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RESPOSTA DOS LINFÓCITOS T γδ A TUMOURES: RECRUTAMENTO, RECONHECIMENTO E FUNÇÕES

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Abbreviations

ACT	Adoptive cell transfer
ADCC	Antibody-dependent cell cytotoxicity
AIDS	Acquired immunodeficiency syndrome
ALL	Acute lymphoblastic leukaemia
AML	Acute myeloid leukaemia
APC(s)	Antigen presenting cell (s)
AVC(s)	Angiogenic vascular cell(s)
β2m	β2-microglobulin
BCG	Bacilli Calmette-Guérin
BrHPP	Bromohydrin pyrophosphate,
CAF(s)	Cancer-associated fibroblast(s)
CAR	Chimeric antigen receptor
CD	Cluster of differentiation
CLL	Chronic lymphocytic leukaemia
CMV	Cytomegalovirus
CRC	Colorectal carcinoma
CTL(s)	Cytotoxic T lymphocyte(s)
CTLA-4	Cytotoxic T-lymphocyte antigen 4
CTX	Cyclophosphamide
CVID	Common variable immunodeficiency
DC(s)	Dendritic cells
DETC(s)	Dendritic epidermal T cells
DLBCL	Diffuse large B cell lymphoma
DMBA	Dimethylbenanthracene
DNAM-1	DNAX accessory molecule-1
EPCR	Endothelial protein C receptor
FACS	Fluoresce-assisted cell sorting
FCS	Foetal calf serum
FDA	Food and drug administration
FITC	Fluorescein
FL	Follicular lymphoma
FoxP3	Forkhead box P3
GITR	Glucocorticoid-induced TNFR family related gene
GM-CSF	Granulocyte-macrophage colony stimulating factor
H60	Histocompatibility antigen 60
HDMA-PP	Hydroxy-dimethyl-allyl-pyrophosphate
HMB-PP	4-hydroxy-3-methyl-but-2 enyl-pyrophosphate
HCMV	Human cytomegalovirus
HPV	Human papilloma virus

IEL(s)	Intraepithelial lymphocyte(s)
IFN	Interferon
IL	Interleukin
IPP	Isopentenyl pyrophosphate
KIR(s)	Killer-cell immunoglobulin-like receptor(s)
M1	“classical” activated macrophage
M2	“alternatively” activated macrophage
MACS	Magnetic-activated cell sorting
MCA	Methylcholanthrene
MDSC(s)	Myeloid-derived suppressor cell(s)
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MICA	MHC class I polypeptide-related sequence A
MICB	MHC class I polypeptide-related sequence B
MMP	Matrix metalloproteinase
mRNA	Messenger RNA
MULT1	Murine UL16-binding protein-like transcript 1
MVA	Mevalonate
N1	“classical” activated neutrophil
N2	“alternatively” activated neutrophil
NCR	Natural cytotoxicity triggering receptor
NHL	Non-Hodgkin´s lymphoma
NK	Natural killer
NKG2A	Natural killer group 2 member A
NKG2D	Natural killer group 2 member D
NKR(s)	Natural killer cell-associated receptor(s)
NKT	Natural killer T cell
NOD	Non-obese diabetic
NSCLC	Non-small cell lung cancer
OKT3	Anti-CD3 antibody, clone OKT3
PBL(s)	Peripheral blood lymphocyte (s)
PBMC(s)	Peripheral blood mononuclear cells (s)
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein 1
PE	Phycoerythrin
Pen/Strep	Penicillin Streptomycin
PerCP	Peridinn chlorophyll
PerCP-Cy5.5	Peridinn chlorophyll-Cy5.5
Pfn	Perforin
PHA	Phytohemagglutinin
PMA	Phorbol 12-myristate 13-acetate
Rae1	Retinoic acid early transcript
RAG	Recombination activating gene

RNA	Ribonucleic acid
RPMI	Roswell park memorial institute cell culture medium
RT-qPCR	Real-time-quantitative polymerase chain reaction
SCID	Severe combined immunodeficiency
SCT	Stem cell transplantation
STAT	Signal transducer and activator of transcription
T10	Thymus leukaemia antigen 10
T22	Thymus leukaemia antigen 22
TAA(s)	Tumour-associated antigens
TAM(s)	Tumour-associated macrophage(s)
TAN(s)	Tumour-associated neutrophil(s)
TCR(s)	T cell receptor(s)
TME	Tumour microenvironment
TGF-β	Transforming growth factor beta
TIL(s)	Tumour-infiltrating lymphocytes
Th1	T helper cell type 1
Th2	T helper cell type 2
Th17	T helper cell type 17
TLR(s)	Toll-like receptor(s)
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
TPA	12-0-tetra-decanoylphorbol
TRAIL	Tumour necrosis factor related apoptosis inducing ligand
TRAMP	Trangenic adenocarcinoma mouse prostate cancer
Treg	Regulatory T cell
ULBP(s)	UL-16 binding proteins
VEGF	Vascular endothelial growth factor
Zol	Zoledronate

Resumo

A teoria da imunovigilância do cancro postula que as células do sistema imunitário são capazes de eliminar células transformadas, da mesma forma que combatem patogénios e células infectadas por patogénios. Esta teoria constitui a base da imunoterapia do cancro, a qual explora as propriedades anti-tumorais do sistema imunitário (tanto do sistema inato como do adaptativo) no tratamento de doenças malignas.

As células T γδ são linfócitos que constitutem uma pequena percentagem (1-10%) dos linfócitos periféricos do sangue humano, mas que representam a maioria das células T em tecidos epiteliais. Estas células apresentam algumas propriedades que as tornam uma boa aposta para protocolos de imunoterapia para o cancro, como por exemplo, citotoxicidade independente da apresentação de antigénios por moléculas do complexo major de histocompatibilidade (MHC) e reactividade selectiva a fosfoantigénios que podem ser sintetizados em larga escala. Contudo, apesar do entusiasmo inicial, o sucesso clínico do uso de células T γδ tem sido limitado dadas as baixas percentagens de resposta clínica objectiva obtidas. Estes resultados revelam a necessidade de mais investigação acerca dos mecanismos que determinam uma interação produtiva entre células T γδ e células tumorais.

Uma das maiores lacunas na biologia das células T γδ são os mecanismos que controlam o reconhecimento de células tumorais. Por esse motivo decidimos analisar quais as moléculas envolvidas na detecção de tumores hematológicos por células Vγ9Vδ2, as quais constituem a maior fracção das células T γδ no sangue periférico humano. Observámos uma grande variabilidade de susceptibilidade de linhas celulares de leucemia e linfoma à citotoxicidade mediada por células Vγ9Vδ2. Verificámos que o receptor NKG2D é necessário, enquanto que o receptor de células T (TCR) é dispensável, para a eliminação de linhas tumorais susceptíveis. Analisámos a expressão de ligandos de NKG2D nas linhas celulares tumorais resistentes e susceptíveis, e observámos que a expressão de ULBP1 (tanto ao nível do mRNA como ao nível da proteína) está associada a tumores susceptíveis. Realizámos ensaios de perda e ganho de função *in vitro* e concluímos que o ULBP1 é um determinante não redundante do reconhecimento de leucemias e linfomas por parte das células T γδ humanas. De realçar que observámos uma grande heterogeneidade de expressão de ULBP1 em amostras primárias de doentes de leucemia e linfoma, o que poderá contribuir para a variabilidade de respostas observadas em ensaios clínicos hematológicos baseados em células T γδ.

Outro aspeto limitante para o sucesso da utilização de células T γδ na imunoterapia do cancro, é a falta de conhecimento dos fatores que controlam a migração e infiltração tumoral das células T γδ . Dado o papel fulcral das quimiocinas e dos seus receptores na migração de leucócitos, investigámos o seu envolvimento o recrutamento tumoral das células T γδ. Usámos o modelo murino pré-clinico de melanoma, baseado na injecção da linha tumoral B16, e comparámos a composição de quimiocinas em extratos proteicos de tumores provenientes de murganhos suficientes (“selvagens”) ou deficientes em linfócitos T γδ (*Tcrd*$^{-/-}$), tendo observado

uma acumulação de ligandos de CCR2 (CCL2 e CCL12) em extratos tumorais de animais *Tcrd*$^{-/-}$. Curiosamente, verificámos também que os murganhos *Ccr2*$^{-/-}$ apresentavam tumores maiores do que os murganhos selvagens. Analisámos a composição leucocitária e concluímos que os tumores de murganhos *Ccr2*$^{-/-}$ e também de murganhos *Ccl2*$^{-/-}$ continham significativamente menos células T γδ infiltrantes do que os murganhos selvagens. Verificámos também que a migração de outras populações de linfócitos T, nomeadamente células CD4^{+} ou CD8^{+}, não foram afetadas. Analisámos as populações mielóides, incluindo macrófagos, neutrófilos e "myeloid-derived suppressor cells" (MDSCs), e observámos (como previsto na literatura) que estas populações leucocitárias se encontravam reduzidas em tumores originários de murganhos *Ccr2*$^{-/-}$. Considerando que a infiltração de tumores por células mielóides está geralmente associada com mau prognóstico, é notável que os murganhos *Ccr2*$^{-/-}$ apresentem tumores maiores em comparação com murganhos selvagens. Adicionalmente, como os murganhos *Tcrd*$^{-/-}$ também apresentaram tumores aumentados (em comparação com murganhos selvagens), os nossos dados sugerem uma nova função protetora da via inflamatória CCR2/CCL2 através do recrutamento de células T γδ citototóxicas.

Dadas as diferenças significativas entre células T γδ de murganho e humanas, e no sentido de aplicar estas descobertas à medicina, investigámos se as células T γδ humanas dependiam de CCR2/CCL2 para a sua migração. Verificámos que a sub-população Vδ1 expressa CCR2 e migra para CCL2 *in vitro*; pelo contrário, a sub-população Vδ2 não expressa CCR2 (mesmo após ativação)e não responde a CCL2. Adicionalmente, observámos uma grande variabilidade de níveis de expressão de CCL2 em vários tipos de tumores humanos; alguns tipos sobrexpressam, enquanto que outros subexpressam CCL2 comparativamente com os respetivos tecidos saudáveis. Estes dados salientam a importância de correlacionar a infiltração de células T Vδ1 com a expressão de CCL2 *in situ* em ensaios clínicos futuros.

Apesar das potentes propriedades anti-tumourais das células T γδ estarem bem estabelecidas, alguns estudos recentes reportaram uma atividade pro-tumoral das células T γδ. Consequentemente, proposemo-nos investigar as condições que determinam as propriedades anti- ou pro-tumorais das células T γδ. Através da comparação de dois modelos murinos de melanoma (linha B16) e de carcinoma do ovário (linha ID8), mostrámos que o efeito pro-tumoral das células T γδ se associa a elevada produção de produção de interleucina-17 (IL-17). Verificámos que murganhos *Tcrd*$^{-/-}$ apresentam sobrevivência reduzida (comparado com murganhos selvagens) após transplante do tumor B16, mas sobrevivência aumentada após transplante do tumor ID8. A acumulação de células T γδ produtoras de IL-17 exclusivamente na cavidade peritoneal de murganhos transplantados com tumores ID8, levou-nos a propôr um efeito pro-tumoral dependente de IL-17. É importante notar que a maior fonte de IL-17 nos tumores ID8 foram as células T γδ, e estas que estas expressaram níveis mais elevados de IL-17 (ao nível de cada célula) em comparação com as células T CD4^{+}.

Resumindo, nesta tese caracterizámos vários aspetos-chave da resposta das células T γδ a tumores , nomeadamente o reconhecimento molecular de linfomas e

leucemias; os mecanismos de migração e infiltração tumoral; e possíveis propriedades pro-tumorais. Acreditamos que estas descobertas contribuem significativamente para o conhecimento da biologia das células T γδ, e esperamos que possam melhorar os protocolos atuais de imunoterapia do cancro baseados da ativação de células T γδ.

Palavras-chave: Células T γδ; ULBP1; NKG2D; leucemia; linfoma; imunoterapia do cancro; CCR2; CCL2; IL-17

Summary

Tumour immunosurveillance postulates that immune cells are able to eliminate transformed cells, as much as they eliminate pathogens or pathogen-infected cells. This theory constitutes the basis of cancer immunotherapy which explores anti-tumour properties of the immune system (both innate and adaptive) for the treatment of human malignancies.

γδ T cells are innate-like lymphocytes that account for a small percentage (1-10%) of human peripheral blood lymphocytes, but represent the majority of T cells in epithelial tissues. Several properties make γδ T cells attractive targets for cancer immunotherapy, namely their MHC-unrestricted cytotoxicity and unique responsiveness to clinical grade available (phospho) agonists. Despite the promise of γδ T cells in cancer immunotherapy, the clinical success has been limited by low percentages of objective responses. This urges more research on the mechanisms that govern the interactions between γδ T cells and tumours.

One of the major gaps in the γδ T cell field is the lack of mechanistic knowledge on tumour cell recognition. We decided to analyze which molecules determine haematological tumour recognition by Vγ9Vδ2 T cells, the major γδ T cell subsets in human peripheral blood. We observed widely variable susceptibility of leukaemia and lymphoma cell lines to Vγ9Vδ2 T cell-mediated cytotoxicity. For those tumours that were efficiently targeted by Vγ9Vδ2 T cells, we found that this required the NK receptor NKG2D, but not the signature T cell receptor (TCR). We then analyzed the expression of NKG2D ligands in susceptible or resistant tumour cells lines, and observed that ULBP1 expression (both at the mRNA level and protein level) segregated with susceptible targets. Through a series of loss- and gain-of-function assays, we demonstrated that ULBP1 constitutes a non-redundant determinant of leukaemia and lymphoma cell recognition by human γδ T cells. Importantly, we observed a dramatic heterogeneity of ULBP1 expression in primary samples obtained from leukaemia and lymphoma patients, which can thus contribute to the highly variable outcomes of γδ T cell-based clinical trials in haematological tumours.

Another important limitation to the modulation of γδ T cells in cancer immunotherapy is the lack of molecular cues that direct γδ T cell migration to tumours. Given the pivotal role played by chemokines and their receptors in leukocyte migration, we investigated which chemokines could determine γδ T cell recruitment to tumours. We used the “gold standard” pre-clinical transplantable B16 melanoma model and compared chemokine composition of tumour extracts from WT and TCRδ-deficient mice, and observed an accumulation of the CCR2 ligands, CCL2 and CCL12, in tumour extracts from TCRδ-deficient animals. Interestingly, the comparison of WT and CCR2-deficient hosts revealed increased tumour growth in $CCR2^{-/-}$ mice. Critically, we showed that tumours from $CCR2^{-/-}$ (as well as $CCL2^{-/-}$) mice contained significantly less infiltrating γδ T cells compared to WT tumours, whereas other T cell populations, particularly $CD4^{+}$ and $CD8^{+}$ T cells, were not affected. We also analysed myeloid populations, namely macrophages, neutrophils and myeloid-

derived suppressor cells (MDSCs), and observed that, as expected from previous literature, these leukocyte populations were reduced in tumours from $CCR2^{-/-}$ mice. Considering that myeloid cell infiltration into tumours is usually associated with poor prognosis, it is noticeable that $CCR2^{-/-}$ mice displayed increased tumour growth when compared to WT mice. Furthermore, as TCRδ-deficient mice also showed increased tumour burden (compared to WT mice), our data suggests a novel protective role for the CCR2/ CCL2 chemokine pathway through the recruitment of cytotoxic γδ T cells.

Considering the significant differences between murine and human γδ T cells, and trying to translate these findings into the human setting, we also investigated if human γδ T cells relied on CCR2 for migration. We observed that the Vδ1 subset expresses CCR2 and migrates towards CCL2 *in vitro*; by contrast with the Vδ2 population that lacks CCR2 expression, even after activation. Moreover, we observed a dramatic variety of CCL2 levels in several cancer types, with some overexpressing and other downmodulating CCL2 when compared to healthy tissue controls. These data collectively highlight the importance of correlating Vδ1 T cell infiltration with CCL2 expression *in situ* in future cancer clinical studies.

While the potent anti-tumour properties of γδ T cells have been widely demonstrated, some recent reports have implied a pro-tumour role for γδ T cells. We therefore set out to investigate which conditions determine the anti- versus pro-tumour properties of γδ T cells. By comparing two different murine tumour models, B16 melanoma and ID8 ovarian carcinoma, we proposed that the pro-tumour role of γδ T cells may be determined by IL-17 production. TCRδ-deficient mice showed reduced survival (compared to WT) when challenged with B16 tumours, but enhanced survival when challenged with ID8 tumours. We demonstrated an accumulation of IL-17-producing γδ T cells in the peritoneal cavity of ID8 tumour bearing mice that seemed to dominate over anti-tumour IFN-γ production. Importantly, the major source of IL-17 in the ID8 tumour model were γδ T cells, and these expressed higher levels of IL-17 (on a per cell basis) than their $CD4^+$ T cell counterparts.

In summary, in this thesis we have characterized some key features of γδ T cells in tumour immunosurveillance, namely leukaemia/ lymphoma cell recognition; migration and tumour infiltration; and putative pro-tumour properties. We believe these findings make an important contribution to our understanding of γδ T cell function, and may help to improve current cancer immunotherapy protocols based on γδ T cell activation.

Keywords: γδ T cells; ULBP1; NKG2D; leukaemia; lymphoma; tumour immunotherapy; CCR2; CCL2; IL-17

CHAPTER I:

General Introduction

1.1 Immune response to tumours

1.1.1 Tumour immunosurveillance theory

Cell transformation arises as a consequence of accumulating genetic alterations affecting intrinsic cellular programs, for example, cell cycle check point control, programmed cell death, differentiation or metabolism. However, not all transformed cells lead to cancer because tumour cell growth and dissemination is highly dependent upon reciprocal interactions between genetically modified cells and the dynamic microenvironment that surrounds them.

The idea that the immune system was part of the tumour microenvironment, and could potentially control tumour cells, was postulated by Paul Ehrlich in the early 1900s. He suggested that cells of the immune system are able to eliminate tumours, much like they destroy pathogens or pathogen-infected cells. However, so little was known about the composition and function of the immune system at that time, that it was not possible to assess the validity of its prediction. It took nearly 50 years for MacFarlane Burnet and Lewis Thomas to conceive their tumour immunosurveillance theory (Figure 1), which proposed that adaptive immunity was responsible for preventing cancer development in immunocompetent hosts, by recognizing and eliminating continuously arising, transformed cells [1].

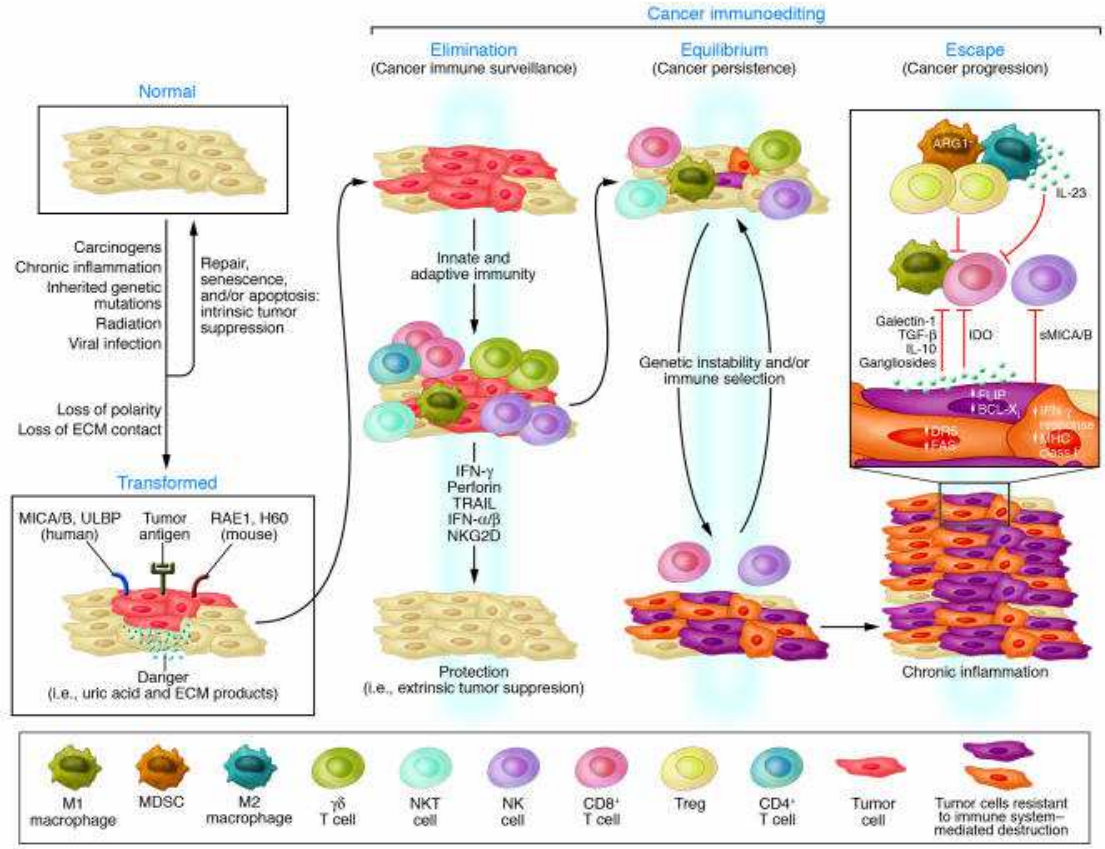

Figure 1 - Tumour immunosurveillance theory and Cancer immunoediting hypothesis. Adapted from Swann& Smyth 2007[2]

However, the first *in vivo* evidence for tumour immunosurveillance was only demonstrated in the 1990´s and derived from the observation that transplanted tumours grew more robustly in mice treated with neutralizing monoclonal antibodies specific for interferon-γ (IFN-γ)[3]. By that time with the advent of genetically modified immunodeficient mice, the relative contribution of the several components of the immune system for tumour immunosurveillance was examined[4] . For example, lymphocyte-deficient mice, such as: recombination activating gene *Rag1*$^{-/-}$ and *Rag*2$^{-/-}$, severe combined immunedeficient (SCID), *Tcrb*$^{-/-}$, *Tcrd*$^{-/-}$ and nude mice all display an increased susceptibility to tumour induction after methylcholanthere (MCA) treatment[5-9]. Moreover, immunodeficient mice show increased spontaneous tumour development with age. One striking example is the incidence of immunogenic B cell lymphomas in aged mice (> 1 year) on either C57BL/6 or BALB/c backgrounds, which increases from 0-6% in wild-type mice, to 40-60% in perforin-deficient mice[10, 11]. The genetic absence of other lymphocyte cytotoxic pathways, such as TRAIL or FasL, also increases the susceptibility of mice to spontaneous lymphomas[12, 13]. All together, these data provided strong evidence that lymphocytes (mainly due to critical cytotoxic molecules they produce) protect hosts from tumour development[4].

