757

Diefenbach A, Jamieson AM, Liu SD, Shastri N, Raulet DH (2000) Ligands for the murine NKG2D receptor: expression by tumour cells and activation of NK cells and macrophages. *Nat Immunol* **1:** 119–126

Diefenbach A, Jensen ER, Jamieson AM, Raulet DH (2001) Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature* **413:** 165–171

Dunn GP, Bruce AT, Ikeda H, Old LJ, Schreiber RD (2002) Cancer immunoediting: from immunosurveillance to tumour escape. *Nat Immunol* **3:** 991–998

Fujii S, Shimizu K, Kronenberg M, Steinman RM (2002) Prolonged IFN-γ-producing NKT response induced with α-galactosylceramide-loaded DCs. *Nat Immunol* **3:** 867–874

Giaccone G *et al* (2002) A phase I study of the natural killer T-cell ligand α-galactosylceramide (KRN7000) in patients with solid tumours. *Clin Cancer Res* **8:** 3702–3709

Girardi M, Oppenheim DE, Steele CR, Lewis JM, Glusac E, Filler R, Hobby P, Sutton B, Tigelaar RE, Hayday AC (2001) Regulation of cutaneous malignancy by γδ T cells. *Science* **294:** 605–609

Girardi M, Glusac E, Filler RB, Roberts SJ, Propperova I, Lewis J, Tigelaar RE, Hayday AC (2003) The distinct contributions of murine T cell receptor (TCR)γδ$^+$ and TCRαβ$^+$ T cells to different stages of chemically induced skin cancer. *J Exp Med* **198:** 747–755

Godfrey DI, MacDonald HR, Kronenberg M, Smyth MJ, Van Kaer L (2004) NKT cells: what's in a name? *Nat Rev Immunol* **4:** 231–237

Groh V, Wu J, Yee C, Spies T (2002) Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature* **419:** 734–738

Guillaudeux T, Rodriguez AM, Girr M, Mallet V, Ellis SA, Sargent IL, Fauchet R, Alsat E, Le Bouteiller P (1995) Methylation status and transcriptional expression of the MHC class I loci in human trophoblast cells from term placenta. *J Immunol* **154:** 3283–3299

Gumperz JE, Roy C, Makowska A, Lum D, Sugita M, Podrebarac T, Koezuka Y, Porcelli SA, Cardell S, Brenner MB, Behar SM (2000) Murine CD1d-restricted T cell recognition of cellular lipids. *Immunity* **12:** 211–221

Hayakawa Y, Rovero S, Forni G, Smyth MJ (2003) α-Galactosylceramide (KRN7000) suppression of chemical- and oncogene-dependent carcinogenesis. *Proc Natl Acad Sci USA* **100:** 9464–9469

Hurks HM, Valter MM, Wilson L, Hilgert I, van den Elsen PJ, Jager MJ (2001) Uveal melanoma: no expression of HLA-G. *Invest Ophthalmol Vis Sci* **42:** 3081–3084

Ibrahim EC, Morange M, Dausset J, Carosella ED, Paul P (2000) Heat shock and arsenite induce expression of the nonclassical class I histocompatibility HLA-G gene in tumour cell lines. *Cell Stress Chaperones* **5:** 207–218

Ishikawa A, Motohashi S, Ishikawa E, Fuchida H, Higashino K, Otsuji M, Iizasa T, Nakayama T, Taniguchi M, Fujisawa T (2005) A phase I study of α-galactosylceramide (KRN7000)-pulsed dendritic cells in patients with advanced and recurrent non-small cell lung cancer. *Clin Cancer Res* **11:** 1910–1917

Kawano T *et al* (1998) Natural killer-like nonspecific tumour cell lysis mediated by specific ligand-activated Vα14] NKT cells. *Proc Natl Acad Sci USA* **95:** 5690–5693

Kikuchi-Maki A, Yusa S, Catina TL, Campbell KS (2003) KIR2DL4 is an IL-2-regulated NK cell receptor that exhibits limited expression in humans but triggers strong IFN-γ production. *J Immunol* **171:** 3415–3425

Kinjo Y, Wu D, Kim G, Xing GW, Poles MA, Ho DD, Tsuji M, Kawahara K, Wong CH, Kronenberg M (2005) Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature* **434:** 520–525

Kubin M, Cassiano L, Chalupny J, Chin W, Cosman D, Fanslow W, Mullberg J, Rousseau AM, Ulrich D, Armitage R (2001) ULBP1, 2, 3: novel MHC class I-related molecules that bind to human cytomegalovirus glycoprotein UL16, activate NK cells. *Eur J Immunol* **31:** 1428–1437