In humans, the importance of immune surveillance against tumour emergence and progression was reinforced with the observation that immune deficiency states, including iatrogenic immune suppression, severe combined immunodeficiency (SCID), common variable immunodeficiency (CVID), and acquired immunodeficiency syndrome (AIDS), greatly increased the susceptibility of patients to many types of malignancies[14].

Collectively, these were the foundations for the development of tumour immunotherapy as a “discipline”, and cancer immunotherapy as its clinical application.

1.1.2 Cancer immunoediting hypothesis

A hallmark study in 2001 showing that the immune system not only protects the host against tumour formation, but also shapes tumour immunogenicity[7] prompted a major revision of the tumour immunosurveillance theory. Robert Schreiber postulated the cancer immunoediting hypothesis, which stresses the dual host-protective and tumour-promoting actions of the immune system in developing tumours.

The cancer immunoediting hypothesis[15] states that tumour development is a dynamic process composed of three sequential phases: elimination, equilibrium and escape (Figure 1). In the elimination phase, innate and adaptive immunity destroy developing tumours before they become clinically apparent. If this phase goes to completion, the host remains free of cancer, and elimination thus represent the full extent of the process. If, however, a cancer cell variant is not destroyed in the elimination phase, it may then enter the equilibrium phase, in which tumour cells

are maintained in a state of functional dormancy. This phase is dependent on the adaptive immunity and it´s when editing of tumour immunogenicity occurs. Equilibrium may also represent an end stage of the cancer immunoediting process. However, as a consequence of constant immune selection pressure placed on genetically unstable tumour cells held in equilibrium, tumour cell variants may emerge that (i) are no longer recognized by adaptive immunity (antigen loss variants or tumour cells that develop defects in antigen processing or presentation), (ii) become insensitive to immune effector mechanisms, or (iii) induce an immunosuppressive state within the tumour microenvironment. These tumours may then enter the escape phase, in which their outgrowth is no longer blocked by immunity. These tumours emerge to cause clinically apparent disease.

1.1.3 Tumour microenvironment

Tumour immunosurveillance relies on the presence of leukocytes, most notably lymphocytes, in the tumour microenvironment. There, tumour growth, invasiveness, and metastasis are dynamic processes that involve the interaction of cancer cells with the extracellular matrix, the vasculature, and various types of non-cancerous host cells that form the tumour stroma. Commonly, the tumour microenvironment (TME) is constituted by: angiogenic vascular cells (AVCs), cancer-associated fibroblastic cells (CAFs) and infiltrating leukocytes[16].

1.1.3.1 Tumour-infiltrating leukocytes

Within leukocytes, lymphocytes have gathered most attention given the phenotypes of lymphocyte-deficient mice and cancer patients[14], and the prognosis value of their infiltration into tumours (see ahead 1.1.4.). However, it has become clear that a wide variety of leukocyte subtypes is present in the tumour microenvironment and play important anti-tumour roles, but also sometimes pro-tumour roles therein.

1.1.3.1.1 The traditional players: NK, $CD8^+$ T and Th1 cells lymphocytes

It has been known for three decades that NK cells and $CD8^+$ T lymphocytes, including those extracted from tumour biopsies, can efficiently kill transformed cells. Collectively, these killer lymphocytes recognize two important types of tumour antigens (among others): processed peptides presented by MHC class Ia proteins via TCR$\alpha\beta$; and non-classical (class Ib) MHC proteins via NKG2D[17]. The latter, which is expressed on NK, $CD8^+$ and also $\gamma\delta$ T cells, has been recently shown to be a key genetic determinant of cancer immunosurveillance[18].

NK and $CD8^+$ cells provide highly complementary anti-tumour strategies. Indeed, as demonstrated by the seminal work of Kärre and Kiessling, the downregulation of MHC class Ia, which is a common mechanism of evasion against $CD8^+$ cells, renders tumours more susceptible to NK cell-mediated lysis. This “missing

self" recognition by NK cells is based on a set of MHC class Ia-specific inhibitory receptors that include killer cell immunoglobulin-like receptors (KIRs) in humans, lectin-like Ly49 molecules in mice, and CD94/NKG2A heterodimers in both species; in fact, NK cells express a complex repertoire of inhibitory and activating receptors that calibrate this anti-tumour function, while ensuring self-tolerance[19, 20]. In result, NK cells eliminate tumours that lack MHC class Ia expression; or that overexpress ligands for activating NK receptors like NKG2D or the natural cytotoxicity receptors NKp30, NKp44 and NKp46[20]. Furthermore, NK cells express high levels of low-affinity Fc receptor for IgG (CD16), which allows them to mediate antibody-dependent cell-mediated cytotoxicity (ADCC)[21].

NK cells have been described to infiltrate various types of tumours in the skin, lung, gut and kidney[18]. Recent data on human NK cells infiltrating highly aggressive non-small cell lung cancers (NSCLC) showed a profound alteration of their phenotype, with decreased ability to degranulate and to produce IFN-γ, when compared with NK cells from distal lung tissues or blood from the same patients or from healthy donors[22]. This functional impairment of NK-TILs correlated with decreased expression of NKp30, NKp80, DNAM-1, CD16 and ILT2 receptors. Interestingly, among these, NKp30 has been shown to affect the prognosis of gastrointestinal stromal tumours through a specific pattern of alternative splicing[23].

Various immunotherapeutic strategies have been proposed to tackle the common defects of NK cell activity in cancer patients[20]: activation of endogenous NK cells (with cytokines like IL-2, IL-15 and IL-18), NK-cell adoptive immunotherapy, NK-cell-based donor lymphocyte infusions and allogenic stem cell transplantation (SCT)[21]. Although globally the objective responses have been disappointing, some data from allogenic and, more recently, haploidentical hematopoietic SCT have shown clinical (in the absence of adverse) effects mediated by NK cells[20]. This inspires further translational studies aimed at enhancing NK cell recruitment to tumours and their functional activity *in situ*.

With regard to $CD8^+$ T cell-based immunotherapy, many recent efforts have focused in activating and expanding $CD8^+$ tumour-infiltrating lymphocytes (TILs) *ex vivo* and then re-infusing them into the cancer patients - adoptive cell therapy (ACT). ACT of $CD8^+$ TILs into lymphodepleted metastatic melanoma patients has shown very high objective response rates, ranging from 50% up to 81%[24]. In fact, TIL-ACT (combined with high doses of IL-2) has mediated cancer regression in 49-72% of melanoma patients, and durable complete responses, beyond 3-7 years, are currently ongoing in 40% of the patients[25].

In pre-clinical models, adoptively transferred naïve $CD8^+$ cells were shown to infiltrate melanoma lesions, be activated *in situ* and differentiate into functional cytotoxic T lymphocytes (CTLs)[26]. The naïve status of the infused population appeared to be an important parameter, as the differentiation stage of CTLs inversely correlated with their anti-tumour efficacy *in vivo*[27]. The enhanced anti-tumour function of naïve T cells was related to sustained effector cell development, prolonged cytokine production, and increased expansion *in vivo*.

Transduction of tumour antigen-specific TCRs[28] or chimeric antigen receptors (CARs)[29, 30] represent exciting prospects to increase the efficacy of cytotoxic ACT.

These strategies have thus far enabled cancer regression in patients with metastatic melanoma, synovial sarcoma, neuroblastoma and refractory lymphoma or leukaemia[25].

In addition to cytotoxicity, IFN-γ secretion is a key anti-tumour function of $CD8^+$ and NK cells, who share this property with various other lymphocyte populations, most notably "helper type 1" (Th1) $CD4^+$ cells. These were first described 25 years ago in the context of the "Th1/ Th2" paradigm of immunity to infection, and since then clearly implicated in promoting anti-tumour responses: Th1 cells enhance the cytotoxic functions of NK and $CD8^+$ cells, upregulate MHC class I expression in tumour cells (a direct effect of IFN-γ), and support $CD8^+$ cell proliferation through the secretion of IL-2[31]. Moreover, Th1 cells condition the antigen-presenting capacity of DCs and macrophages, thus shaping the CTL response. In fact, the combination of Th1 cell therapy with local radiation therapy augmented the generation of tumour-specific CTL at the tumour site and induced a complete regression of subcutaneous tumours[32].

1.1.3.1.2 "New" effectors : γδ T, NKT and Th17 lymphocytes

The immune response to tumours by γδ T cells is the main focus of this thesis, so it will be discussed in the next chapter.

The "Th1/ Th2" paradigm for $CD4^+$ T cell differentiation has been recently revised with the addition of Th17 cells, characterized by the production of interleukin-17 (IL-17). IL-17-deficient mice were shown to be more susceptible (than wild type animals) to tumour growth and lung metastasis[33, 34]. Adoptive transfer studies from the Restifo lab showed that *in vitro* generated Th17 cells were more efficient at eradicating tumours than Th1 cells[35], and this was recently associated with stem cell-like properties of Th17 cells[36]. Importantly, adoptively transferred Th17 cells gave rise *in vivo* to Th1-like effector cell progeny[36], and IFN-γ was actually necessary for the protective effects of adoptively transferred Th17 cells[35]. These data suggest that acquisition of Th1-like properties is required for an anti-tumour function by Th17 cells.

In stark contrast to the previous studies, IL-17-deficient mice presented reduced tumour growth in other models such as B16 melanoma and MB49 bladder carcinoma[37], DMBA/TPA-induced skin carcinoma[38], or in a spontaneous intestinal tumour model (driven by a mutation in the tumour suppressor gene APC)[39].

The pro-tumour functions of IL-17 have been tightly linked to angiogenesis: IL-17 has been shown to act on endothelial, stromal and tumour cells to induce the expression of pro-angiogenic factors like VEGF, Angiotensins, PGE2 and IL-8, and thus promote tumour vascularization[40]. The precise conditions that determine pro- versus anti-tumour functions of Th17 TILs remain unclear and require further investigation.

Although Th17 cells are important providers of IL-17, this cytokine can be abundantly produced by other tumour-infiltrating leukocyte populations. Namely, murine γδ T cells can be the major source of IL-17, not only in homeostatic

conditions[41], but also upon infection or tumour challenge[42, 43]. Like for Th17 cells, the role of IL-17 produced by γδ cells within the tumour microenvironment is controversial: it has been associated both with angiogenesis and promotion of tumour growth[42, 44]; and with $CD8^+$ T cell recruitment and the therapeutic effects of chemotherapy against several subcutaneous tumour lines[43, 44].

NKT cells also employ NK receptors, as well as CD1d-restricted TCRs to recognize tumour targets. The vast majority of these T cells are canonical or invariant NKT (type I NKT) cells that possess a specific TCRα rearrangement (Vα14Jα18 in mice; Vα24Jα18 in humans), associated with Vβ chains of limited diversity. All the other NKT cells that are CD1d-restricted and do not express this invariant TCR are called type II NKT cells[45, 46]. Although CD1d-deficient mice showed increased susceptibility to MCA-induced sarcomas[47], there is evidence of functional heterogeneity also within NKT cells: while type I NKT cells seem to be protective, type II NKT cells mostly suppress tumour immunity[46, 48].

In terms of cytokine production, activated NKT cells are potent providers of IFN-γ and IL-4 (and, to lesser extent, of IL-17). In the B16 metastatic melanoma model, a dual role of NKT cells was linked to immune suppressive IL-4 production by the thymus-derived subpopulation; and protective IFN-γ production by liver-derived type I NKT cells[49].

Based on the pre-clinical evidence for an anti-tumour role of type I NKT cells, and the availability of a specific TCR agonist, α-Gal-Cer, several clinical trials have attempted to activate endogenous iNKT cells, or – more promising given by relative rarity of NKT cells in humans – perform ACT with (*ex vivo* expanded) type I NKT cells. However, the clinical effects of a-Gal-Cer or NKT ACT have been very limited[46], thus illustrating the difficulty in translating findings from animal models of cancer into improved immunotherapies.

1.1.3.1.3 The inflammatory phagocytes: TAMs and TANs

Macrophages and neutrophils are important myeloid cells of the innate immune system and major drivers of inflammatory responses. Given the long-established association between cancer and inflammation, it is not surprising that tumour-associated macrophages (TAMs) and neutrophils (TANs) can have great impact on the course of tumour progression. While most studies have associated TAM and TAN infiltration with promotion of tumour cell growth, some other reports have proposed some anti-tumour roles. Once again, these opposing behaviors may be explained by heterogeneous TAM and TAN phenotypes, with distinct intra-tumour dynamics in various models.

Mirroring Th1/ Th2 polarization of $CD4^+$ T cells, two distinct subsets of macrophages have been recognized: the "classical" activated (M1) macrophage phenotype and the "alternatively" activated (M2) macrophage phenotype[50]. IFN-γ drives the polarization towards M1 macrophages, which are characterized by abundant production of TNF-α, IL-12 and IL-23, CXCL9 and CXCL10, reactive nitrogen

and oxygen species; and by high expression of MHC class II and costimulatory molecules (making them efficient antigen-presenting cells)[51]. Conversely, IL-4 polarizes macrophages towards the M2 phenotype, which is associated with low levels of IL-12 but high levels of IL-10, IL-1RA and IL-1 decoy receptor. M2 cells also produce CCL17, CCL22 and CCL24, which results in the recruitment of Tregs and Th2 cells, eosinophils and basophils[51].

The balance between M1 and M2 phenotypes seems to be controlled by NFkB signaling. Thus, NFkB targeting switched macrophages from an M2 to an M1 phenotype and led to ovarian tumour regression *in vivo*[52]. Nonetheless, the most frequent TAM phenotype seems to be M2[50]. Consistent with this, TAM depletion was associated with improved anti-tumour immunity in models of metastatic breast, colon and non-small lung cancers[53]. The pro-tumour roles of M2 macrophages derive from various molecular mechanisms, including the production of the pro-angiogenic mediator semaphoring 4D[54] and the invasive proteases cathepsins B and S[55].

In the case of neutrophils, besides secreting cytokines and chemokines (such as IL-1b, IL-8, and IL-12), they produce large amounts of proteinases that remodel the extracellular matrix and promote the release of pro-angiogenic VEGF, thus supporting tumour cell growth and invasiveness[56]. Particularly important neutrophil proteinases are elastase[57] and matrix metalloproteinases MMP-8 and MMP-9[58].

Despite being widely accepted as pro-tumour mediators based on multiple pre-clinical and clinical studies[57], a dual nature of tumour-infiltrating neutrophils has also been suggested recently[59, 60]. Thus, anti-tumour N1 and pro-tumour N2 subsets were described and modulated within tumours by TGF-β[59] or IFN-β[61]. Consistent with such a complex neutrophil activity within the tumour microenvironment, the concentration of reactive oxygen species also seems to determine either pro-tumour (genotoxicity at modest concentrations) or anti-tumour (cytotoxicity at high concentrations) effects[57]. Consequently, the depletion of total neutrophils can lead to either reduced[59] or increased[62] tumour burden, further illustrating the globally paradoxical roles of tumour-infiltrating leukocytes.

1.1.3.1.4 Immunosuppressive leukocytes: Treg and MDSCs

Myeloid-derived suppressor cells (MDSCs) represent a heterogeneous population of myeloid progenitors and precursors of macrophages, granulocytes and dendritic cells, which are better characterized by their strong capacity to inhibit both innate and acquired immunity[63] particularly T cell responses[64]. Murine MDSCs can be identified by the expression of Gr1 (includes Ly6C and Ly6G, macrophage and neutrophil markers, respectively) and CD11b (characteristic of macrophages). In humans, MDSCs are characterized by a $CD11b^+$ $CD33^+$ $CD34^+$ $CD14^-$ $HLA\text{-}DR^-$ phenotype. Tumours produce various factors that promote MDSC expansion, such as IL-6, VEGF or GM-CSF, whereas they get further activated by local IFN-γ, IL-1β or Toll-like receptor (TLR) signal[64].

MDSCs use a diversity of mechanisms to suppress T cell function, including the uptake of arginine and cysteine (essential amino acid for T cell activation) and

the nitration of the TCR[63]. In addition, MDSCs have been recently shown to directly support tumour growth by promoting the epithelial-to-mesenchymal transition in melanocytes[65].

The possibility of improving anti-tumour immune responses by targeting MDSCs has been explored in pre-clinical models. One of the chemical drugs that seem to be more effective for MDSC depletion was 5-fluorouracil (5-FU). In a model of thymoma EL4 cells transplanted subcutaneously, tumour-bearing mice treated with 5-FU showed reduced number of MDSC in tumour lesions. This associated with prolonged mouse survival and enhanced intratumoral $CD8^+$ T cell antigen-specific capacity to produce IFN-γ[66]. Interestingly, combination therapy with an agent (cyclophosphamide, CTX) that reduces Tregs led to a synergistic protective effect. Consistent with this, another study showed that inhibition of MDSC and Treg function within B16 melanomas using blocking antibodies to CTLA-4 (already in clinical use - ipilimumab - in late-stage melanoma) and to PD-1 reduced tumour development and increased mouse survival[67].

$Foxp3^+$ Tregs are well known to suppress the activation, proliferation and effector functions (such as cytokine production) of a wide range of immune cells, including $\alpha\beta$ and $\gamma\delta$ T cells, NK and NKT cells, B cells, macrophages and DCs. Suppressive functions displayed by Tregs include contact-dependent mechanisms, such as those that involve CTLA-4, PD-1 and GITR; and cytokine-mediated mechanisms such as TGF-β, IL-10 and IL-35[68]. TGF-β is particularly critical since, besides being strongly immunosuppressive, creates a potent positive feedback mechanism by instructing the differentiation of "inducible" Tregs[69].

Experimental Treg depletion has been usually accomplished using anti-CD25 monoclonal antibodies, since there is a good correlation between CD25 and Foxp3 expression within $CD4^+$ T cells (although activated effector cells also upregulate CD25). Prophylactic Treg depletion in renal cell carcinoma and MCA carcinoma was shown to reduce tumour growth, with protection being dependent on $CD8^+$ and NK cells[70, 71].

While most studies have concentrated on the immunosuppressive function of Tregs, two recent reports have shown that they can also act by directly promoting tumour growth and dissemination. Thus, Treg TILs in ovarian cancer they secrete VEGF that promotes endothelial cell proliferation[72]; and in breast cancer they produce RANKL, which associates with lung metastasis[73]. Importantly, the latter study is one of many that demonstrates that Treg accumulation within tumours is a marker for poor clinical outcome[74].

1.1.3.2 Other cells (non-leukocytes)

Historically, tumour angiogenesis was thought to be regulated by cancer cells expressing proangiogenic factors, which is indeed one mechanism; however there is now abundant evidence that stromal cells in the tumour microenvironment are instrumental in switching on and sustaining chronic angiogenesis in many tumour

types[16]. The angiogenic switch was reported to be accompanied by reduced apoptosis and increased proliferation of cancer cells; the vascularization of tumours serves to attenuate cell death that would otherwise result from hypoxia and lack of serum-derived nutrients and survival factors[75, 76].

Recently, angiogenic vascular cells have been implicated in local supply of growth-promoting trophic factors that are expressed by endothelial cells, potentially acting to stimulate, in a paracrine way, proliferation of tumour cells[77]. To hamper neovascularisation to tumours, potent angiogenic inhibitors have been developed, mainly acting at the vascular endothelial growth factor (VEGF) and other proangiogenic signalling pathways. This notwithstanding, the clinical responses are typically transitory, and survival benefit limited to duration of the treatments[16].

A variety of cancer-associated fibroblasts can be recruited and/or be activated at the tumour microenvironment to contribute to tumour progression. For example, cancer-associated fibroblasts can produce mitogenic epithelial growth factors and also orchestrate the epithelial-to-mesenchymal transition (EMT) via secretion of TGF-β[78]. Moreover, cancer-associated fibroblasts in different tumour microenvironments can produce a number of proangiogenic factors, namely VEGF and IL-8/CXCL8[79].

Interestingly, platelets have been also shown to be able to promote tumour progression. By physically associating with cancer cells, platelets secrete TGF-β and induce transitory epithelial-to-mesenchymal transition, facilitating extravasation and seeding of metastases[80].

1.1.3.3 Key molecular players: Cytokines and Chemokines

The cells that compose the tumour microenvironment produce a number of cytokines that may modulate the immune response and tumour progression. The hallmark cytokines that show potent anti-tumour effect are IFN-γ and TNF-α. IFN-γ is mainly produced by lymphocytic cytotoxic cells, including $CD8^+$ and γδ T cells, NK cells, and as well as by Th1 $CD4^+$ T cells. The most well characterized function of IFN-γ is the upregulation of MHC class I molecules to aid the priming and presentation of antigens in APCs[81]. Moreover, IFN-γ regulates all aspects of Th1-mediated immune responses and activates macrophages[82]. IFN-γ has been used in the clinical management of a variety of malignancies, including bladder carcinoma, colorectal cancer, ovarian cancer, and adult T-cell leukaemia[83].

TNF-α is primarily produced by macrophages, but also by a variety of other cells, including NK cells, T lymphocytes, smooth muscle cells, fibroblasts. The biological effect of TNF-α binding depends on the type of receptor activated (TNFR1 or TNFR2) and the cellular stage during activation, but the late stage event in TNF-α stimulation is cell apoptosis[84]. The potential use of TNF-α as a therapeutic agent was intensively studied *in vitro* and *in vivo* studies. These studies highlighted its possible role as an anticancer agent and prompted support for the numerous phase I and

phase II studies that followed[85]. Nevertheless, systemic TNF-α has not yet been translated to a patient therapy mainly due to elevated toxicity and lack of efficacy.

Although the anti-tumoral roles of IFN-γ (type II interferon) are well described, type I interferons (IFN-α and IFN-β) also play a role in tumour immunosurveillance. These are ubiquitously expressed and signal through the interferon type I receptor. Preclinical mechanistic experiments revealed that host type I IFN signalling was necessary upstream for spontaneous $CD8^+$ T cell response against tumour antigens in mice[86]. The mechanism of this effect was predominantly through the action of host type I IFNs on $CD8\alpha^+$ dendritic cells (DCs), the subset involved in cross-presentation of antigens to $CD8^+$ T cells. The therapeutic effect of IFN-α was explored in several cancer clinical trials, most importantly in melanoma[87]. A meta-analysis of the available published data from randomized clinical trials reported an event-free survival and overall survival in patients with high-risk melanoma treated with IFN-α adjuvant therapy[88]. To date, the mechanism of the therapeutic effects of IFN-α is not completely known but the examination of the nodal tumour taken before and after treatment, revealed increased infiltration of the tumour tissue by dendritic cells and T cells; and a striking ablation of STAT3 expression that is typically constitutively active in melanoma[89].

TGF-β is a potent cytokine that regulates cell proliferation, differentiation and apoptosis. During tumourigenesis, two distinct roles have been reported for this cytokine: it has been reported as a tumour suppressor at early stages of the disease, and as a tumour promoter at later stages. These dual effects may be determined by the cell type where TGF-β acts as well as by the tumour microenvironment[90]. Another important cytokine that modulates immune responses at the tumour microenvironment is IL-10. The biological role of IL-10 in cancer is quite complex; however its role in promoting tumour progression is evidenced by the presence of IL-10 in advanced metastases and by the correlation of serum IL-10 levels to progression of disease[91]. Several suppressive cells, including TAMs, MDSCs and Tregs are important sources of TGF-β and IL-10.

The control of leukocyte trafficking is determined by a complex network of interactions involving tissue-specific integrins and chemokines, with selectins and chemokine receptors expressed on the leukocytes. It is now widely accepted that leukocytes are localized both in the tumour-supporting stroma and the tumour areas (and actually may account for up to 50% if the tumour mass) and that their migration from lymphoid tissues to tumours is determined by chemokines (Fig 2).

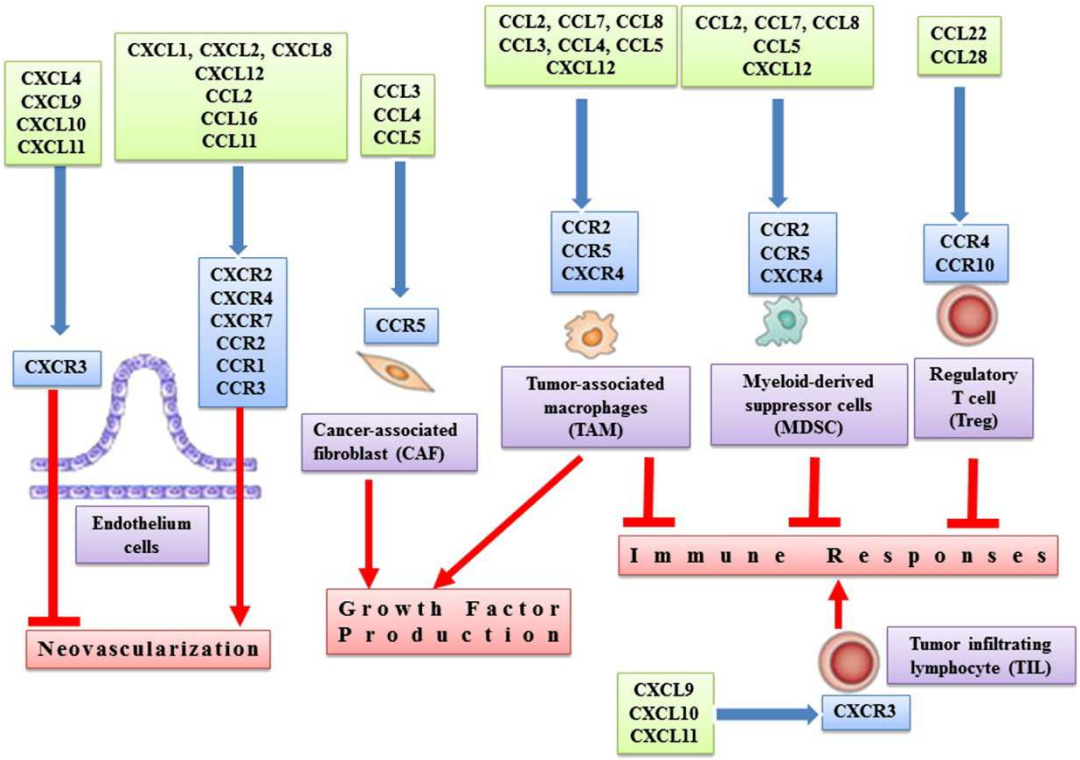

Figure 2 - Chemokine-mediated interaction between tumour cells and stromal cells. Adapted from Mukaida *et al.* 2012[92]

Several reports support a critical role for CXCR3, a receptor for CXCL9/10/11 chemokines, in T cell recruitment to tumours. In mouse and human melanoma, CXCR3 ligands and CCL5 synergize to promote T-cell infiltration into cutaneous lesions[93]. In hepatocarcinoma, inflammatory cytokines such as TNF-α and IFN-γ, or TLR ligands, induce the production of CXCL10 and CCL5 in tumour epithelial cells or tumour-infiltrating immune cells[94]. Importantly, CXCL9/10 were associated with better survival for colorectal cancer patients[95] and positive outcome in ovarian cancer patients[96].

By contrast to CXCR3 ligands, usually correlated with good prognosis for cancer patients, some chemokines predict a poor outcome mostly due to the attraction of potentially suppressive cells. Several lines of evidence indicate that CCL2 plays an important role in TAMs and MDSCs recruitment. For example, CCL2 recruitment of TAMs to breast, prostate and colon carcinoma correlated with negative prognostic value for patients[97]. Also, CCL2 seems to recruit MDSCs in several types of mouse tumour models, including Lewis lung carcinoma, methA sarcoma, melanoma and lymphoma[98].

A large number of Treg cells often infiltrate into tumours and systemic removal of Treg cells enhances natural as well as vaccine-induced anti-tumour T cell immunity. Tregs usually express CCR4, and its ligand, CCL22, regulates intratumoral Treg infiltration into various tumours[99].

Overall, chemokines are thought to be promising candidates for immunomodulatory strategies in cancer, and several clinical trials are underway[100].

1.1.4 TILs as prognostic factors

Although a favorable association of high numbers of TILs in primary tumours had been generally reported for decades in many human cancers, TILs had never reached the level of recognized prognostic marker (or proof for tumour immunosurveillance) probably due to their phenotypic and functional heterogeneity[101]. The recent observations that specific immune parameters have better prognostic value than standard staging systems, highlights the importance of the endogenous immune response in determining the clinical outcome. This may help to modify current classifications, and - most importantly - to identify the patients who would benefit the most from adjuvant immunotherapy (Table 1).

Considering the data reviewed above, it is tempting to assume that good prognosis associates (for example) with $CD8^+$ and NK cells, whereas bad prognosis is linked to the accumulation of Tregs and MDSCs. Moreover, given the functional heterogeneity within many leukocyte populations, clearly distinct outcomes could be expected from Th1 versus Th2 or Th17 $CD4^+$ subsets, M1 versus M2 macrophages, N1 versus N2 neutrophils. This level of refinement is obviously incompatible with traditional immunohistochemistry of cancer patient samples, thus requiring additional techniques like flow cytometry and molecular biology to provide an adequate characterization of tumour-infiltrating leukocytes. Furthermore, detailed imaging may also be important as to define the localization of TILs within the tumour mass. For example, in a pre-clinical model, $CD8^+$ T cells were recently shown to be trapped in the stroma and thus excluded from the core of tumour due to post-translational modifications (nitration) of the chemokine CCL2[102]. Interestingly, novel drugs that inhibited CCL2 nitration facilitated $CD8^+$ T cell infiltration and tumour regression

Table 1 - Tumour-infiltrating leukocytes associated with prognosis for cancer patients. Adapted from Lança & Silva-Santos 2012[103]

	Good prognosis		Bad prognosis	
TIL	**Cancer type**	**Reference**	**Cancer type**	**Reference**
$CD8^+$	Colorectal cancer Hepatocellular carcinoma Esophageal carcinoma Breast cancer	104 105 106 94 107 108 105, 106, 109		
Th1 ($CD4^+$)	Colorectal cancer Hepatocellular carcinoma Breast cancer	110 94 111		
Th2 ($CD4^+$)			Pancreatic cancer	112
Th17 ($CD4^+$)	Esophageal carcinoma	109	Colorectal cancer Hepatocellular carcinoma Prostate cancer	110 113 114
Tregs ($CD4^+$)			Colorectal cancer Hepatocellular carcinoma Ovarian carcinoma Breast Cancer	115 107 116 117
γδ T cells	Ovarian carcinoma	118	Breast Cancer	119
B cells	Breast cancer	120		
NK cells	Esophageal carcinoma Hepatocellular carcinoma	109 94		
MDSCs			Esophageal, pancreatic and gastric cancer	121
Macrophages			Breast cancer	122
Neutrophils			Renal cell carcinoma	123

The most comprehensive clinical studies correlating tumour-infiltrating leukocytes with disease outcome have been performed in colorectal cancer, where the general conclusion has been that disease free overall survival is positively associated with a coordinated Th1/ $CD8^+$ T cell infiltration[101] (Table 1). A similar result was reached for breast cancer[120, 124] and for hepatocellular carcinoma, where NK markers and the chemokines CCL2, CCL5 and CXCL10 were additional immune signatures predictive of patient survival (at early stages of the disease)[94].

By contrast, Treg infiltration has been generally associated with poor prognosis (Table 1). In ovarian carcinoma, melanoma, breast cancer, Hodgkin lymphoma and glioblastoma, the presence and frequency of Tregs correlated with tumour grade and with reduced patient survival[74]. These studies also highlighted the potential role for CCL17 and CCL22 (ligands for the chemokine receptor CCR4) in recruiting Tregs into tumours. The combined value of quantifying both $CD8^+$ and Treg TIL (antagonistic) subsets as prognostic of disease-free survival was demonstrated in hepatocellular carcinoma[107, 108] and colorectal cancer[104]. However, in some cancer types, such as colorectal[115] and head and neck carcinomas[125], Treg accumulation within tumours has been associated with favorable prognosis. This was suggested to be due to a dominant effect in suppressing infection-associated inflammation at mucosal interfaces[124]. Nonetheless, positive associations between survival and Treg numbers were also observed upon immunohistochemical analysis of biopsies from four types of lymphoma patients[126, 127].

Whereas Th2 infiltration has been associated with poor prognosis in pancreatic cancer[112], the role of Th17 TILs in human cancer is much more controversial. On one hand, Th17 cell infiltration has been correlated with poor prognosis in prostate cancer[114] and in hepatocellular carcinoma[113]; on the other, it has been associated with better overall survival in ovarian cancer[96] and in esophageal squamous cell carcinoma[109]. While the reasons for these discrepancies are unclear, it may be interesting to assess the co-production of IL-17 and IFN-γ by Th17 cells, as well as their association with $CD8^+$ T cell recruitment.

Finally, a recent study attempted to integrate the prognostic values of Th1, Th2 and Th17 TILs by hierarchical clustering of signature gene transcripts in colorectal tumour specimens. The results showed that: the Th2 cluster did not correlated with prognosis; patients with high expression of the Th1 cluster had prolonged disease-free survival; and patients with high expression of Th17 cluster had poor prognosis[110]. In the future, we believe that will be highly informative and important to collect similar data in many other cancer types.

1.1.5. Principles of cancer immunotherapy

The first serious attempts to actively use the immune system to eradicate tumours were applied as early as in the 1890s by Coley, who treated sarcoma patients with bacterial preparations, commonly referred to as “Coley´s toxin” (Coley WB. The treatment of malignant tumours by repeated inoculations of erysipelas: with a report of ten original cases. Am J Med Sci. 1893). Acute activation of the

patient's immune system with "Coley´s toxin" was reported to lead to tumour regression in some cases. Coley´s strategy was lather abandoned due to very real risks associated with the administration of infectious agents to already weakened cancer patients (Fig. 3).

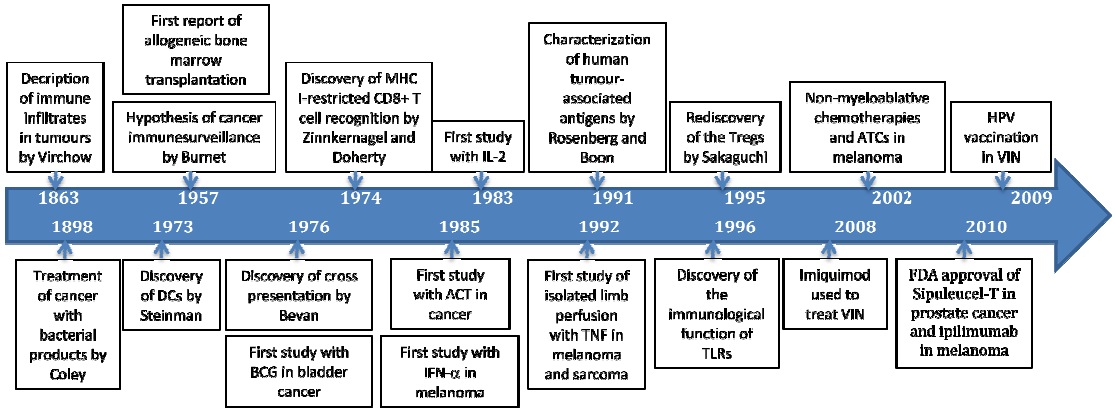

Figure 3 - Timeline: the history of cancer immunotherapy. Important basic immunological discoveries and key clinical trials are shown. DC, dendritic cells; MHC, major histocompatibility complex; ACT, adoptive cell transfer; IFN-α, interferon-α; BCG, bacilli Calmette-Guérin; IL-2, interleukin 2; TNF, tumour necrosis factor; Tregs, T regulatory cells; TLRs, Toll-like receptors; VIN, vulvar intraepithelial neoplasia; HPV, human papillomas virus. Adapted from Lesterhuis *et al.* 2011[128]

More recently, inspired by the discovery of tumour-specific antigens or tumour-associated antigens, which can be recognized by αβ T cells, several groups have attempted to activate adaptive immune cells in order to elicit anti-tumour immune responses[129]. Efforts have focused on generating MHC-restricted, tumour specific αβ T cells, through vaccination, *ex vivo* activation or expansion of cytotoxic lymphocytes, or various methods of redirected cytolysis. Nevertheless, despite the promise of immunotherapeutic strategies and numerous clinical trials that have utilized immunobased therapies for the treatment of patients with cancer, the elicitation of consistent clinical responses has been disappointing for almost 30 years. The efficacy of conventional αβ T cell therapy continues to be limited because tumour neoantigens are usually weak immunogens (except in some cases of viral transformation where virus antigens are expressed on malignant cells). Further, MHC molecules, tumour-associated antigens and costimulatory molecules can be downregulated to evade detection, and tumours can inactivate responding T cells. However, the tide has finally changed due to the success of recent proof-of-concept clinical trials[130] using three main clinical strategies: antibody therapy, vaccines and adoptive cell transfer (ACT).

A key development in cancer immunotherapy was use of the monoclonal antibody anti-CTLA-4, Ipilimumab. Results from phase III trials in late-stage melanoma showed not only a clear survival advantage for the patient group with no other therapeutic option, but notably, it was achieved with an agent whose

mechanism of action involves modulation of endogenous T-cell responses[130]. Like CD28, CTLA-4 binds to B7 receptors (CD80 and CD86) on antigen-presenting cells. It initiates inhibitory effects upon binding, including cell cycle arrest and decreased cytokine production. More importantly, some forms of the B7 receptor show dramatically increased binding affinity for CTLA-4 over CD28, as a single CTLA-4 receptor can bind as many as eight B7 molecules. A balancing act thus occurs between CD28/B7 mediated activating signals and CTLA-4/B7 inhibitory signals that strongly impacts on T cell function[131].

Programmed death 1 (PD-1) protein is another T-cell inhibitory receptor with a structure similar to that of CTLA-4 but with distinct biologic functions and ligand specificity. PD-1 has two known ligands, PD-L1 (B7-H1) and PD-L2 (B7-DC). In contrast to CTLA-4 ligands, PD-L1 is selectively expressed on many tumours[132]. Inhibition of the interaction between PD-1 and PD-L1 can enhance T-cell responses *in vitro* and mediate preclinical anti-tumour activity[133]. Recent phase I studies using blocking antibodies for PD-1L[134] and for PD-1[135], showed a striking anti-tumoral effect when blocking this pathway, in patients with advanced cancer, including non-small-cell lung cancer, melanoma and renal-cell cancer.

The results obtained so far in the clinic, with antagonists for CTLA-4 and PD-1 (or PD-1L), open a new perspective in cancer immunotherapy, by using T cell costimulation blockade.

Although antibody therapy targeting T cell costimulation is now giving his first steps, passive immunotherapy that uses cancer-associated antigens monoclonal antibodies, has achieved broad therapeutic efficacy. These include Her2/neu, VEGF, CD20, CD52 and CD33 which are approved by FDA, for treatment of solid and haematological malignancies[130].

Cancer vaccines come in two formats: prophylactic and therapeutic. Prophylactic (or preventive) vaccines have been used with considerable success for the prevention of cancers of viral origin, such as hepatitis B virus and human papillomavirus (HPV), where the aetiological agent is known. In contrast, the development of therapeutic vaccines to treat existing cancers has proved problematic[130]. An exception exists for the first therapeutic vaccine that was approved by the FDA, Sipuleucel-T. This vaccine was approved based on results from a placebo-controlled Phase II randomized trial. Sipuleucel-T is used for the treatment of metastatic castration-resistant prostate cancer and consists of autologous PBMCs, which are activated *in vitro* with a fusion protein (PA2024) of the prostate antigen prostatic acid phosphatise and the immunostimulant GM-CSF[128].

Other strategies, using the administration of immunostimulatory cytokines, such as IL-2 (Proleukin, FDA approved) and GM-CSF are also under clinical trials.

The adoptive cell transfer (ACT) of tumour antigen-specific T cells is also under clinical evaluation. Usually the protocol includes the infusion of *ex vivo* expanded cells (autologous or allogeneic) that may traffic into the tumour and mediate its destruction. These immunotherapeutic protocols can be based on the adoptive transfer of naturally occurring or gene engineered tumour-specific T

cells[136]. ACT has emerged as the most effective treatment for patients with metastatic melanoma. Of patients with metastatic melanoma refractory to all treatments, 50% will experience an objective response, some with complete responses[137]. The success of ACT had a decisive improvement after the introduction of an immunodepleting preparative regimen given before the adoptive transfer[138].

One way to engineer tumour-specific T cells is by transducing them with CARs (chimeric antigen receptors)[136]. This technology consists on the cloning of variable regions of antibody genes fused with intracellular domains that are capable of activating T cells. Thus, CARs recognize MHC-unrestricted structures on the surface of target cells, whereas TCRs recognize mainly intracellular antigens that have been processed and presented as peptides complexes with MHC molecules[136]. Recently, the feasibility of this technology was demonstrated in the treatment of a patient suffering from chronic lymphocytic leukaemia (CLL) with CAR autologous-modified T cells[29]. The CAR was specific for the B-cell antigen CD19 and was coupled with CD137 (a costimulatory receptor in T cells (4-1BB) and CD3-zeta (a signal-transduction component of the T-cell antigen receptor. Reinfused engineered T cells expanded *in vivo,* persisted at high levels for six months and the patient experienced remission that lasted at least ten months after treatment[29].

Still, these protocols are technically challenging and the promising data obtained in terms of objective clinical responses from small clinical trials will need to be further confirmed in large multicenter clinical trials[136].

We believe the best hope to eradicate cancer relies on a combinatorial treatment strategy, where intrinsic pathways regulating neoplasic cell survival are targeted, in combination with therapies (as the ones mentioned before) affecting both extrinsic pathways that boost effective cytotoxic T cell responses, and limit or normalize harmful pro-tumoral immunity.

1.2 Role of γδ T cells in tumour immunity

The γδ lineage of T lymphocytes was first described in the mid 1980´s with reports of a new heterodimeric T cell receptor that was associated with CD3[139-141]. Since then, γδ T cells have been intensively studied, in a global effort to unravel their development, antigen recognition, activation and function.

γδ T cells are typically regarded as a "bridge" between the innate and adaptive immune responses[142 143] . On one hand, γδ T cells may be considered a component of the adaptive immune system in that they rearrange TCR genes to produce junctional diversity and antigen-specific immune response concomitant with the develop a memory phenotype. On the other hand, various γδ T cell subsets may also be considered part of the innate immune system, due to restricted oligoclinal TCR usage and very rapid responses upon primary challenge.

The physiological roles fulfilled by γδ T cells are varied and include protective immunity against extracellular and intracellular pathogens, tumour surveillance, and modulation of innate and adaptive immune responses, tissue healing and epithelial cell maintenance[144]. These non-redundant roles rely on the provision of an immediate source of cytokines, chemokines, cytotoxicity and other functions that can substantially affect downstream responses[143]. Moreover, a combination of antigen specificity, tissue distribution and functional properties, rather than in any of these individually is essential for the pleiotropic γδ T cell immune response[144].

Throughout this thesis we will use the Vγ gene nomenclature of Heilig and Tonegawa[141] for rodent γδ T cells; and Lefranc and Rabbits[145] for human γδ T cells.

1.2.1 Thymic development of γδ T cells

Murine γδ T cells develop in the thymus in waves that sequentially populate different tissues by regulated expression of appropriate chemokines receptors (Figure 4). Mouse thymocytes bearing and invariant canonical Vγ5Vδ1 TCR at embryonic day E15-17 are the first to leave the mouse fetal thymus, giving rise to skin associated dendritic epidermal T cells (DETCs); thymocytes bearing a Vγ6Jγ1Cγ1 TCR at E16-18 give rise to the γδ T cells in the tongue and reproductive tract; peri- and postnatal thymocytes bearing Vγ1Cγ1 and Vγ4Cγ1 TCRs give rise to systemic γδ T cells. This sequential generation of γδ T cells at different stages of ontogeny is a fixed developmental program, and the disruption of the generation of γδ T cells in the early foetal thymus by the administration of an anti-γδ-TCR antibody into pregnant mice, resulted in the selective absence of DETCs in adult mice[146].