LeMaoult J, Krawice-Radanne I, Dausset J, Carosella ED (2004) HLA-G1-expressing antigen-presenting cells induce immunosuppressive CD4$^+$ T cells. *Proc Natl Acad Sci USA* **101:** 7064–7069

Mattner J *et al* (2005) Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* **434:** 525–529

Morandi F, Levreri I, Bocca P, Galleni B, Raffaghello L, Ferrone S, Prigione I, Pistoia V (2007) Human neuroblastoma cells trigger an immunosuppressive program in monocytes by stimulating soluble HLA-G release. *Cancer Res* **67:** 6433–6441

Mouillot G, Marcou C, Rousseau P, Rouas-Freiss N, Carosella ED, Moreau P (2005) HLA-G gene activation in tumour cells involves *cis*-acting epigenetic changes. *Int J Cancer* **113:** 928–936

Mouillot G, Marcou C, Zidi I, Guillard C, Sangrouber D, Carosella ED, Moreau P (2007) Hypoxia modulates HLA-G gene expression in tumour cells. *Hum Immunol* **68:** 277–285

Nieda M, Okai M, Tazbirkova A, Lin H, Yamaura A, Ide K, Abraham R, Juji T, Macfarlane DJ, Nicol AJ (2004) Therapeutic activation of Vα24$^+$Vβ11$^+$ NKT cells in human subjects results in highly coordinated secondary activation of acquired and innate immunity. *Blood* **103:** 383–389

Ogasawara K, Hamerman JA, Hsin H, Chikuma S, Bour-Jordan H, Chen T, Pertel T, Carnaud C, Bluestone JA, Lanier LL (2003) Impairment of NK cell function by NKG2D modulation in NOD mice. *Immunity* **18:** 41–51

Oppenheim DE, Roberts SJ, Clarke SL, Filler R, Lewis JM, Tigelaar RE, Girardi M, Hayday AC (2005) Sustained localized expression of ligand for the activating NKG2D receptor impairs natural cytotoxicity *in vivo* and reduces tumour immunosurveillance. *Nat Immunol* **6:** 928–937

Ponte M, Cantoni C, Biassoni R, Tradori-Cappai A, Bentivoglio G, Vitale C, Bertone S, Moretta A, Moretta L, Mingari MC (1999) Inhibitory receptors sensing HLA-G1 molecules in pregnancy: decidua-associated natural killer cells express LIR-1 and CD94/NKG2A and acquire p49, an HLA-G1-specific receptor. *Proc Natl Acad Sci USA* **96:** 5674–5679

Rajagopalan S, Fu J, Long EO (2001) Cutting edge: induction of IFN-γ production but not cytotoxicity by the killer cell Ig-like receptor KIR2DL4 (CD158d) in resting NK cells. *J Immunol* **167:** 1877–1881

Raulet DH (2003) Roles of the NKG2D immunoreceptor and its ligands. *Nat Rev Immunol* **3:** 781–790

Rodgers JR, Cook RG (2005) MHC class Ib molecules bridge innate and acquired immunity. *Nat Rev Immunol* **5:** 459–471

Rouas-Freiss N, Moreau P, Ferrone S, Carosella ED (2005) HLA-G proteins in cancer: do they provide tumour cells with an escape mechanism? *Cancer Res* **65:** 10139–10144

Salih HR, Rammensee HG, Steinle A (2002) Cutting edge: down-regulation of MICA on human tumours by proteolytic shedding. *J Immunol* **169:** 4098–4102

Seliger B, Abken H, Ferrone S (2003) HLA-G and MIC expression in tumours and their role in anti-tumour immunity. *Trends Immunol* **24:** 82–87

Smyth MJ, Thia KY, Street SE, Cretney E, Trapani JA, Taniguchi M, Kawano T, Pelikan SB, Crowe NY, Godfrey DI (2000) Differential tumour surveillance by natural killer (NK) and NKT cells. *J Exp Med* **191:** 661–668

Smyth MJ, Crowe NY, Godfrey DI (2001) NK cells and NKT cells collaborate in host protection from methylcholanthrene-induced fibrosarcoma. *Int Immunol* **13:** 459–463

Smyth MJ, Crowe NY, Pellicci DG, Kyparissoudis K, Kelly JM, Takeda K, Yagita H, Godfrey DI (2002) Sequential production of interferon-γ by NK1.1$^+$ T cells and natural killer cells is essential for the antimetastatic effect of α-galactosylceramide. *Blood* **99:** 1259–1266