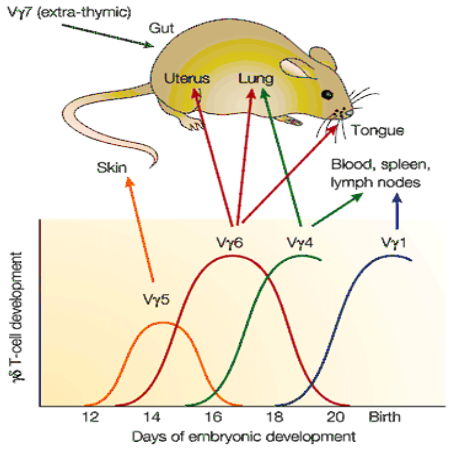

Figure 4 - Mouse γδ cell generation is developmentally programmed. Adapted from Carding & Egan 2002[147]

Similarly, in the human fetal thymus, the first γδ T cells to emerge use the Vδ1 chain (paired with various Vγ chains), an these preferentially populate epithelial tissues such as the intestine[148]. Latter, from a different set of progenitors, the human peripheral blood Vγ9Vδ2^{+} subset emerges, and expands significantly in the peripheral blood during childhood.

For γδ T cells (unlike for αβ T cell thymic development) the criteria for negative selection are poorly defined. Many of the known γδ T cell antigens in both human and mice are self or ubiquitous molecules, making negative selection for these cells questionable and unresolved.

1.2.2 TCRγδ repertoires and functions

γδ T cells express a unique type of TCR which has been strongly conserved across 400-500 million years of evolution of jawed vertebrates: cartilaginous but not extant jawless fish have it. Despite the TCRγ and TCRγ genes being highly conserved in terms of general organization, Vγ genes diverge considerably between species: the TCRγ locus in mice contains seven commonly utilised genes; as it does in humans (Table 2). On the contrary, there are twenty to thirty chicken Vγ chain gene segments, and more than six Vγ families in skate[142]. The complexity of TCRγδ genes correlates with the abundance of γδ T cells: in adult mice they account for 0.5-2% of peripheral lymphocytes, in human blood they can range between 1.5-15%, whereas in young ruminants they can account for more than 70% of the peripheral CD3^{+} cells, declining to 5-25% with age[142].

Although, theoretically, a great diversity of TCRγδ can be produced in rodents and humans, the set of TCRs detected on peripheral γδ T cells is far more limited. Individual γδ T cell subsets in particular tissue locations show biased use of certain

TCR V gene segments and, in some cases, express "invariant" TCR with identical (canonical) junctional sequences[144] (Table 2).

Table 2- Frequency, distribution and repertoires of γδ T cells. Adapted from Bonneville *et al* 2010[144]

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 Vγ7Vγ6	Intermediate
	Uterovaginal epithelial	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

1.2.2.1 Mouse γδ T cell subsets

It is thought that the highly restricted TCRs expressed by different subsets of γδ T cells enable them to recognize ligands that are specifically expressed in infected or stressed cells in particular anatomical sites where these cells populate.

For example, epidermal intraepithelial Vγ5Vδ1+ (DETCs) cells have been shown to carry out distinct functions which are not typical of other γδ T cells, such as production of keratinocyte growth factor, which plays an important role in wound healing. These cells form a dendritic network that is unique among T cells, but similar to that of Langerhans cells, the antigen-presenting cells of the epidermis. In physiological states, DETCs constitute more than 90% of the epidermal T cells, with virtually no TCR diversity[149].

Vγ6Vδ1+ T cells comprise the vast majority of the intraepithelial lymphocytes of the tongue and reproductive tract. Moreover, these cells seem to play an important role in tissue remodelling at the maternal-fetal interface[150].These cells were also shown to mainly produce IL-17 during pulmonary inflammation thus preventing lung fibrosis[151].

Cells that express the Vγ7 TCRγδ (usually paired with Vδ4 or Vδ5) are typically found as IELs (intestinal epithelial lymphocytes), in gut epithelia and show cytoprotective, immunomodulatory and antibacterial functions. These protective

functions are associated with the production of epithelial cell trophic factors, inflammatory cytokines (such as IL-2 and IFN-γ) and cytotoxic molecules[152].

Cells that express Vγ1 and Vγ4 constitute the major peripheral re-circulating γδ T cell subsets of the blood and lymphatics. Vγ1 cells are capable of killing Listeria-infected macrophages via Fas/Fas ligand[153], and were also shown to promote mouse chronic granulomatous disease[154]. The Vγ4 population tend to be IL-17 biased whereas the Vγ1 population tend to produce IFN-γ[155].

1.2.2.2 Human γδ T cell subsets

Human γδ T cells use three main Vδ and at most six Vγ region genes to make their TCRs[142]. Nevertheless, the actual peripheral γδ TCR combinatorial diversity is even more limited because the TCR V region repertoire of human γδ T cells, as in rodents, is highly skewed in particular tissue locations[156].

The two main populations of human γδ T cells are the Vδ1 and the Vγ9Vδ2 subsets. γδ T cells expressing the Vδ1 chain are most often found in mucosal tissues, where they are thought to be involved in maintaining epithelial tissue integrity on a setting of damage, infection, or transformation[142]. A second population of γδ T cells expresses Vγ9Vδ2 TCR and comprises about 1%-10% of circulating lymphocytes (60-95% of total γδ T cells) in healthy adults. This Vγ9Vδ2 pairing is only present in humans and nonhuman primates[142, 157]. Interestingly, studies comparing the genotypes of γδ T cell clones derived from the thymus with those derived from peripheral blood found that thymic clones possessed nearly all possible Vγ-Vδ combinations, with the Vγ9Vδ2 pairing making up only 5%, indicating selective chronic expansion of this cell population[158]. Such extensive peripheral expansion can be driven by antigens present in environmental microbes and certain edible plants that are encountered by Vγ9Vδ2 during childhood.

Vδ2 and non-Vδ2 (Vδ1 and Vδ3) subsets differ in several aspects. Most Vδ2 cells display a memory phenotype acquired during perinatal life, whereas non-Vδ2 are mainly naïve in young adults[159]. Vδ2 cells express more genes involved in promoting inflammation, such as TNF-α, IFN-γ and IL-21, whereas the Vδ1 cells, express higher levels of L-selectin and CCR7, which suggests that they can home to non-inflamed tissues, whereas Vδ2 cells express higher levels of IL-12 receptor and CCR5, meaning that they can home to sites of inflammation[160]. Vδ1 cells have been found to infiltrate various human epithelial tumours (colorectal, intestinal, breast and esophageal)[161-164]. Moreover, most Vγ9Vδ2 T cells react against a set of non-peptidic, phosphorylated compounds, whereas non-Vδ2 cells seem to recognize heterogeneous poorly defined antigens unrelated to Vδ2 agonists.

Vγ9Vδ2 cells were recently shown to display several features of professional APCs, namely the capacity to present antigens either on MHC-II, or cross present antigens on MHC-I; up-regulation of CD80, CD86, or CD40; the ability to phagocytise

and process antigens; and the ability to activate naïve αβ T cells[165, 166]. Moreover, it was reported that Vγ9Vδ2 cells were more efficient in antigen cross-presentation than monocyte-derived DCs[167]. Nevertheless, all these observations have been made from studies *in vitro*, thus it remains to be determined when and where γδ T cell-APC functions are physiological relevant.

1.2.3.γδ T cell activation: TCRγδ ligands

Immunologists have been searching for TCRγδ ligands for about two decades. However, this proved to be a very difficult task, likely due to the low affinity interactions that prevent biochemical purification of the putative ligands. An important characteristic of γδ T cells is that they do not recognize classical TCR ligands (peptides derived from processed proteins) and do not depend on MHC-presentation, which markedly distinguishes them from αβ T cells.

It is postulated that γδ T cells recognize a diversity of "stress-associated" molecules complexed or not with an antigen-presenting element. As more TCRγδ ligands will become elucidated, it will be interesting to determine whether they comprise molecules whose major function is to regulate immunity (as we conventionally view MHC) or molecules with intrinsic function(s) related to cellular dysregulation, e.g., heat shock proteins[143].

The crystal structure of two independently derived murine TCRγδ (Vγ4 subset) complexed with the T10/T22 (thymus leukaemia, TL, antigens) molecule has been solved[168]. T10 and T22 are murine non-classical MHC class I molecules that are expressed by highly activated cells. Although MHC-I related, T10 and T22 do not present peptides or lipids, being instead recognized as intact proteins via contacts with an extended complementary-determining region (CDR)3 loop of TCRγδ[168, 169].

Human Vγ9Vδ2 cells can be activated through the TCR by small amounts of phosphate containing compounds, called phosphoantigens. These are produced through the isoprenoid biosynthetic pathway[170]. The natural phosphoantigen with highest known bioactivity is 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 *Protozoan* but not by eukaryotes[171].

The intracellular mechanisms of HMB-PP-mediated Vγ9Vδ2 activation were described by our lab[172]. HMB-PP activates MEK/Erk and PI-3K/Akt pathways as rapidly as OKT3 (CD3ε agonist), and induces an almost transcriptional profile, leading to γδ T-cell activation, proliferation and anti-tumour cytotoxicity.

Vγ9Vδ2 T cells can also be activated by much higher concentrations of intermediates of the mevalonate (MVA) pathway used by mammalian cells (and some bacteria), such as isopentenyl pyrophosphate (IPP). IPP activates Vγ9Vδ2 at concentrations 10 to 100 000-fold higher than those molecules derived from microbial peptides[173], which may allow the efficient detection of infected cells

producing very small amounts of microbial phosphoantigens, while preventing activation by normal cells that express basal levels of the weakly stimulatory mammalian metabolites.

Aminobisphosphonates are not themselves able to activate Vγ9Vδ2 T cells; they rather inhibit farnesyl pyrophosphate synthase, an enzyme acting downstream isopentenyl pyrophosphate (IPP) synthesis along the MVA pathway, promoting IPP intracellular accumulation[173, 174].

How precisely the Vγ9Vδ2 cells are activated by phosphoantigens remains elusive. Antibody blocking and gene transfer experiments showed that Vγ9Vδ2 TCR expression is required for activation[175, 176], nevertheless, attempts to show cognate interactions between recombinant soluble Vγ9Vδ2 TCRs and phosphoantigens have not been successful. This notwithstanding, it was observed that respiratory burst and Ca^{2+} signaling events only occur in pelleted Vγ9Vδ2 cells but not when they are maintained in suspension, which denotes that phosphoantigens may not be recognized per se but instead may be recognized in association with a yet undefined surface presenting receptor. One possible presenting molecule may be Ecto-F1 ATPase[177]. Direct binding of the Vγ9Vδ2 TCR to this mithocondrial enzyme ectopically expressed at the cell complexed with a serum protein, Apolipoprotein A1 (ApoA-1) was described by Champagne and collaborators[177].

Surprisingly, the NKG2D ligand ULBP4 was recently reported to bind not only to NKG2D but also to the Vγ9Vδ2 TCR[178]. This study highlighted the importance of this non-classical MHC molecule in γδT-cell-mediated killing of tumour cells and virus infected cells.

Dual recognition of stress and infection is also achieved by human Vδ1 cells, as TCR-dependent response towards both epithelial cell-derived tumours and cells infected have been shown[179]. MICA has been proposed as an important tumour antigen, with recognition of MICA-positive tumour cells by Vδ1-lymphocytes infiltrating colon carcinomas[162, 180, 181]. Nevertheless, the very low affinity of MICA-Vδ1TCR interactions estimated by surface plasmon resonance analyses raises doubts about the functional relevance of MICA recognition by Vδ1 TCRs[182].

Increased circulating numbers of both Vδ1 and Vδ3 subsets are observed in immunosuppressed patients undergoing cytomegalovirus (CMV) reactivation, and a large fraction of clonally expanded Vδ1 and Vδ3 cells from these patients recognizes in a TCR-dependent fashion CMV- infected fibroblast *in vitro*[183, 184]. It was suggested that the putative Vδ1/Vδ3 antigens are not virally encoded but instead correspond to endogenous stress-induced ligands possibly shared by CMV-infected cells and several colon tumours[179]. Moreover, a human Vγ4Vδ5 clone was shown directly bound endothelial protein C receptor (EPCR), which allowed γδ T cells to recognize both endothelial cells targeted by CMV and epithelial tumours. EPCR is a major histocompatibility complex-like molecule that binds lipids analogously to the antigen-presenting molecule CD1d[185].

1.2.4 NKG2D and its ligands

1.2.4.1. NKG2D receptor

NKG2D, natural killer group 2 member D, is an activating C-type lectin receptor expressed on the surface of NK cells, γδ T cells and $CD8^+$ T cells[186] (Table 3).

Table 3 - Expression of NKG2D by immune cells. Adapted from Raulet 2003[186]

Cell type	Cell surface expression pattern	
	Mouse	Human
NK cells	100%	100%
$CD8^+$ αβ T cells	Before activation: absent After activation: ≈100% Ag specific memory cells: ≈100%	Before activation: ≈100% After activation: ≈100% Ag specific memory cells: ≈100%
$CD4^+$ αβ T cells	Rare or absent	Normally absent
γδ T cells	Splenic: ≈25% IELs: absent DETCs: ≈100%	PBL: ≈100% IELs: ≈100%
Macrophages	Before activation: absent After activation (LPS, IFN-α/β/γ): ≈100%	Absent in monocytes and macrophages

NKG2D itself does not possess signaling capacity. In humans, NKG2D exists on the cell surface in complex with the DAP10 adaptor protein that contains a YxxM motif that, upon tyrosine phosphorylation after NKG2D/DAP10 ligation, couples the receptor complex to the PI3K/Grb2-Vav pathway[187, 188]. Murine NKG2D is encoded by two splice variants[189]. The long isoform (mNKG2D-L) associates only with DAP10, whereas the short isoform (mNKG2D-S) associates with DAP10 or DAP12[189, 190]. Moreover, it was shown that TCR ligation in $CD8^+$ T cells upregulates NKG2D/DAP10 cell surface expression[191].

Several mechanisms are known to regulate the cell surface expression of the human NKG2D receptor, including the differential action cytokines. IL-2 and IL-15 stimulation increases NKG2D surface expression[192, 193] by increasing DAP10 mRNA and large upregulation of DAP10 protein synthesis. By contrast, TGF-β1[194-196] and IL-21[197]treatment leads to downregulation of NKG2D expression on human NK and $CD8^+$ T cells.

NKG2D signaling is best described in NK cells, where its crosslinking (on murine NK cells) was shown to trigger several effector mechanisms, such as, intracellular Ca^{2+} mobilization, Th1 cytokine production (IFN-γ, GM-CSF, TNF-α) and release of cytotoxic granules[198, 199].

NKG2D is commonly viewed as a primary activator for NK cells and a costimulator for $CD8^+$ and γδ T cells. Nevertheless, the role of NKG2D in T cells seems controversial, with some authors arguing that NKG2D has solely a costimulatory function whereas others argue that NKG2D signals can activate T cells in the absence

of TCR engagement. Some studies reported the ability of Vγ9Vδ2 T cells to trigger effector responses through NKG2D stimulation alone[200, 201]. However, others have failed to show any Vγ9Vδ2 T cell NKG2D-induced activation without coincident TCR stimulation[202, 203]. For human CTLs, some reports also showed that NKG2D-DAP10 can mediate cytolysis independently of TCR engagement when CTLs are exposed to IL-15 or high dose IL-2[193, 204-206]. The observed discrepancies may be due to different activation status of T cells as a result of different cell culture conditions. Moreover, it was showed recently, in γδ T cells 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 cytotoxicity activity. By contrast, the production of IFN-γ was unaffected by NKG2D costimulation[203].

NKG2D is a critical determinant of tumour immunosurveillance, since NKG2D-deficient mice show increased tumour growth of epithelial and lymphoid tumours in two transgenic models of de novo tumourigenesis[18].

1.2.4.2 NKG2D ligands

NKG2D ligands belong to the MHC class Ib proteins family (also known as non-classical major compatibility complex family), which are usually upregulated on transformed, stressed or infected cells. The MHC class Ib molecules are structurally related to class Ia proteins in they show typical (α1-α2) MHC fold on a single polypeptide, which, in the case of Ib, does not pair obligatorily with β2-microglobulin. Furthermore, although many MHC Ib gene are 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[17]. MHC class I b molecules do not share amino acid sequence similarity across species (are not orthologous), and members include peptide-binding ligands for conventional TCRs, ligands for NK receptors and unconventional TCR ligands[17].

Mouse NKG2D binds to retinoic acid early transcript (Rae1), histocompability antigen 60 (H60) and murine UL16-binding protein-like transcript 1 (MULT1). Human NKG2D binds to MHC I chain-related (MIC) peptides A and B (MICA and MICB), and to UL16-binding proteins (ULBP, members 1-6)[17, 207]. MICA/B, H60 and MULT1 are transmembrane proteins, ULBP1-5 and Rae1 localize to the cell surface using glycosylphosphatidylinositol (GPI) linkages and ULBP6 locates intracellular[207]. None of the NKG2D ligands bind to additional – peptide or lipid- antigens, but rather interact directly with the receptor. In addition, NKG2D ligands do not associate with β2-microglobulin[186], unlike some other members of the MHC class Ib (e.g. HLA-G or CD1d).

NKG2D ligands are usually induced by a variety of signals associated with cellular stress, namely oxidative stress, ionizing radiation, DNA damaging agents, viral infections and intracellular bacterial infections[208]. Nevertheless, the various NKG2D ligands have distinct patterns of expression, indicating that they cannot be considered simply redundant in function.

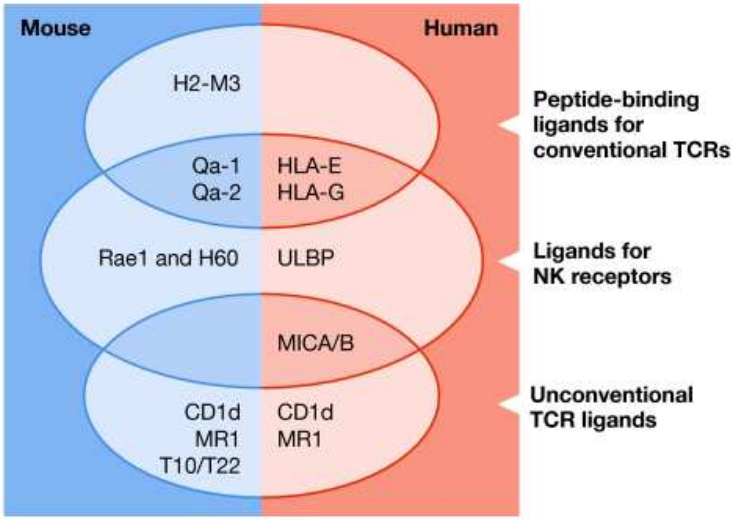

Figure 5 – Non-classical MHC molecules and their receptors. Conventional T-cell receptors (TCRs) are those of polyclonal αβ T cells; unconventional TCRs correspond to oligoclonal T-cell subsets, such as NKT cells (for CD1d), γδ T cells (T10/T22, MICA/B) or gut-associated T cells (MR1). Proposed functional 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. Adapted from Gomes *et al* 2007[17].

Despite the marked differences in their amino-acid sequences, the different ligands interact with NKG2D similarly, and the receptor does not seem to undergo marked conformational changes to accommodate different ligands[209]. So far there is no evidence that the different ligands induce qualitatively distinct biological effects in responding cells, though this remains a possibility. Minimally, the various ligands would be predicted to differ quantitatively in their effects based on the marked differences in their affinity for NKG2D. At present, the relevance of such differences has not been documented.

The murine ligands Rae1 and H60 are rare in healthy adult tissues, but their transcription is strongly induced in keratinocytes after their exposure to carcinogens *in vivo*[8], and they are overexpressed in the cutaneous papillomas and carcinomas that subsequently develop, as well as in various tumour cell lines[189, 210]. The expression of Rae1 or H60 by target cells was shown to enhance cytolysis and the production of IFN-γ by CTLs[211] and γδ T cells[8] leading to tumour rejection in vivo. Moreover, transduction of Rae1, H60 or Mult1 into NK-cell-resistant target cells made them susceptible to NK-cell-mediated killing and stimulated IFN-γ secretion[211, 212].

In contrast with the other mouse ligands (Rae1 and H60), Mult1 is expressed at marked levels by various normal cells at the mRNA level[213], but cell-surface expression is low or has not been documented. For example, C57BL/6 thymocytes contain high levels of Mult1 mRNA, but stain poorly with NKG2D tetramers[214]. However, Mult1 is expressed at functional levels on the cell surface of numerous

tumour cell lines, indicating that these molecules might be regulated at a level other than transcription[214].

The human MICA and MICB proteins show 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[180, 215]. Upregulation of MICA and MICB expression by these cells seems to result from activation of heat-shock transcription elements in the promoters of the corresponding genes, an event known to accompany transformation[180]. Interestingly, heat-shock elements have not been implicated in regulating the expression of Rae1, H60, Mult1 or ULBPs. Atypically for MHC Ib molecules, the MIC genes are highly polymorphic: there are 61 MICA and 30 MICB alleles[186].

Whereas the membrane-bound form of MICA provides stimulatory signals to killer lymphocytes, soluble forms that shed from the cell surface, may downregulate surface NKG2D and impair tumour cytolysis, constituting a an important immune evasion mechanism[216, 217]. Moreover, NKG2D ligands can be expressed by tumour-released exosomes[218] that promote downregulation of surface NKG2D expression by NK and CD8+ T cells. Interestingly, a similar phenomenon occurs in human placenta to avoid immunosupression during pregnancy[219].

Distantly related to the MIC proteins are the members of the ULBP family (Table 4). In contrast with Rae1 or MICA, ULBPs are expressed at significant levels in a wide range of healthy tissues and cell lines of both epithelial or nonepithelial origin[220, 221].

Table 4 – Percentage of amino acid identities between human NKG2D MICA/B and ULBPs. Adapted from Cosman *et al* 2001[220]

	ULBP1	**ULBP2**	**ULBP3**	**MICA**	**MICB**
ULBP1	100	60	55	23	23
ULBP2		100	55	26	25
ULBP3			100	25	27
MICA				100	83
MICB					100

Ectopic expression of ULBP1 or ULBP2 on murine EL4 or RMA tumour cells elicits potent anti-tumour responses to syngeneic B6 and SCID mice, recruiting NK, NKT and T cells to the tumour[222]. Similarly, tumour cells that are insensitive to NK cells can be lysed effectively when transfected with ULBPs[223]. Moreover, tumour cell susceptibility to current first line treatment to non-Hodgkin lymphoma, Ritumixab, was shown to greatly depend on ULBP 1-3 expression[224].

Cancer cells can also shed proteins of the ULBP family. ULBP2 is secreted both from tumour cell lines and primary tumour cells from patients and sera soluble ULBP2 was shown have poor prognosis value in melanoma patients[225]. Other studies also correlate NKG2D ligands expression with cancer clinical prognosis, for example loss of ULBP1 in hepatocellular carcinoma correlates with tumour progression and

early recurrence[226] whereas expression of MICA/B and ULBP2 in breast cancer is an independent prognostic parameter for relapse free period[227].

The expression of human NKG2D ligands seems to be modulated by proteasome regulation. For example, in head and neck squamous cell carcinoma (HNSCC), Bortezomib (an approved drug for treatment for plasma cell myeloma) and other proteasome inhibitors with distinct mechanisms of action, dramatically and specifically up-regulated ULBP1mRNA and cell surface protein expression. In different types of tumours, such as hepatocellular carcinoma, low dose proteasome inhibitor drugs caused cells to up-regulate MICA and MICB, but not ULBP1-3[228]. In contrast, other report showed that several proteasome inhibitor drugs increased ULBP2 levels on Jurkat surface T cells, whereas MICA, MICB, ULBP1, 3 and 4 were not affected[229].

Moreover, both murine and human non-tumour cell lines may up-regulate NKG2D ligands in response to DNA-damaging agents and DNA synthesis inhibitors. The activation of the DNA damage pathway is frequently activated in tumour cell lines, possibly due to the greater genomic instability of these cells compared with transformed cells[208].

Other mechanisms of NKG2D ligand expression regulation include: differences in promoter sequences of the several ligands[230] ; cytokine treatment, for example, TGF-β decreased transcription of MICA, ULBP2 and ULBP4 in human gliomas[231, 232] and IFN-γ decreased MICA message levels in melanoma[233]; and induction of p53, that lead to upregulation of ULBP1 and ULBP2 at the tumour cell surface[234].

An open question in the field is why there are so many ligands for the NKG2D receptor. It is possible that the several ligands stimulate NKG2D positive cells to respond to different forms of stress because they are able of being expressed independently of each other[220, 221, 230], and because they engage NKG2D with different affinities, suggesting that NKG2D ligands may not be functionally equivalent.

1.2.5 Other important receptors in γδ T cell activation

Although the TCR and NKG2D play central roles in the activation of γδ T cells, their response to tumours may involve other receptors, particularly Natural Cytotoxicity receptors (NCRs). These are activating receptors that include NKp46[235, 236], NKp30[237] and NKp44[238, 239].

A recent study from our lab, demonstrated that human Vδ1 T cells can be selectively induced to express NKp30, NKp44 and NKp46[240]. Importantly, specific gain-of–function and loss-of-function experiments showed that NKp30 makes the most important contribution to TCR-independent leukaemia cell recognition.

Moreover, the Vδ1 NKp30$^+$ subset is able to target primary haematological tumours highly resistant to fully activated Vγ9Vδ2 PBLs.

NKp44 seems to be involved in Vγ9Vδ2 cytotoxicity against multiple myeloma cell lines lacking expression of NKG2D ligands. However, the percentage of NKp44$^+$ γδ T cells in culture was very low[241], thus raising the question about the biological importance of NKp44 expression on Vγ9Vδ2 T cells.

Another important NK receptor is DNAX accessory molecule-1 (DNAM-1 or CD266), a transmembrane glycoprotein that associates with LFA-1. Its ligands include PVR and Nectin-2. In NK cells, DNAM-1 has a role in tumour cell recognition together with NCRs and to a lesser extent with NKG2D[242]. Moreover, the Vγ9Vδ2 subset was shown to express DNAM-1, and upon recognition of ligands expressed by hepatocellular carcinoma cells, DNAM-1 signals increase Vγ9Vδ2 cell cytotoxicity and IFN-γ secretion[243].

Outside the NK receptor family, the TNF receptor superfamily member, CD27, has also been implicated in γδ T cell activation. Surface expression of CD27 increases upon T cell activation through TCR/CD3 complex, with either phytohemagglutinin (PHA) or anti-CD3 monoclonal antibodies[244]). The ligand for CD27 is CD70, and the interaction between these molecules provides a potent second signal for cytokine production, induction of activation markers and proliferation of primed and unprimed peripheral blood lymphocytes[245]. Recent work from our lab[246] demonstrated that CD70-CD27 interactions in Vγ9Vδ2 T cells associates with enhanced survival and proliferation upon activation, and promoted Th1-like responses (IFN-γ, TNF-α and LT-α) of Vγ9Vδ2 T cells. These data were also validated in mice, where CD27 costimulation was shown to control the expansion of IFN-γ-producing γδ T cells *in vivo*[155].

1.2.6 γδ T cell response to tumours

1.2.6.1 Anti-tumour properties

γδ T cells can kill transformed cells, through pathways that involve the engagement of death-inducing receptors, such as CD95 (also known as FAS) and TNF-related apoptosis-inducing ligand receptors (TRAILR), and the release of cytotoxic effector molecules such as perforin and granzymes[171]. Murine IELs, activated DETCs, and human Vγ9Vδ2 cells primarily express granzymes A and B at levels substantially higher than conventional CD8$^+$T cells. Moreover, a significant fraction of Vγ9Vδ2 cells express intermediate levels of CD16 and thus γδ T cells can improve antibody-dependent cell mediated cytotoxicity (ADCC)[247].

The importance of murine γδ T cells in tumour immunosurveillance was first described in 2001 by a seminal paper from the Hayday lab. They showed that γδ-deficient mice were highly susceptible to multiple regimens of cutaneous

carcinogenesis. Moreover, they observed that the γδ T cell response in WT mice was determined by NKG2D recognition of Rae-1 and H60 molecules, expressed by skin tumour cells. This work further revealed that γδ T cells not only inhibited the early stages of papillomas development but also limited their progression to carcinomas[8].

Table 5 - Mouse tumour models implicating γδ T cells in tumour immunosurveillance. MCA, methylcholanthrene; DMBA, dimethylbenanthracene; TPA, 12-O-tetra-decanoylphorbol; β2m, β-2 microglobuin; pfn, perforin; TRAMP, transgenic adenocarcinoma mouse prostate cancer

Spontaneous tumours	Chemical carcinogen-induced-tumours	Transplantable tumour cell lines	Tumour type	Reference
	MCA, DMBA+TPA	PDV	Skin fibrosarcoma Squamous cell carcinoma	8
	MCA	B16-F0	Skin fibrosarcoma Squamous cell carcinoma	248
	DMBA +TPA		Squamous cell carcinoma	249
b2m$^{-/-}$ *pfn* $^{-/-}$			Spontaneous B cell lymphomas	250
TRAMPx *Tcrd* $^{-/-}$			Prostate carcinoma	251
	MCA		Skin fibrosarcoma	252

In the murine B16 melanoma model, γδ T cells were shown to infiltrate tumour lesions already at day 3 post-transplantation and to provide a critical early source of IFN-γ[248]. By using bone marrow chimeras and fetal liver reconstitution experiments, the authors showed that IFN-γ production by γδ T cells seems to be required to control the growth of both MCA-induced tumours and B16 melanoma tumours. This ability of γδ T cells to produce IFN-γ was crucial for the subsequent αβ T cell activation and differentiation. Thus, depletion of γδ T cells resulted in significantly reduced IFN-γ production by both $CD4^+$ and $CD8^+$ T cells upon challenge with tumour lysates[248]. The direct comparison of protective properties of γδ T cells and αβ T cells was addressed in other chemical carcinogen induced tumours, namely squamous cell carcinoma[249]. While papilloma development was comparable in WT and *Tcrb*$^{-/-}$ mice, it was highly accelerated in *Tcrd*$^{-/-}$ and in the double-knockout mice, *Tcrb*$^{-/-}$*d*$^{-/-}$. This study revealed that γδ T cells are strongly protective, whereas the contribution of αβ T cells for tumour progression control is more modest.

Subsequent studies also using carcinogen–induced- skin tumours reinforced the non-redundant anti-tumoral role of γδ T cells[252-254]. Moreover, by backcrossing TCRδ$^{-/-}$ mice with TRAMP (transgenic adenorcarcinoma mouse prostate cancer) mice,

Liu and colleagues showed that γδ T cells limit the development and progression of spontaneously arising mouse prostate cancer[251]. The authors also assessed the possibility of developing an adoptive cell therapy, by treating TRAMP-C2 sub-cutaneous tumour bearing mice, with adoptively transferred γδ T cells. Treated mice with supraphysiological numbers of WT γδ T cells develop measurably less disease compared with untreated mice[251].

γδ T cells were also characterized as prototypic anti-tumour mediators in B cell lymphomas. Peng and colleagues showed that B cell lymphomas arose with higher frequency in Fas mutant lpr mice that were additionally deficient for γδ T cells[255]. Moreover, γδ T cells were present in great numbers around B cell tumour masses in the spleens of *pfp*$^{-/-}$ mice[250]. Also, in this work, both γδ T cells and NK cells were shown to display potent cytotoxicity against spontaneously arising MHC class I-deficient B cell lymphomas.

Studies in mice have thus provided important clues to the physiological roles of γδ T cells, but owing to the differences between mouse and human γδ T cell subsets, these studies have not generally predicted the behavior of human γδ T cells[144].

This notwithstanding, both main subsets of human γδ T cells, Vγ9Vδ2 and Vδ1 cells, have been shown to lyse *in vitro* a broad range of tumour cell lines. The Vγ9Vδ2^{+} subset has been more widely studied than the Vδ1 subset, probably do the easiness of isolation, as they comprise most of the γδ-PBLs. They have been shown to display potent cytotoxicity towards several cell lines of different origins, e.g. B-cell lymphoma[256], esophageal carcinoma[164], multiple myeloma[257], prostate[251], breast cancer[258] and recently also towards cancer stem cells[259, 260]. However, the frequency of Vδ2 cells within lymphocytes infiltrating solid tumours is generally low, even within Vγ9Vδ2-suscepible tumours such as renal and colon carcinomas[261, 262] . By contrast, Vδ1 cells are quite frequent within T cells infiltrating solid tumours[262, 263].

Vδ1-tumour infiltrating lymphocytes from colorectal cancer were shown to lyse autologous and allogeneic colorectal, renal and pancreatic tumour cell lines[161]. Moreover, circulating Vδ1 from chronic lymphocytic leukaemia patients were shown to lyse B-CLL cells expressing ULBP3[264]. Interestingly, Vδ1 cells shared reactivity towards CMV infected cells and tumour intestinal epithelial cells[179]. Our lab has also recently demonstrated that fully-activated (with PHA and IL-2) Vδ1 cells display stronger cytotoxicity against B-CLL cells than the corresponding Vδ9Vδ2 counterparts, which was attributed to the selective induction of NCR expression (see 1.2.5) in Vδ1 cells[240].

1.2.6.2 Pro-tumour properties

The potent anti-tumoral properties of γδ T cells have been widely shown for more than 15 years. This notwithstanding, some recent studies imply a pro-

tumourigenic role for γδ T cells, e.g. γδ T cell depletion reduced papilloma incidence[265] and breast tumour infiltrating-γδ T cells suppressed naive and effector T cell responses and blocked maturation and function of dendritic cells[163].

This controversial function of γδ T cells may rely on their production of IL-17; while a study showed that IL17-producing γδ T promote tumour growth in a murine fibrosarcoma tumour model[42], it was shown that IL17-producing γδ T cells are necessary for BCG treatment of bladder cancer[266] and for chemotherapeutic efficacy in sub-cutaneous tumour models[43]. Actually, the role of IL-17 in tumour surveillance is itself paradoxical. IL-17 production has been associated with enhanced tumour development/ progression in murine models of intestinal[39], skin[38], bladder[37] and ovarian carcinoma[267]. By contrast, IL-17 deficient mice were more susceptible to the development of lung melanoma[33] and lung metastasis[34].

A more detailed characterization of γδ-TILs, in a wider set of preclinical tumour models is thus required to clarify the role of IL-17-producing γδ T cells in tumour immunosurveillance. This should take into account the two functional γδ T cell subsets recently identified in our laboratory: $CD27^+$ γδ T cells make IFN-γ but no IL-17, whereas IL-17 production is restricted to $CD27^-$ γδ T cells[155].

1.2.6.3 Cancer clinical trials

Several features of γδ T cells make them attractive targets for cancer immunotherapy: abundant IFN-γ secretion; potent, broad and MHC-unrestricted cytotoxicity; and the availability of clinical grade agonists for Vγ9Vδ2 T cells.

Vγ9Vδ2 T cells can be directly activated *in vivo* with TCR agonists or be expanded *in vitro* and then reinfused into patients (adoptive cell therapy) (gomes aq 2010 cancer res). Clinical grade agonists used so far include the phosphoantigen bromohydrin pyrophosphate (BrHPP) and the aminobisphosphonates zolendronate and pamidronate. In most clinical trials, recombinant IL-2 (rIL-2; a fundamental cytokine for γδ T cell expansion) was used in combination with TCR agonists (Table 6).

The anti-tumour activity of γδ T cells was first tested in a clinical trial in 2003 in which rIL-2 was combined with pamidronate for the treatment of non-Hodgkin lymphoma and multiple myeloma[268]. The combination of pamidronate and low-dose rIL-2 was well tolerated and partial responses were observed in 33% of the patients (Table 6).

More recently, a complete remission of a metastatic renal cell carcinoma patient was reported[269]. The patient underwent 6 monthly cycles of autologous γδ-PBLs, activated and/or expanded *in vitro* with HMBPP plus rIL-2, combined with the infusion of zoledronate plus rIL-2 low-dose. This response was associated with a sharp increase of in IFN-γ-producing Vγ9Vδ2 T cells following adoptive transfer, and the patient has been disease free for 2 years without any additional treatment.

Globally, the clinical trials completed to date, particularly those stimulating γδ T cell *in vivo*, have show objective responses in the range of 10 to 33% (Table 6). While in some patients there was clearly insufficient expansion of Vγ9Vδ2 T cells[268, 270, 271], in other patients this could not explain for the absence of objective response. It is therefore critical to further clarify the basic mechanisms of γδ T cell recruitment to tumours, and their local recognition of transformed cells, in order to design more efficient γδ T cell-based protocols or cancer immunotherapy.

Table 6 – Clinical trials involving γδ T cells. PD, progressive disease; SD, stable disease; PR, partial remission; CR, complete response ;RCC, renal cell carcinoma; ZOL, zolendronate; ND, not determined. Adapted from Hannani *et al* [272].

Immuno-therapy	Cancer type	Treatment	N	% PD	% SD	% PR	% CR	Reference
Autologous γδ T cell infusions	Metastatic RCC	+ rIL-2 + BrHPP	10	40	60			273
	Metastatic RCC	+ rIL-2 + ZOL	1				100	269
	Solid tumours	+ rIL-2 + BrHPP	28	ND	ND	ND	ND	274
	Non-small-cell lung cancer	+ rIL-2 + ZOL	8	63	37	0		275
	Non-small-cell lung cancer	+ rIL-2 + ZOL	15	60	40			276
	Solid tumours	γδ T alone combination	5 20	40 30	40 5	 15		277
	Solid tumours		18	61	17	11	6	278
Bisphos--phonates *in vivo*	Refractory low-grade non-Hodgkin lymphoma and multiple myeloma	rIL-2 d6-d8 no preselection rIL-2 d1-d6 preselection	10 9	80 44	10 22	 33		268
	Metastatic hormone-refractory prostate cancer	Phase I ZOL Phase I rIL-2+ZOL	9 9	78 33	11 44	11 44		270
	Advanced stage IV breast cancer	Phase I rIL-2 + ZOL	10	70	20	10		271

1.3 Objectives of the Thesis

The anti-tumour properties of γδ T cells have prompted the development of clinical trials based on their stimulation by the administration of small non-peptidic phosphoantigens or bisphosphonates in combination with rIL-2. Despite the promise of tumour immunosurveillance by γδ T cells, these trials have shown a low and variable degree of efficacy, which urges new fundamental knowledge of γδ T cell responses to tumours. In this thesis we set out to investigate three key aspects pf tumour immunology associated with γδ T cells.

1. *Tumour cell recognition by γδ T cells*

Given the fact that the greatest enigma in γδ T cell biology is their target cell recognition, as very few tumour-associated antigens for γδ T cells have been identified, our goal was to define novel tumour antigens expressed in malignant cells that trigger γδ T cell cytotoxicity. Based on the evidence that both TCRγδ and NKG2D were implicated in Vγ9Vδ2 T cell-mediated killing of epithelial tumours, we tested the relative contribution of both receptors for haematological tumour recognition. Moreover, we extensively studied the expression of NKG2D ligands and other non-classical MHC molecules in human leukaemia and lymphoma cell lines and patient biopsies.

2. *Recruitment of γδ tumour-infiltrating lymphocytes*

One serious limitation reported for conventional T cell-based therapies results from the inability of T cells to infiltrate tumours. Knowing that and owing to the fact that the migration properties of γδ T cells are poorly defined, we aimed to analyze which chemokine signals would determine γδ T cell migration and infiltration into tumours *in vivo.* We used the pre-clinical B16 melanoma tumour model and validated the results on human γδ T cell migration *in vitro*.

3. *Promotion of tumour growth by γδ T cells*

After observing that TCR$\delta^{-/-}$ mice displayed lower ovarian carcinoma (ID8) tumour burden than WT mice, we decided to test the potential pro-tumour properties of γδ T cells. Based on the evidence that γδ T cells are innate producers of IL-17 and that this cytokine has been shown to promote tumour progression, we hypothesized that IL17 producing γδ T cells could promote ID8 tumour growth *in vivo.*

In summary, this thesis aimed at elucidating the mechanisms of anti-tumour γδ T cell tumour cell recognition and their molecular mechanisms of migration and tumour cell infiltration. Additionally, we addressed the potential role of IL-17-producing γδ T cells in promoting tumour growth. We consider this fundamental knowledge of great importance for the modulation of γδ T cells in cancer immunotherapy strategies.

CHAPTER II:

Tumour cell recognition by γδ T cells

The MHC class Ib protein ULBP1 is a nonredundant determinant of leukaemia/lymphoma susceptibility to γδ T cell cytotoxicity

2.1 Abstract

On the path to successful immunotherapy of hematopoietic tumours, γδ T-cells offer great promise due to their HLA-unrestricted targeting of a wide variety of leukaemias/ 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 non-redundant, highlighting a thus far unique physiological relevance for tumour recognition by γδ T-cells. Importantly, we observed a very wide spectrum of ULBP1 expression levels in primary biopsies obtained from lymphoma and leukaemia patients. We suggest this will impact on the responsiveness to γδ T-cell-based immunotherapy, and therefore propose ULBP1 to be used as a leukaemia/ lymphoma biomarker in upcoming clinical trials.

2.2 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 anti-tumour activity toward a variety of malignant cell types in both mice[8] and humans[279], with special emphasis on lymphomas and leukaemias[280]. Unlike their αβ counterparts, γδ T-cells are not restricted by classical MHC presentation, but share many characteristics with NK cells, including the expression of "NK receptors", most notably NKG2D[142, 186]. Most (60-95%) human γδ peripheral blood lymphocytes (γδ - PBL) express a Vγ9Vδ2 TCR[281] and are specifically activated by non-peptidic prenyl pyrophosphate intermediates of isoprenoid biosynthesis ("phosphoantigens")[172, 282] which constitutes the basis of current cancer immunotherapy strategies involving γδ T-cells[268, 270, 279].

Although several molecules have been proposed to play a role in tumour-Vγ9Vδ2 cell interactions, from phosphoantigens[282, 283] to a F1-ATPase-related structure complexed with delipidated apolipoprotein A-I[177] and, more recently, DNAM-1-ligands[243] or the non-classical MHC protein ULBP4[178], a consensus about γδ T-cell recognition of tumours, particularly upon physiological (non-ectopic) conditions, is yet to be reached.

Here we set out to determine the mechanism of leukaemia/ lymphoma cell recognition by γδ T-cells, particularly relevant as previous γδ T-cell-based clinical trials have shown a variable degree of success amongst patients[268]. The establishment an *in vitro* model representative of this clinical scenario and the quantification and manipulation of candidate gene expression allowed us to demonstrate a non-redundant role for ULBP1 in determining the susceptibility of leukaemia/ lymphoma cells to γδ T-cell-mediated cytolysis.

2.3 Results

2.3.1 Expanded and activated γδ-PBL efficiently kill leukaemia cells (Molt-4) *in vitro* and *in vivo*

The capacity of peripheral blood γδ T cells to target multiple tumour cell lines is well documented[164, 251, 258], namely cells from hematopoietic origin[256, 257]. In our laboratory we have develop an *in vitro* cytotoxicity assay to determine γδ-PBL capacity to lyse human tumour cells. We expanded γδ-PBL from total human peripheral blood mononuclear cells (PBMCs) by culturing them for 2-3 weeks in complete media supplemented with the phosphoantigen HMB-PP and rIL-2. By the end of the culture, the percentage of Vγ9$^+$ cells was determined and confirmed to be in the range of 85-90%. Before incubating tumour cells with γδ-PBL, they were stained with DDAO-SE, a dye that able us to distinguish tumour cells (DDAO-SE$^+$) from γδ-PBL. After 3 hours of co-culture, total cells were stained for Annexin V in order to score for apoptotic cells.

Figure 1A shows the fluorescence -assisted cell sorting (FACS) plots obtained from the *in vitro* killing assay of the acute lymphoblastic leukaemia cell line, Molt-4. In the presence of γδ-PBL, the percentage of tumour cell death is as high as 95%, which denotes the effective capacity of γδ-PBL to kill leukaemia cells *in vitro*. We used this killing assay throughout the experiments (Fig.2A, 2B, 3A, 4B and 4C).