Sriram V, Cho S, Li P, O'Donnell PW, Dunn C, Hayakawa K, Blum JS, Brutkiewicz RR (2002) Inhibition of glycolipid shedding rescues recognition of a CD1$^+$ T cell lymphoma by natural killer T (NKT) cells. *Proc Natl Acad Sci USA* **99:** 8197–8202

Steele CR, Oppenheim DE, Hayday AC (2000) γδ T cells: non-classical ligands for non-classical cells. *Curr Biol* **10:** R282–R285

Street SE, Hayakawa Y, Zhan Y, Lew AM, MacGregor D, Jamieson AM, Diefenbach A, Yagita H, Godfrey DI, Smyth MJ (2004) Innate immune surveillance of spontaneous B cell lymphomas by natural killer cells and γδ T cells. *J Exp Med* **199:** 879–884

Sullivan LC, Hoare HL, McCluskey J, Rossjohn J, Brooks AG (2006) A structural perspective on MHC class Ib molecules in adaptive immunity. *Trends Immunol* **27:** 413–420

Sutherland CL, Rabinovich B, Chalupny NJ, Brawand P, Miller R, Cosman D (2006) ULBPs, human ligands of the NKG2D receptor, stimulate tumour immunity with enhancement by IL-15. *Blood* **108:** 1313–1319

Swann J, Crowe NY, Hayakawa Y, Godfrey DI, Smyth MJ (2004) Regulation of antitumour immunity by CD1d-restricted NKT cells. *Immunol Cell Biol* **82:** 323–331

Tabaczewski P, Shirwan H, Lewis K, Stroynowski I (1994) Alternative splicing of class Ib major histocompatibility complex transcripts *in vivo* leads to the expression of soluble Qa-2 molecules in murine blood. *Proc Natl Acad Sci USA* **91:** 1883–1887

Terabe M *et al* (2003) Transforming growth factor-β production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumour immunosurveillance: abrogation prevents tumour recurrence. *J Exp Med* **198:** 1741–1752

Terabe M, Swann J, Ambrosino E, Sinha P, Takaku S, Hayakawa Y, Godfrey DI, Ostrand-Rosenberg S, Smyth MJ, Berzofsky JA (2005) A nonclassical non-Vα14Jα18 CD1d-restricted (type II) NKT cell is sufficient for down-regulation of tumour immunosurveillance. *J Exp Med* **202:** 1627–1633

Thedrez A, Sabourin C, Gertner J, Devilder MC, Allain-Maillet S, Fournie JJ, Scotet E, Bonneville M (2007) Self/non-self discrimination by human γδ T cells: simple solutions for a complex issue? *Immunol Rev* **215:** 123–135

Ungchusri T, Chiang EY, Brown G, Chen M, Tabaczewski P, Timares L, Stroynowski I (2001) Widespread expression of the nonclassical class I Qa-2 antigens in hemopoietic and nonhemopoietic cells. *Immunogenetics* **53:** 455–467

Wu DY, Segal NH, Sidobre S, Kronenberg M, Chapman PB (2003) Cross-presentation of disialoganglioside GD3 to natural killer T cells. *J Exp Med* **198:** 173–181

Wu J, Groh V, Spies T (2002) T cell antigen receptor engagement and specificity in the recognition of stress-inducible MHC class I-related chains by human epithelial γδ T cells. *J Immunol* **169:** 1236–1240

Yan J, Parekh VV, Mendez-Fernandez Y, Olivares-Villagomez D, Dragovic S, Hill T, Roopenian DC, Joyce S, Van Kaer L (2006) *In vivo* role of ER-associated peptidase activity in tailoring peptides for presentation by MHC class Ia and class Ib molecules. *J Exp Med* **203:** 647–659

Ye SR, Yang H, Li K, Dong DD, Lin XM, Yie SM (2007) Human leukocyte antigen G expression: as a significant prognostic indicator for patients with colorectal cancer. *Mod Pathol* **20:** 375–383

Zhou D *et al* (2004) Lysosomal glycosphingolipid recognition by NKT cells. *Science* **306:** 1786–1789

Zidi I, Guillard C, Marcou C, Krawice-Radanne I, Sangrouber D, Rouas-Freiss N, Carosella ED, Moreau P (2006) Increase in HLA-G1 proteolytic shedding by tumour cells: a regulatory pathway controlled by NF-κB inducers. *Cell Mol Life Sci* **63:** 2669–2681

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