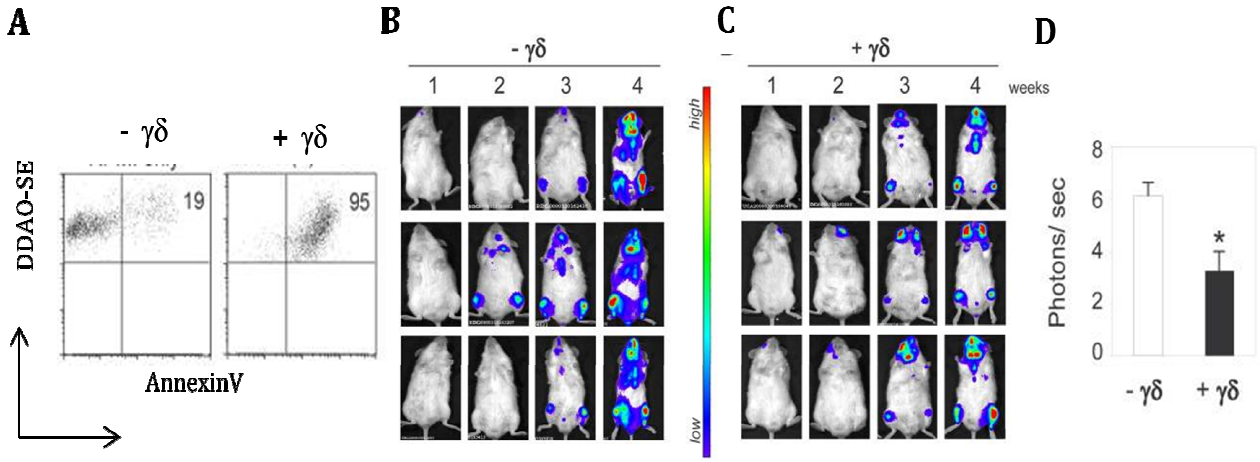

Figure 1 - Expanded and activated γδ-PBL efficiently kill Molt-4 acute leukaemia cells (A) *in vitro* lysis of Molt-4 leukaemia cells. Expanded and activated γδ-PBL (of which 85-90% Vγ9$^+$) were co-incubated with DDAO-SE-labelled Molt-4 cells for 3h in media devoid of activating compounds. Samples were then stained with Annexin V to identify dying (Annexin V$^+$) tumour (DDAO-SE$^+$) cells by flow cytometry. (B-C) Bioluminescent imaging of NOD/SCID mice inoculated with luciferase$^+$ Molt-4 leukemic cells, with (C) or without (B) co-injection of pre-activated γδ PBL, analyzed weekly as described in Material and Methods (D) Living Image quantification of photon signals (tumour load) collected at day 28 of experiment

illustrated in (B-C). Comparison of γδ–treated and control animals (n=5, $p<0.05$). Data in this figure is representative of three independent experiments

In order to translated these findings into a *in vivo* setting, we adapted a model of transplantation of human tumours into lymphopenic SCID mice, previously used with human γδ T-cells by Kabelitz and colleagues[284], and added bioluminescent analysis of tumour development, which allows early detection of tumours and temporal evaluation throughout the course of treatment, in live animals and in real-time[285].

Four weeks after Molt-4 tumour cell transplantation, mice that had received HMB-PP plus IL-2-treated (activated and expanded over twelve days) γδ-PBL showed significantly reduced tumour burden compared to control mice that did not receive γδ T-cells (Figure 1 B,C). Furthermore, while most control mice had to be sacrificed at week four due to excessive body weight loss, γδ-PBL treated animals resisted wasting for longer, up to week six (Figure 1 B,C) and data not shown). These results attest the capacity of γδ-PBL (HMB-PP-expanded and activated) to induce anti-tumour responses *in vivo*, and support the use of this pre-clinical *in vivo* model to evaluate immunotherapeutic strategies based on γδ T cells.

2.3.2 Differential susceptibility of leukaemia and lymphoma cells to γδ–PBL cytotoxicity

In our laboratory we have studied a panel of twenty tumour cell lines of hematopoietic origin, including acute lymphoblastic leukaemia (ALL) (Jurkat, Molt-4, RCH-ACV, 697, CEM, TOM-1, RS4-11, B15, REH, BV173) and acute myeloid leukaemia (AML) (HL-60, HEL, THP-1) cell lines; and non-Hodgkin Burkitt´s (Daudi, Raji, Ramos), follicular (DOHH2) and lymphoblastic (Oz) lymphoma. *In vitro* cytotoxic assays (as described in 2.3.1) to all the twenty cell lines were performed using γδ-PBL of the same blood donor[286] . We observed that while some cell lines were quite susceptible to γδ-PBL mediated lysis (e.g. Molt-4, DOHH2 and Jurkat), a substantial fraction of cell lines (e.g. Raji, 697 and Bv173) were strikingly resistant to γδ-PBL[286]. For systematic analysis of our killing assay data, we considered tumour samples with over 70% lysis as susceptible to γδ-PBL-mediated lysis ("γδ-susceptible"), and those under 30% lysed as "γδ-resistant".

We selected a group of eight cell lines DOHH2, HL-60, Jurkat, Molt-4, Raji, 697, REH and BV173 (described in Table1) and performed *in vitro* cytotoxicity assay (Fig. 2A) simultaneously with three different γδ-PBL (previously expanded and activated) donors, and observed that the pattern of susceptible/resistant lines was equivalent for the three independent healthy donors. We first considered that tumour resistance to γδ-PBL cytotoxicity could stem from intrinsic anti-apoptotic mechanisms developed by some leukaemia/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 (Figure 2B). 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) (Fig. 2B). 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 tumour/γδ-PBL interactions) that we set out to characterize.

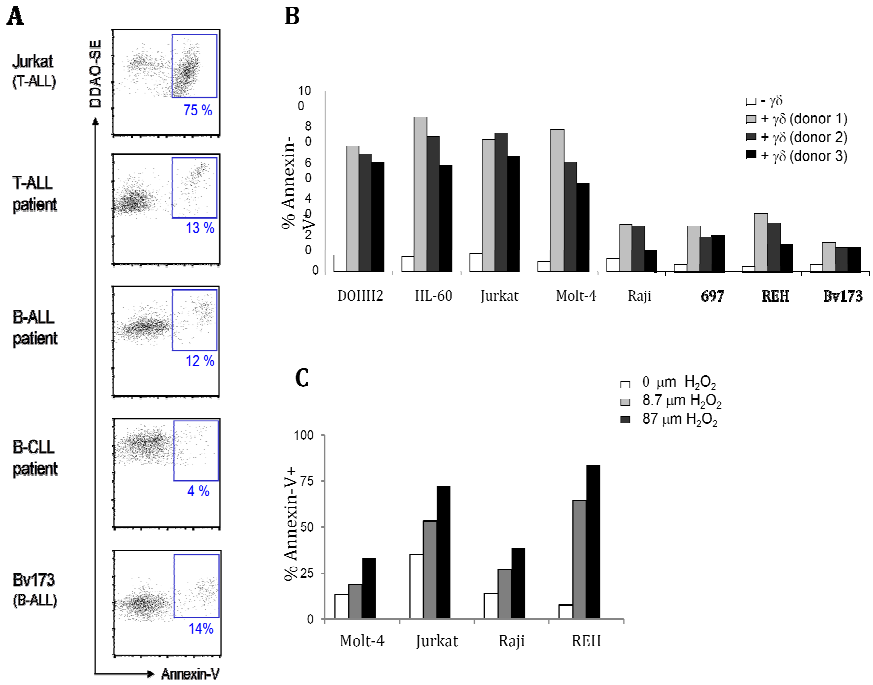

Figure 2 - Differential susceptibility of leukaemia and lymphoma cells to γδ–PBL cytotoxicity Different susceptibility of (A) Jurkat and Bv173 leukaemia cell lines and primary cells from the peripheral blood of leukaemia patient (B) leukaemia and lymphoma cell lines (described in Table1) (C) Effect of increasing concentrations of H_2O_2 on leukaemia/lymphoma cell apoptosis (% Annexin V^+)

Table 1 – Description of the haematopoietic tumour cell lines used in this study

Tumour type	Cell line	Cell type	Diagnosis	PubMed ID
Follicular lymphoma	DOHH2	Mature B cell	Follicular B cell lymphoma	1849602
Burkitt´s lymphoma	Raji	Mature B cell	Burkitt´s lymphoma	14086209
Acute lymphoblastic leukaemia	697	Pre-B cell	Pre-B cell leukaemia	6982733
Acute lymphoblastic leukaemia	REH	Pre-B cell	Pre-B cell leukaemia	197411
Acute lymphoblastic leukaemia	Bv173	Pre-B cell	Pre-B cell leukaemia	6572735
Acute lymphoblastic leukaemia	Jurkat	T cell	T cell leukaemia	68013
Acute lymphoblastic leukaemia	Molt-4	T cell	T cell leukaemia	4567231
Acute myeloid leukaemia	HL-60	Promyeloblast	Promyelocytic leukaemia	288488

2.3.3 NKG2D mediates Vγ9Vδ2 T-cell recognition of haematopoietic tumours that endogenously express ULBP1 and MICA

Despite the great effort of clinical trials to explore γδ T cells for the treatment of lymphoid malignancies, very little is known about the mechanism that determine haematological susceptibility to γδ-PBL. Nevertheless, both γδTCR and NKG2D receptors have been described to play roles in recognition of epithelial tumours. In order to determine the relative contribution of both receptors in haematological tumour cell recognition, we performed *in vitro* killing assays two susceptible cell lines (Molt-4 and Jurkat) in the presence of blocking antibodies for TCRγδ and/or NKG2D (Fig. 3). Surprisingly, we observed that blockade of NKG2D, but not TCRγδ, lead to significant decreased tumour cell lysis by γδ-PBL in both tumour cell lines. We did not observe an additive effect when blocking both receptors. Taking into account that TCRγδ is needed for γδ-T cell activation (dan paper plos one), we propose a model where haematological tumour cell recognition is TCR independent (by contrast with conventional αβ T cells) while NKG2D dependent.

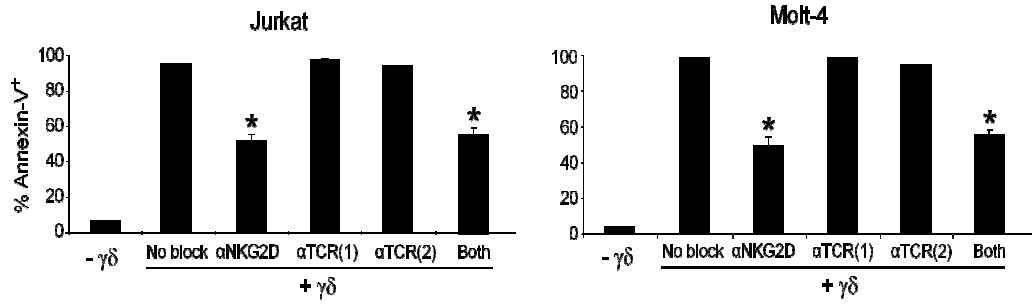

Figure 3 - NKG2D (but not TCR) mediates Vγ9Vδ2 T-cell leukaemia line tumour cell recognition. γδ-PBL were incubated with saturating amounts of anti-NKG2D/ clone 1D11 and anti-TCRγδ clones B1.1 (1) or IMMU510 (2) blocking antibodies, or both anti-NKG2D and anti-TCRγδ for 1h at 4ºC. γδ-PBL were then co-cultured either with Jurkat or Molt-4 leukaemia lines, and tumour cell lysis was assessed as in 1A. Error bars represent SD (n=3, *p<0.05).

After the observation of the dependence of NKG2D to haematological tumour cell recognition by γδ T cells, we proposed that the susceptibility/ resistant pattern we observed in the *in vitro* cytotoxic assays (Fig. 2A), would probably determined by the expression of NKG2D ligands by the tumour cell lines. We therefore analyzed the expression of NKG2D ligands ULBP family (1-4) and MICA/B by RT-PCR (Fig. 4A). Of the six NKG2D ligands analyzed, only ULBP1 expression segregated with tumour cell susceptibility; with susceptible cell lines expressing on average 6 times more ULBP1 mRNA than resistance tumour cell lines. MICA was highly expressed by DOOH2 and HL-60 but not by Jurkat and Molt-4 (susceptible tumour cell lines) while it was slightly expressed by the resistant tumour cell lines. The expression of the other NKG2D ligands did not segregate with the susceptibility-resistant patterns we observed in the *in vitro* killing assays (Fig. 2A).

Because NKG2D recognizes its ligands at the cell surface, we also evaluated the expression of ULBP1 and MICA at the protein level at cell the surface of the

tumour cell lines by flow cytometry (Fig. 4B). We observed that the correlation between mRNA and protein levels was not absolute; nevertheless all susceptible cell lines analyzed expressed high levels of ULBP1 while resistance cell did not. MICA was expressed by three of the susceptible cell lines DOHH2, HL-60 and Jurkat (but not by Molt-4) and was not expressed by resistant cell lines.

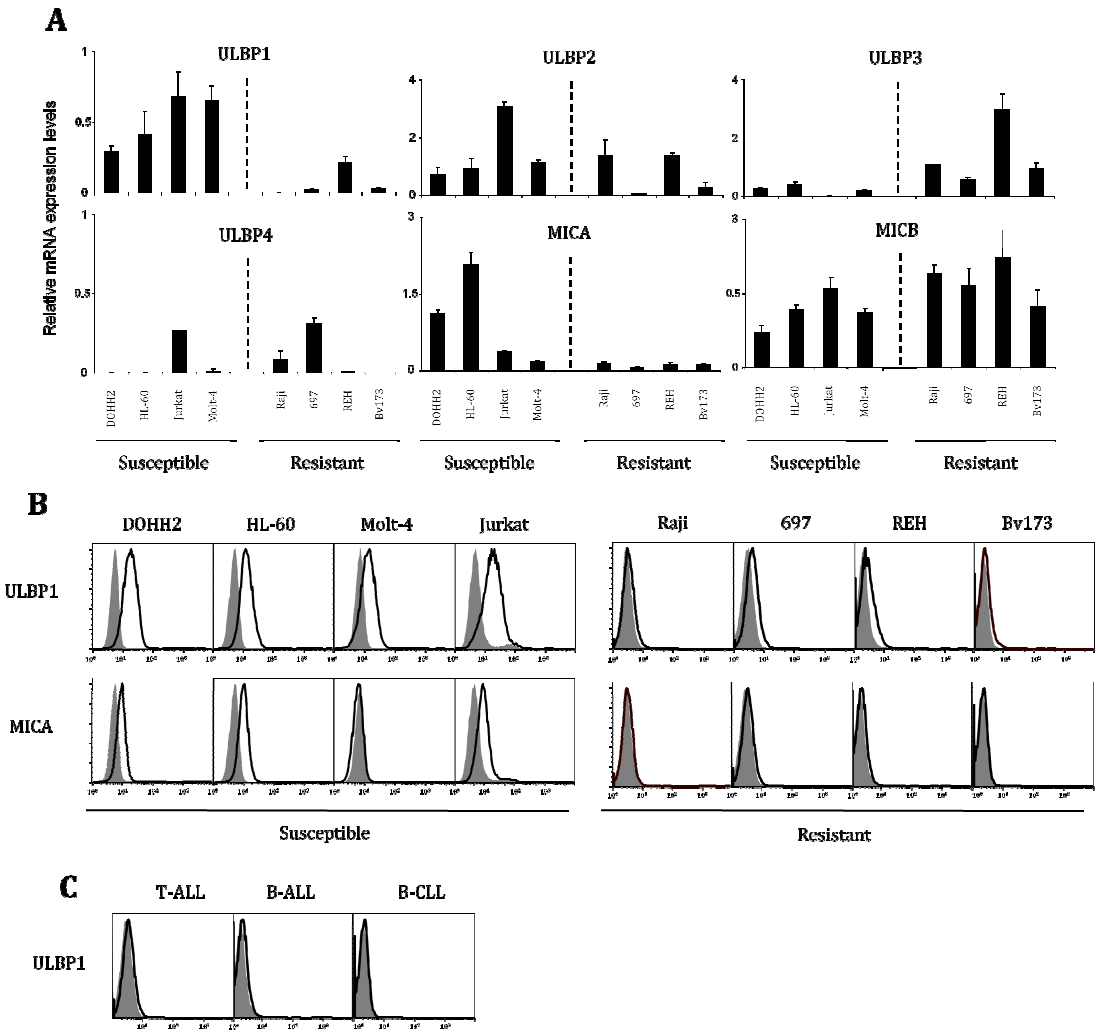

Figure 4 - NKG2D mediates Vγ9Vδ2 T-cell recognition of haematopoietic tumours that endogenously express ULBP1 and MICA (A) RT-qPCR quantification of mRNA levels of NKG2D-ligands in cells lines of (Fig. 2A), normalized to glucuronidase-beta (GUSB) and proteasome subunit beta type 6 (PSMB6) housekeeping genes. (B) Flow cytometry analysis of cell surface expression of ULBP1 and MICA in the leukaemia/ lymphoma lines of (Fig. 2A) and (C) primary cells from peripheral blood of leukaemia patients (Fig 2B) Data presented in this figure are representative of at least three independent experiments with consistent results.

2.3.4 ULBP1 is required for Vγ9Vδ2 T cell-recognition of leukaemia/ lymphoma

To assess the physiological role of ULBP1 and MICA in tumour cell recognition by γδ–PBL we performed a series of loss-of-function assays, targeting the NKG2D ligands by RNA-interference. We used lentiviral vectors expressing short hairpin RNA

(shRNA), obtained from the RNAi Consortium, for the specific silencing of ULBP1 on Molt-4 and Jurkat cell lines, and MICA on Jurkat cell line. The levels of ULBP1 and MICAS were significantly decreased both at the mRNA level (Fig. 5A) and at the protein level (Fig. 5B). Loss of ULBP1 expression lead to a significantly reduction (35-50%) of γδ–PBL mediated cytotoxicity of both Molt-4 and Jurkat cell lines, while MICA expression loss did not alter γδ-PBL mediated lysis to Jurkat cells (Fig 5B). The reduction in γδ-PBL targeting by the loss of ULBP expression essentially "converted "the highly susceptible tumour cell lines in resistant cell lines. In order to try to "convert" resistant cell lines in susceptible cell lines, we overexpressed ULBP1 in Raji tumour cell line by using a lentiviral vector (Figure 6A). We then compared γδ–PBL cytotoxicity to Raji cells transfected with a vector control with Raji cells overxpressing ULBP1, and observed a significant percentage of tumour cell lysis in cells ectopically expressing ULBP1 (Fig. 5D).

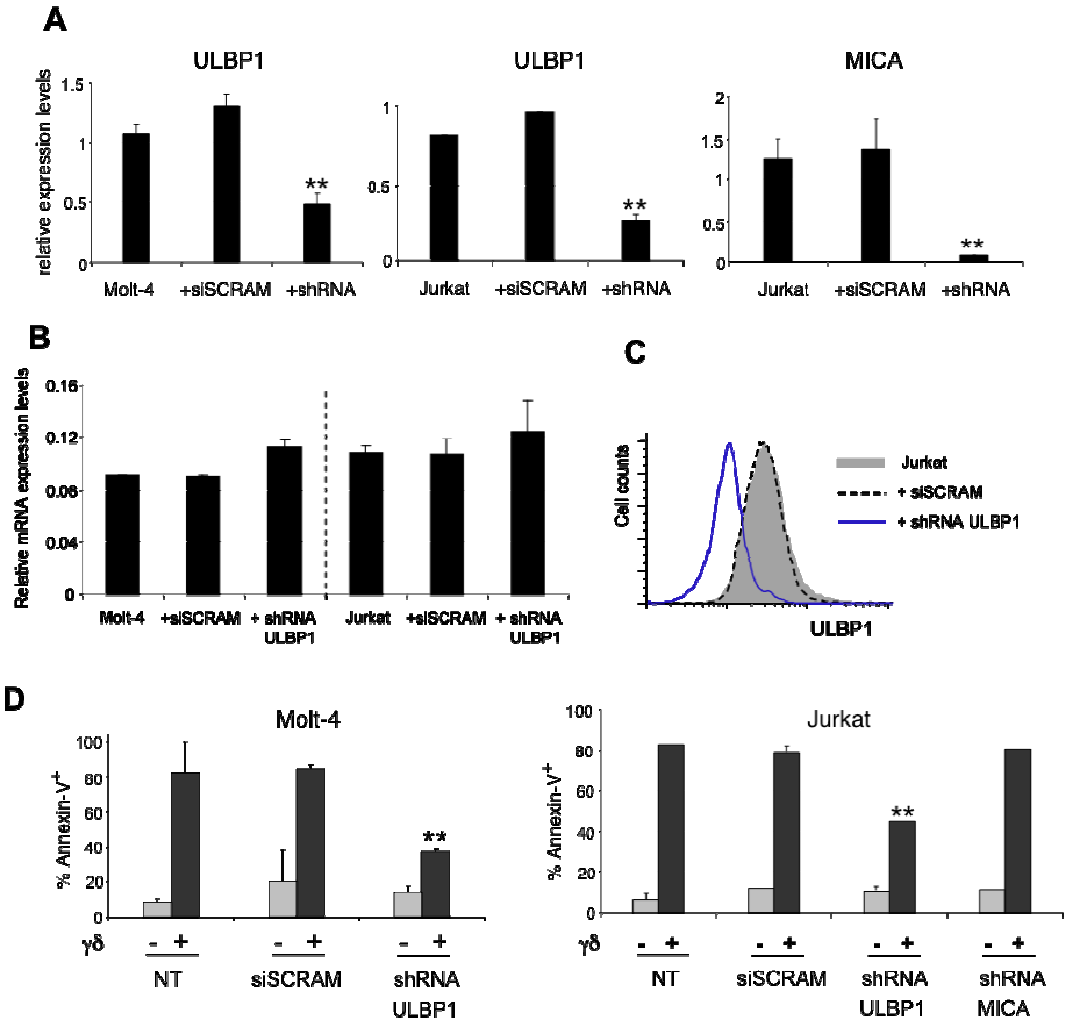

Figure 5 - ULBP1 is required for Vγ9Vδ2 T cell-recognition of leukaemia/ lymphoma cells. (A) Lentiviral shRNA-mediated knock-down of ULBP1 and MICA in Molt-4 or Jurkat leukaemia cells was confirmed by RT-qPCR (A and B) using GUSB and PSMB6 as endogenous references or by flow cytometry (C). Cells were infected with 10µl of high titre virus (10^7 CFU/ml) in media containing polybrene, submitted

to selection 48h later and collected for analysis 96h post-infection. siSCRAM is a shRNA of scrambled (unspecific) sequence, used as an infection control. Error bars represent SD (n=3, **p<0.01). (D) Molt-4 or Jurkat leukaemia cells, subjected to ULBP1 or MICA shRNA knock-down (as in A, B and C), were used in *in vitro* killing assays either in the presence (+) or absence (-) of γδ-PBL (as in Fig. 3). Non-transduced (NT) and siSCRAM-transduced cells were used as controls.

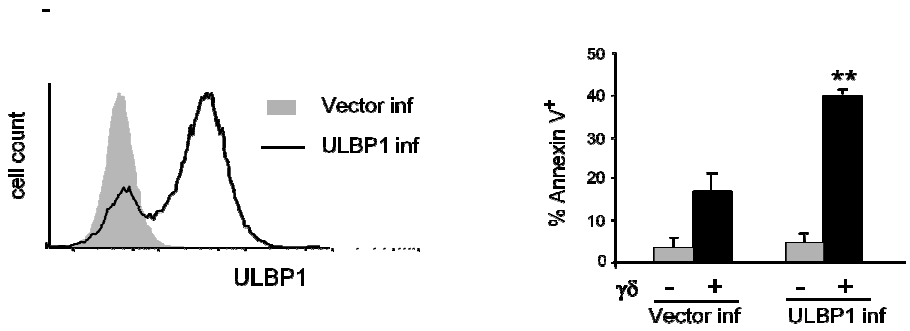

Figure 6 - ULBP1 is required for Vγ9Vδ2 T cell-recognition of leukaemia/ lymphoma cells. Raji lymphoma cells were lentivirally-transduced with ULBP1 (or control vector), and surface expression of ULBP1 was assessed by flow cytometry (A). *In vitro* killing assays were then performed either in the presence (+) or absence (-) of γδ-PBL (B).

2.3.5 ULBP1 displays a highly heterogeneous expression in cancer patients

The observation that ULBP1 expression determines lymphoma/leukaemia *in vitro* susceptibility to γδ-PBL cytolysis, prompted us to evaluate ULBP1 expression on clinical samples. We hypothesised that ULBP1 would be a good biomarker for γδ T cell haematological based clinical trials effectiveness.

Considering this, we analyzed the expression of ULBP1 and other NKG2D ligands (ULBP 2-4) and MICA/B, by RT-PCR in fifteen leukaemia PBMC samples and twelve lymphoma biopsies, which were compared to healthy PBMC and reactive follicles, respectively (Fig. 6A and 6B). Lymphoma biopsies included eight follicular lymphoma (FL) and four diffuse large B cell lymphoma (DLBCL) samples. Leukaemia samples included eight T acute lymphoblastic leukaemia (T-ALL) and seven B acute lymphoblastic leukaemia (B-ALL) PBMC samples.

We observed very low expression of MICA, MICB and ULBP4 mRNA both in lymphoma and leukaemia samples. ULBP2 and ULBP3 were expressed in same samples (mainly in lymphomas biopsies). ULBP1 presented the highest level of expression in both lymphomas and leukaemias, as well as the broadest spectrum of expression levels, as translated by its dramatic variance across clinical samples (Table I). Considering the impact of 2-fold reduction in ULBP1 levels on leukaemia killing *in*

vitro (Figures 4A-C), these results with primary biopsies strongly suggest a large variability in susceptibility to γδ-PBL cytotoxicity in the clinical population.

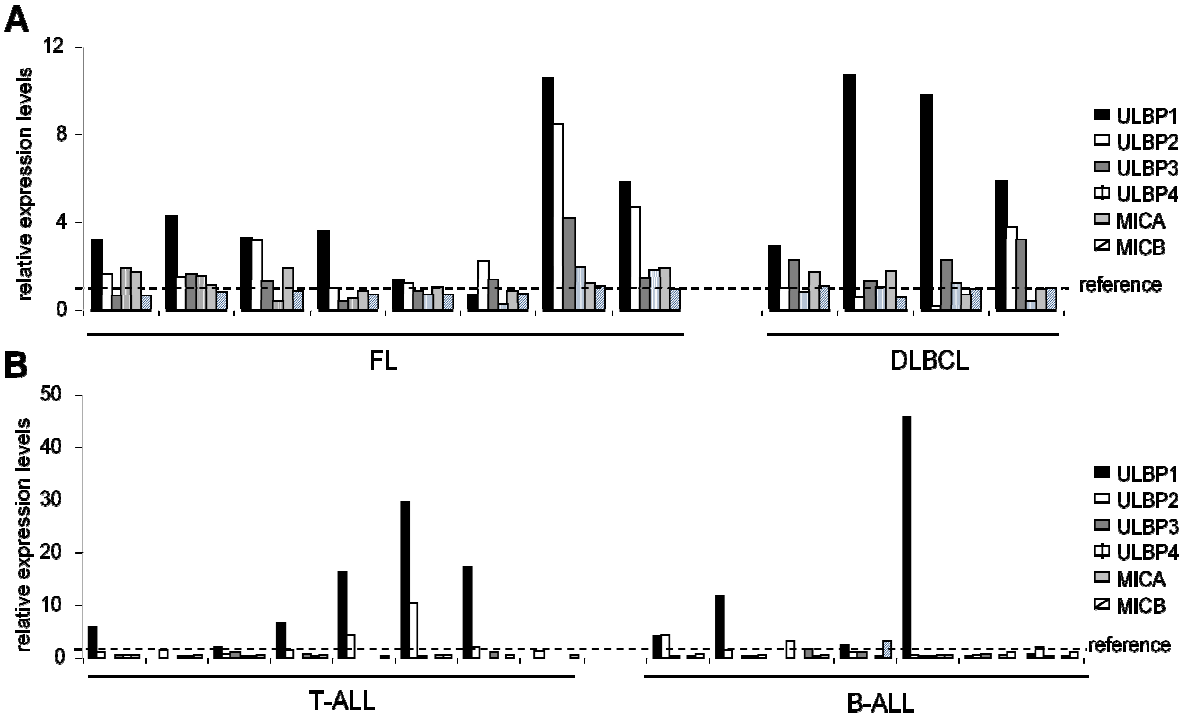

Figure 7 - ULBP1 displays a highly heterogeneous expression in cancer patients. RT-qPCR analysis of mRNA expression of NKG2DLs in (A) eight follicular lymphoma (FL) and four diffuse large B cell lymphoma (DLBCL) biopsies, normalized to housekeeping genes (GUSB and PSMB6) and to a reference sample - reactive follicles – obtained through the same procedure and indicated by the dashed line ; and in (B) eight T acute lymphoblastic leukaemia (T-ALL) and seven B acute lymphoblastic leukaemia (B-ALL) PBMC samples, normalized to housekeeping genes (GUSB and PSMB6) and to reference PBMCs from healthy individuals, indicated by the dashed line.

2.4. Discussion

γδ -PBL, 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[172, 282] were able to mediate efficient killing of only a fraction of leukaemia/ lymphoma cell lines within a large panel established in our laboratory (our unpublished data). Within the group selected for this study, four lines were highly susceptible (60-85% Annexin-V^+) while other four were largely resistant (15-30% Annexin-V^+) to γδ T-cell cytotoxicity (Fig 2A). Furthermore, we observed a consistent resistance of primary leukaemia cells to γδ -PBL cytotoxicity (Fig. 2B), which stresses the importance of understanding the mechanisms of tumour cell recognition by γδ T-cells.

Both TCR γδ and NKG2D have been implicated in Vγ9Vδ2 T-cell-mediated killing of epithelial tumours[261, 287, 288]. To determine their importance in recognition of hematopoietic tumours, we performed specific antibody blockade experiments with HMB-PP-activated γδ -PBL (>90% $V\gamma9^+$) and two susceptible leukaemia lines. We observed a significant reduction through NKG2D inhibition but not via TCR γδ blockade, and no additive effect (Fig. 3), suggesting that, although TCR-mediated activation greatly augments Vγ9Vδ2 T-cell cytolytic capacity[172]), the recognition of leukaemia 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 tumour cell recognition. Thus, γδ T-cell-mediated surveillance of hematopoietic tumours appears to be a two-step process where effector lymphocyte activation is achieved through TCR stimulation (presumably by endogenous phosphoantigens[282, 283] but tumour cell recognition is predominantly mediated by NKG2D.

Based on these results, we hypothesized that the distinct leukaemia/ lymphoma susceptibilities derived from differential expression of NKG2D-ligand(s) (NKG2DL), which was tested by RT-qPCR. ULBP1 expression clearly segregated the best with susceptible versus resistant leukaemia/ lymphomas; on average, ULBP1 mRNA expression was 6-fold higher in susceptible than in resistant lines (Figure 4A). MICA was also highly expressed in some but not all susceptible lines, while ULBP3 was enriched in some resistant cell lines, and the expression of the other NKG2DL did not segregate with susceptibility to γδ -mediated killing (Fig 2A). Of note, ULBP4, recently suggested to be involved in γδ -PBL targeting of some epithelial tumours[178], was very poorly expressed in leukaemia/ lymphomas (Fig. 4A and 7A and 7B). 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 (Fig. 4B).

This prompted us to assess the physiological role of these NKG2DL in leukaemia 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 upon short hairpin RNA (shRNA) infection (Fig. 5A-C). Loss of ULBP1 expression caused a very significant reduction (35-50%) of γδ -PBL mediated lysis of leukaemia lines (Fig. 5D), essentially "converting" these susceptible tumours into γδ -resistant lines. The residual cytotoxicity may be due to other NK-like receptors, such as DNAM-1[243]), which we are currently investigating. Interestingly, MICA down-regulation did not impair γδ-PBL targeting of these tumours (Fig. 5D). These data collectively suggest that ULBP1 plays a crucial and non-redundant role in γδ-PBL recognition of leukaemia. Importantly, this constitutes the first physiological evidence for lymphocyte requirement of NKG2DL expression on tumours, since previous studies[178, 212, 259, 261] have 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 (Fig. 6A and 6B).

These findings suggest that monitoring ULBP1 levels in leukaemia/ 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 leukaemia PBMC samples and 12 lymphoma biopsies, which were compared to healthy PBMC and reactive follicles, respectively. ULBP1 presented the highest degree of over-expression in leukaemias and lymphomas (Fig. 6A-B), as well as the broadest spectrum of expression levels, as translated by its dramatic variance across clinical samples. Taking into account the impact of 2-fold reduction in ULBP1 levels on leukaemia killing *in vitro* (Fig. 5A-D), 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 upregulate ULBP1 expression in carcinoma cells[222] 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 anti-tumours responses[212, 289]. The role we attribute here to ULBP1 in the context of lymphomas and leukaemia is likely not to be universal for γδ T-cell recognition of other tumour types. For example, susceptible epithelial tumours have been shown to express low or undetectable levels of ULBP1[17]. In this context, it is attractive to speculate that ULBP4, recently shown to ectopically trigger Vγ9Vδ2 T-cell cytotoxicity against ovarian and colon carcinomas[178] may play, in epithelial tumours, the equivalent physiological role of ULBP1 in hematopoietic tumours. This would constitute a novel paradigm for tumour recognition, by which stress-inducible, non-classical 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[279]. Furthermore, NGK2D also plays critical roles in anti-tumour NK and CD8 T-cell responses[186, 289], and has been shown to be an essential genetic factor for tumour surveillance in mice[200] . We therefore believe that NKG2D/ NKG2DL modulation entails great promise for cancer immunotherapy.

2.5 Materials and Methods

Vγ9Vδ2 PBL expansion

Peripheral blood was collected from anonymous healthy volunteers, diluted 1: 1.7 (v/v) with PBS (Invitrogen Gibco) and centrifugated in Histopaque in a ratio of 3:4 (3 parts of Histopaque for 4 of diluted blood) for 30 min at 1500 rpm, 25ºC. The interface containing PBMC was collected, washed in PBS and cultured at $1x10^6$ cells/ml at 37ºC, 5%CO_2 in round-bottom 96 well plates with RPMI 1640 with 2mM L-Glutamine (Invitrogen Gibco) supplemented with 10% foetal bovine serum (Invitrogen Gibco), 1mM Sodium Pyruvate (Invitrogen Gibco), 50mg/mL of penicillin/streptomycin (Invitrogen Gibco), in the presence of 100U/mL of rIL-2 (Roche Applied Science) and 1nM of HMB-PP (4-hydroxy-3-methyl-but-2-enyl-pyrophosphate) (a kind gift from H. Jomaa and M. Eberl).

Tumour cell lines culture and *in vitro* killing assays

All tumour cell lines were cultured in complete RPMI 1640 media (supplemented as above) and splitted every 3-4 days. For cytotoxicity assays, tumour cell lines were stained with CellTracer Far Red DDAO-SE (1μm) (Molecular Probes, Invitrogen), and each $3x10^4$ tumour cells were incubated with $3x10^5$ γδ T-cells (expanded and activated) in RPMI devoid of activating compounds, for 3h at 37ºC and 5%CO_2 on a round bottom 96 well plate. Cells were the stained with Annexin V-FITC and analyzed by flow cytometry.

Leukaemia/ lymphoma biopsies

Pediatric B- or T-cell acute lymphoblastic leukaemia cells were obtained from peripheral blood of patients after informed consent and institutional review board approval (Instituto Português de Oncologia, Lisbon, Portugal). Lymphoma cells from lymph node biopsies were frozen in liquid nitrogen and used upon diagnosis (Hospital de Santa Maria-CHLN, Lisbon, Portugal).

RT-qPCR

Total RNA was reverse-transcribed into cDNA using random hexamers and Supercript 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 Primer3v.0.4.0 online programme. Primer sequences are listed in Supplemental Table I. For each transcript, quantification was done using the calibration curve method. Hprt was used as internal control for normalization. All samples were run in triplicate and repeated three times. Analysis of the qPCR results was performed using the ABI SDSv1.1 sequence analysis software (Applied Biosystems).

Antibodies

The following anti-human mAbs were used: ULBP1/ clone IC1380F, MICA/ clone 1300 (R&D systems), NKG2D-PE/ clone 1D11 (Biolegend), TCR γδ / clone B1.1 (eBioscience) and TCR γδ / clone IMMU510 (Beckman Coulter). Goat anti-mouse IgG-PE (Sigma-Aldrich, St Louis, USA) was used as secondary mAb for ULBP1 and MICA staining.

RNA interference and overexpression

Lentiviral vectors expressing shRNAs for the specific silencing of ULBP1 (CCTGGGAAGAACAAACTGAAA) and MICA (CTATGTCCGTTGTTGTAAGAA) were obtained from The RNAi Consortium (TRC) and produced as previously described (Moffat J 2006 cell). For overexpression of ULBP1, its coding sequence was amplified from a human EST clone (GenBank acc. no. BC0356416) by PCR, cloned into pENTR-V5C2 vector, subcloned into pLenti6.2 (Invitrogen), and introduced into the Gateway System (Invitrogen). Lentivirus were then pseudotyped as described (Moffat J 2006 cell).

Bioluminescent imaging of transplanted leukaemia development in SCID mice

$1x10^7$ Molt-4 T-cell leukaemia 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 $5x10^7$ γδ PBL (>80% $V\gamma9^+$), previously expanded and activated *in vitro* with 1nM HMB-PP for 12 days. Treated mice receiver boosts of $5x10^7$ γδ PBL i.v. on day 14 and 10.000 U rIL-2 twice every week. 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).

Ethic 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.

Statistical analysis

Statistical significance of differences between subpopulations was assessed by using Student´s t-test and is indicated when significant as +, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

CHAPTER III:

Recruitment of γδ tumour-infiltrating lymphocytes

Protective role for CCR2/CCL2 through recruitment of tumour-infiltrating γδ T-lymphocytes

3.1 Abstract

Tumour-infiltrating lymphocytes (TILs) are important prognostic factors in cancer progression. Although γδ T-lymphocytes have strong cytotoxic potential and can target many tumour cell types *in vitro*, their manipulation is hampered by our limited knowledge on the molecular cues that determine γδ T-cell migration toward tumours *in vivo*. Here we set out to identify the chemotactic signals that orchestrate tumour infiltration by γδ T-cells. We have used the "gold standard" pre-clinical transplantable B16 melanoma model to profile chemokines in tumour lesions and assess their impact on γδ TIL recruitment. We show that the inflammatory chemokine CCL2 and its receptor CCR2 are necessary for the accumulation of γδ TILs in B16 lesions *in vivo*. Strikingly, the lack of γδ TILs in TCRδ-deficient but also in CCR2-deficient mice enhances tumour growth, thus revealing an unanticipated protective role for CCR2/CCL2 through the recruitment of γδ T-cells. Importantly, we demonstrate that activated human Vδ1 T-cells, but not their Vδ2 counterparts, express CCR2 and migrate to CCL2, whose expression is strongly deregulated in multiple human tumour types. This work identifies a novel protective role for CCL2/ CCR2 in the tumour microenvironment, while opening new perspectives for modulation of human Vδ1 T-cells in cancer immunotherapy.

3.2 Introduction

The recent success of the adoptive transfer of T-lymphocytes to cancer patients has provided a great boost to immunotherapy[25].Consequently, there is increasing interest in manipulating lymphocyte populations capable of destroying tumour cells. Among such populations are γδ T-lymphocytes, which are highly cytolytic upon engagement of their TCR and/ or NK receptors, most notably NKG2D[290]. Importantly, γδ T-cells recognize their tumour targets independently of antigen processing and presentation via MHC, thus bypassing some important immune evasion mechanisms developed by tumours.

Human γδ T-cells, which constitute 1-10% of peripheral blood leukocytes, are known to infiltrate multiple tumour types[103, 272]. Furthermore, based on the available pre-clinical and clinical data, γδ T-cells are considered important players in cancer immune surveillance[272, 290] . Notwithstanding, this has thus far translated in relatively poor objective responses to immunotherapy protocols based on the activation of γδ T-cells either *in vivo* (using chemical agonists) or *ex vivo* (followed by re-injection in the patient)[272, 290].

One key determinant of therapeutic success is likely to be the migration and homing of activated lymphocytes to the tumour site[291]. However, the mechanisms that direct circulating γδ T-cells towards tumours remain largely unknown. Here we have used the "gold standard" pre-clinical experimental tumour model, based on the transplantable B16 melanoma cell line, to identify the key chemokines and chemokine receptors involved in γδ T-cell recruitment *in vivo*. These data were then

validated in highly controlled *in vitro* activation and migration assays using both mouse and human γδ T-cells, which allowed us to establish a key role for the CCR2/CCL2 pathway in γδ T cell migration towards tumours, as well as its potential impact on Vδ1 T-cell-based immunotherapy.

3.3 Results and Discussion

3.3.1 Murine cytotoxic γδ T cells play non-redundant anti-tumour role in B16 lesions

Palpable tumours were detected 7-9 days after subcutaneous injection of the B16 melanoma cell line. Tumour infiltrates were analyzed between days 9 and 14 days. Approximately 1:50 tumour-infiltrating lymphocytes were γδ T-cells, of which most expressed either Vγ4 or Vγ1 TCR chains (Fig. 1A), alike γδ T-cells found in lymph nodes (but not in the skin)[144]. Moreover, γδ tumour-infiltrating lymphocytes (γδ-TILs) expressed CD27 (Fig. 1B) and *ifng* (Fig. 1C), which are characteristics of the most abundant peripheral γδ T-cell subset[155]. IFN-γ (but not IL-17) production was also detected in tumour-draining lymph nodes (Supplemental Fig. 1). Importantly, γδ-TILs also expressed the cytotoxic effector molecules *pfn* and *gzmb* (Fig. 1C), and were able to kill B16 tumour cells *in vitro* (Fig. 1D). These properties suggested an anti-tumour role for γδ T-cells against B16 tumours, which was directly tested *in vivo* by comparing tumour growth in wild-type (WT) and TCRδ-deficient mice. As shown in Fig. 1E, mice lacking γδ T-cells developed significantly larger tumours than WT controls at each time point, thus indicating accelerated tumour cell growth. These results are consistent with those by Gao *et al.*[248]) and demonstrate a non-redundant protective role for γδ TILs in the B16 tumour model. We therefore chose this system to dissect the tumour-chemotactic requirements of γδ T-cell recruitment.

Based on the tumour phenotype of TCRδ-deficient mice (Fig. 1E), we hypothesized that their B16 lesions would selectively accumulate chemokines that would normally be highly consumed by γδ T-cells during tumour infiltration. We therefore employed a sensitive chemokine array to analyze protein extracts derived from B16 tumours isolated (at day 14) from WT or TCRδ-deficient mice. Among the 25 chemokines profiled (Fig. 1F, Supplemental Fig. 2 and not shown), the CCR2 ligands CCL2 and CCL12 were significantly overexpressed in TCRδ-deficient mice (Fig. 1F). This pattern was highly selective: in particular, did not extend to CXCL9 (Fig. 1F), CXCL11 or CXCL16 (Supplemental Fig. 1), all implicated in $CD8^+$ TIL recruitment[291] ; or to CXCL12 (Fig. 1F) or CCL19 (Supplemental Fig. 2), typically associated with LN homing. Furthermore, CCL25 and CCL27, previously shown to control γδ T-cell migration under homeostatic or inflammatory conditions[292, 293], were also equally expressed in WT and TCRδ-deficient mice (Supplemental Fig. 2). These data suggested a potential role for CCR2 ligands in controlling γδ T-cell recruitment to B16 tumours. Importantly, Penido *et al*. had previously shown that CCL2 controlled γδ T-cell migration in allergic[294] or pathogen-induced inflammation[295].

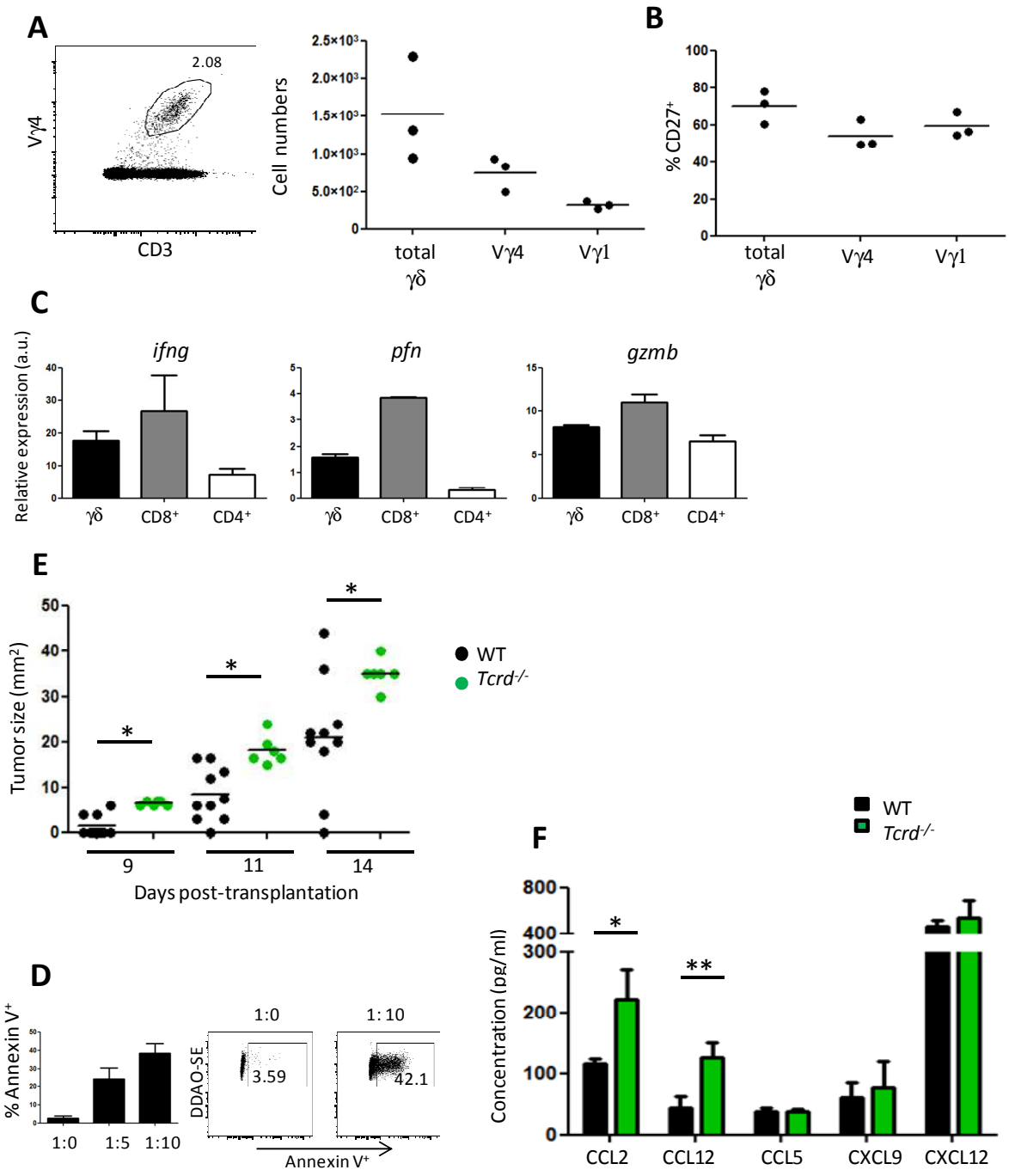

Figure 1 - Murine cytotoxic γδ T-cells play non-redundant anti-tumour role in B16 lesions. Mice were injected subcutaneously with $5x10^4$ B16-F0 melanoma cells and tumour size was measured every 2-3 days up to 14 days. Tumours were harvested and digested for analysis of leukocyte infiltrates. **(A-B)** Representative flow cytometry plot and numbers of total γδ or $Vγ4^+$ or $Vγ1^+$ lymphocytes in day 14-tumours from C57Bl/6 "wild-type" (WT) mice **(A)**, and fraction of $CD27^+$ cells within each subset **(B)**. **(C)** Total γδ, $CD8^+$ or $CD4^+$ T-cells were sorted 9 days post-transplantation and analyzed by quantitative RT-PCR for expression of *ifng*, *pfn* and *gzmb*, normalized to *hprt* and expressed in arbitraty units. Bars indicate SD (n=3). **(D)** *In vitro* killing assay with FACS-sorted γδ T-cells and DDAO-SE-labelled B16-F0 tumour cells (at indicated tumour:effector ratios). Annexin-V staining was performed after

3h of co-incubation and analyzed by flow cytometry. Bars indicate SD (n=3). **(E)** Tumour growth in WT or *Tcrd*$^{-/-}$ mice. Each dot represents one mouse. Average tumour size and statistical differences (*p<0.05) are indicated. **(F)** Chemokine concentrations in protein extracts from day 14-tumours from WT or *Tcrd*$^{-/-}$ mice, as measured by Quantibody Chemokine Mouse Array. Bars indicate SD (n=3; *p<0.05; **p<0.01).

3.3.2 CCR2/CCL2 pathway is required for γδ T cell recruitment to B16 tumours *in vivo*

We observed that 20-30% of γδ T-cells expressed CCR2 (Fig. 2A) and responded to CCL2 by fluxing calcium (Fig. 2B) and migrating in a transwell system (Fig. 2C). Most importantly, γδ T-cells migrated towards B16 protein extracts *in vitro* and this was significantly inhibited by neutralizing anti-CCL2 monoclonal antibodies (Fig. 2D). To test the involvement of CCL2 and CCR2 in the *in vivo* migration of γδ T-cells (and other leukocytes) toward tumours, we analyzed the infiltrating populations (gating strategies shown in Supplemental Fig. 3) in B16 lesions of CCL2-deficient and CCR2-deficient mice (these being additionally impaired in the response to other CCR2 ligands). We observed a significant reduction of γδ T-cell infiltrates in *Ccl2*$^{-/-}$ (Fig. 2E) and *Ccr2*$^{-/-}$ (Fig. 2F) mice. This contrasted with the normal numbers of CD8+ and CD4^{+} T-cells (Supplemental Fig. 4A). On the other hand, and as expected, myeloid populations (macrophages, neutrophils and myeloid-derived suppressor cells) were largely absent in tumours from *Ccr2*$^{-/-}$ mice (Supplemental Fig. 4B). Most importantly, the overall tumour growth rate was higher in *Ccr2*$^{-/-}$ mice, thus suggesting a dominance of CCR2/CCL2-dependent anti-tumour functions (as those mediated by γδ T-cells) over the well-established CCR2/CCL2-dependent tumour-promoting activities of myeloid cells[97, 296, 297]. Collectively, these data demonstrated an unexpected protective role for CCR2/CCL2 through the recruitment of cytotoxic (and IFN-γ-producing) γδ T-cells.

3.3.3 Human CCR2^{+}Vδ1 T cells migrate towards CCL2 which is deregulated in multiple cancer types

Building on these results in mice, we next investigated the potential role of CCR2/CCL2 in the migration of human γδ T-cells. Total γδ peripheral blood lymphocytes (PBLs) responded to CCL2 (Fig. 3A). Since human γδ-PBL contain two major subsets that express either Vδ1 (5-30%) or Vδ2 (60-95%) chains, we determined CCR2 expression in both populations. Interestingly, only Vδ1 PBLs expressed CCR2 either constitutively (Fig. 3B) or after activation with the mitogen phytohemaglutinin in the presence of IL-2 (Fig. 3C). By contrast, Vδ2 PBLs lacked CCR2 expression under both conditions, as well as upon activation with the specific Vγ9Vδ2-TCR agonist, 4-hydroxy-3-methyl-but-2-enylpyrophosphate (HMB-PP) (Fig. 3C, lower panel). Consistent with these data, Vδ1 (but not Vδ2) PBLs migrated

(chemotaxis index > 1) towards CCL2 *in vitro* (Fig. 3D). These data strongly suggest that CCR2/CCL2 may play a key role in the recruitment of Vδ1 PBLs to tumours. Importantly, Vδ1-PBL expansions have been widely reported in cancer patients[161, 162, 298]. Moreover, we have recently shown that Vd1 PBL are unique endowed, among γδ T-cells, with the potential to express natural cytotoxicity receptors that enhance their killing capacity against haematological[240] and solid (our unpublished data) tumours. This highlights the importance of dissecting the (CCR2/CCL2-mediated) mechanisms of Vδ1 T-cell migration and recruitment to tumours.

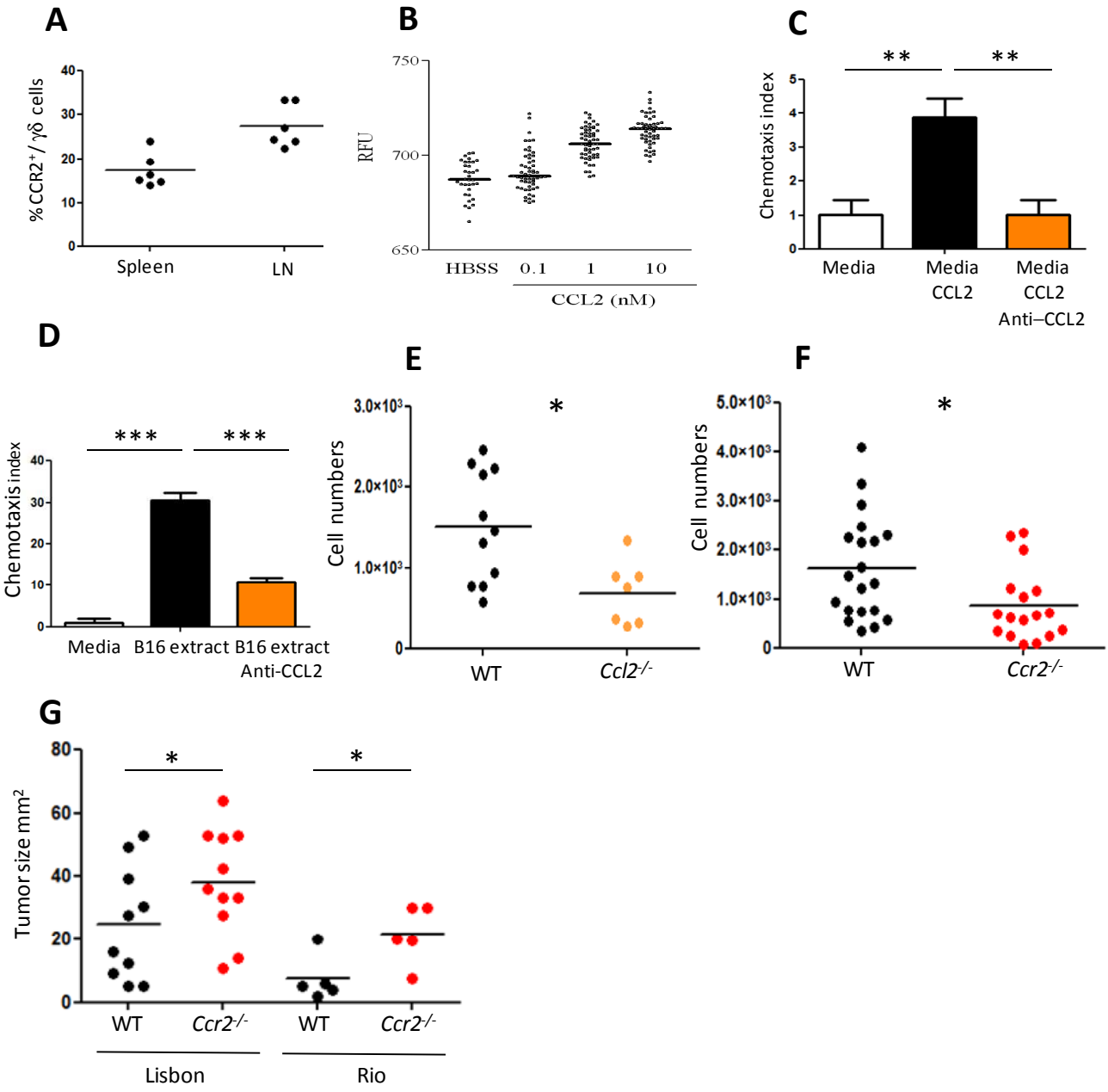

Figure 2 - CCR2/CCL2 pathway is required for γδ T-cell recruitment to B16 tumours *in vivo*. **(A)** CCR2 expression in splenic and lymph node γδ T-cells from WT C57Bl/6

mice was assessed by flow cytometry. Each dot represents one mouse. **(B)** Calcium influx assay on MACS-sorted γδ T-cells exposed to indicated concentrations of recombinant CCL2 in HBSS buffer. RFU, relative fluorescence units. **(C-D)** *In vitro* chemotaxis assays of MACS-sorted γδ T-cells toward recombinant CCL2 **(C)** or B16 tumour protein extracts **(D)** in transwell plates. Where indicated, anti-CCL2 neutralizing antibody was added. Bars indicate SD; **p<0.01; ***p<0.001. (E-G) WT, *Ccl2*$^{-/-}$ or *Ccr2*$^{-/-}$ mice were injected s.c. with 5x10^4 B16-F0 cells and sacrificed after 14 days. **(E-F)** Numbers of γδ tumour-infiltrating lymphocytes. **(G)** Tumour size, in experiments carried out in two animal facilities in Lisbon (IMM) and Rio de Janeiro (FOC). Each dot represents one tumour/ one mouse; *p<0.05

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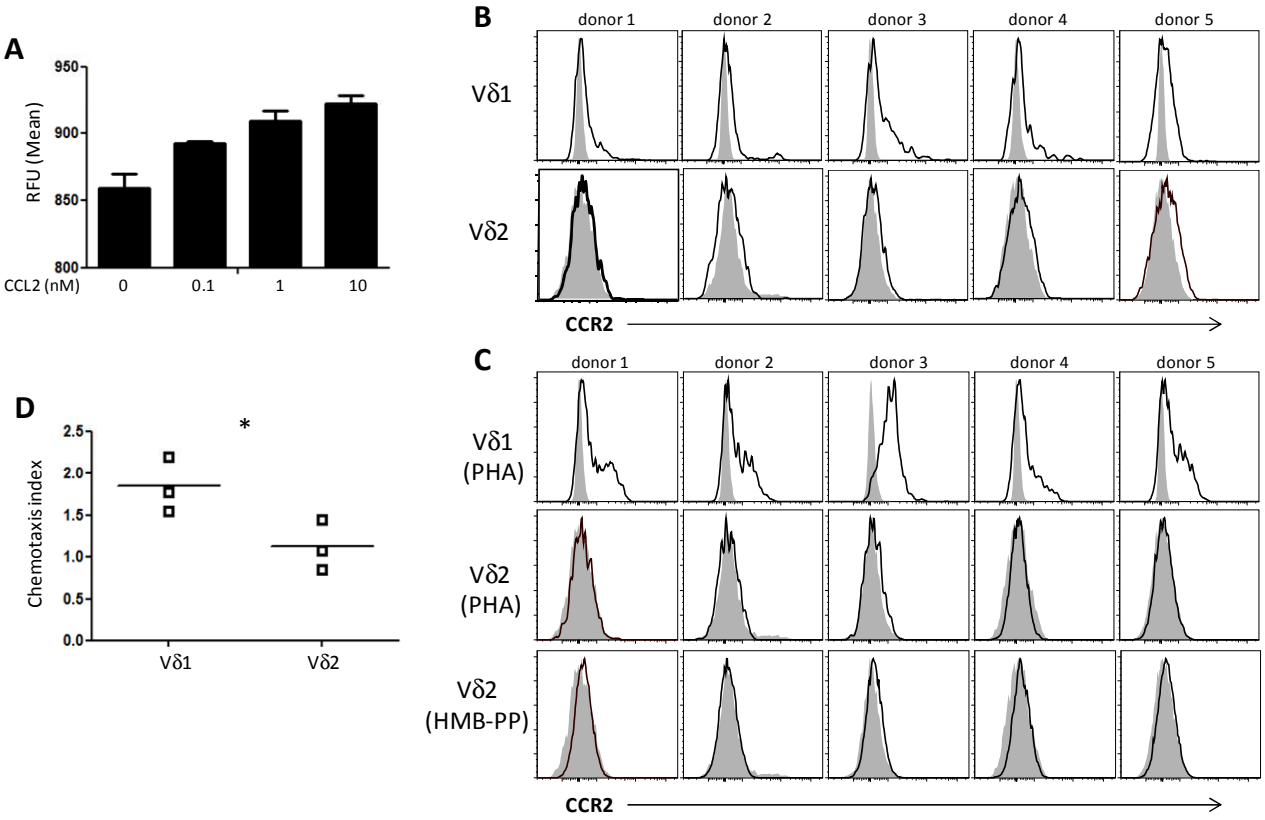


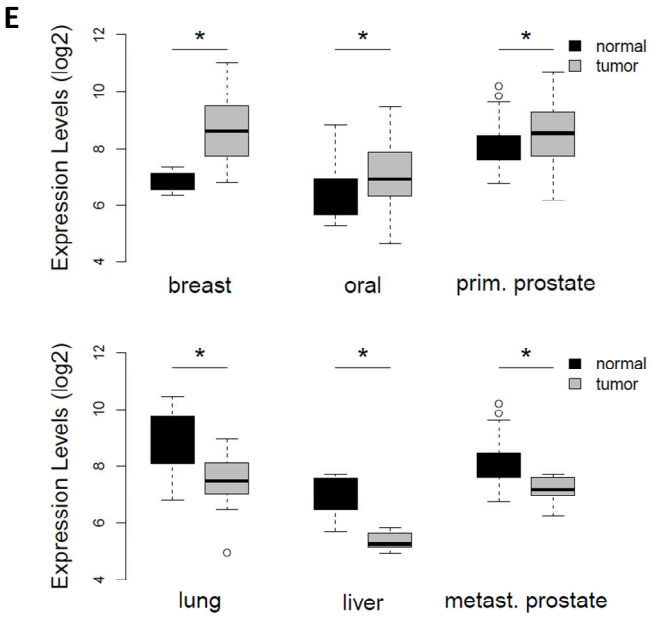


Figure 3 - Human CCR2+ Vδ1 T-cells migrate towards CCL2 that is deregulated in multiple cancer types. (A) Relative fluorescence units (RFU) from calcium influx assays on *in vitro*-expanded γδ T-cells from human peripheral blood exposed to increasing concentrations of recombinant CCL2. **(B-C)** CCR2 expression on freshly-isolated **(B)** or activated **(C)** Vδ1 and Vδ2 T-cells from five healthy donors. Activation was performed for 7 days with either phytohemagglutinin (PHA) or 4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP), always in the presence of IL-2. **(D)** *In vitro* chemotaxis (transwell) assays of FACS-sorted Vδ1 or Vδ2 T-cells toward recombinant human CCL2. **(E)** *Ccl2* expression levels for several human tumour types (grey) and corresponding normal tissue (black) estimated according according to Human Exon 1.0 ST microarray data (see Methods). Tumour types showing significant upregulation (breast cancer, oral squamous cell carcinoma and primary prostate cancer) and downregulation (lung adenocarcinoma, liver cancer, metastatic prostate cancer) are displayed in upper and lower panel, respectively (*p<0.05).

Finally, we investigated how *Ccl2* levels were regulated in human malignancies. We took a bioinformatics approach to inquire public genome-wide transcriptomic data.. Comparison of Ccl2 expression levels in tumour and corresponding normal tissue using Human Exon 1.0 ST microarray data, revealed a significantly strong deregulation of *Ccl2* levels in most (6 out of 9) tumour types analyzed. On one hand, oral squamous cell carcinoma, breast and primary prostate cancer showed significant upregulation of *Ccl2* expression (in comparison with healthy tissue profiled in the same analysis) (Fig. 3E). This may make these tumours particularly permissive to Vδ1 T-cell recruitment, which should be examined in future clinical studies. On the other hand, metastatic prostate cancer, liver and lung cancer displayed reduced *Ccl2* levels, which is likely to confer resistance to Vδ1 T-cell infiltration. In fact, it is interesting to speculate that dowregulation of *Ccl2*

expression may constitute an evasion strategy against Vδ1 T-cell-mediated immunosurveillance. Along these lines, the prostate cancer series showed a strong reduction of *Ccl2* levels between primary and metastatic tumours (Fig. 3E, compare upper and lower right panels). Future studies should test the association between Vδ1 T-cell infiltrates and *Ccl2* expression *in situ*.

Previous research on CCR2/CCL2 had established a key pro-tumour role for this inflammatory chemokine pathway through recruitment of myeloid cells, particularly monocytes/macrophages[97, 296, 297]. Moreover, our analysis of tumour infiltrates in *Ccr2*$^{-/-}$ mice also showed a striking reduction in MDSCs, which can actively suppress anti-tumour CD8^{+} [299, 300] and γδ (our unpublished data) T-cell responses.

However, other reports had revealed a paradoxical anti-tumour role for CCL2 by mediating the recruitment of CD8^{+} T-cells[301, 302]. This was recently supported by an elegant study showing that intratumoral reactive nitrogen species induce CCL2 nitration that prevents CD8^{+} T-cell infiltration[102].

Building on this dichotomy, our results identify a novel anti-tumour role for CCL2 based on cytotoxic γδ T-cell recruitment, thus adding to the pleiotropic functions of the CCL2 in the tumour microenvironment. We propose CCL2 as a major target for manipulation of Vδ1 T-cells, particularly in adoptive transfer settings. Of note, studies with human CD8^{+} T-cells injected into Nude mice bearing human tumour xenografts identified a pivotal role for CCL2 in CD8^{+} T-cell homing to tumours[301]

3.4 Material and Methods

Mice

Female mice were used at 6-10 weeks of age. C57BL/6 mice were obtained from Charles River; *Tcrd*$^{-/-}$ and *Ccl2*$^{-/-}$ mice were obtained from Jackson Laboratory; *Ccr2*$^{-/-}$ mice were generated and kindly provided by Dr. Kunkel (Michigan, USA).

In vivo B16 tumour model

5x10^{4} B16-F0 melanoma cells (American Type Culture Collection) were injected (in 50ml PBS) subcutaneously into the back of isoflurane-anesthetized mice. Tumour size was measured in two perpendicular dimensions every 2-3 days.

Tumour-infiltrating leukocytes

Tumours were harvested, finely chopped and treated for 30min (in shaker at 37ºC) with 5mg/ml Collagenase D (Roche) and 200mg/mL DNAse (Roche). Tissues were mashed through 40μm filter to generate single-cell suspensions. After red-blood cell lysis, samples were stained for 20min at 4ºC with the relevant fluorescently-labeled monoclonal antibodies (mAbs): γδTCR-PE, CD3-PerCPCy5.5, CD27-PeCy7, CD8-FITC,

CD4-PB, CD45-APC-Cy7, CD11b-APC, Gr1-PE, F4/80-FITC and CD11c-PerCPCy5.5 (all from eBioscience). CCR2-APC (R&D systems), Vγ4 PE (Biolegend) and Vγ1 FITC (kind gift from Pablo Pereira, Pasteur Institute, Paris). Samples were analyzed by flow cytometry on a FACSFortessa, or sorted on a FACSAria III (BD Bioscience).

In vitro killing assay

FACS-sorted γδ T-cells were activated for 48h with 1 μg/mL plate-bound anti-CD3 mAb (BD Pharmingen). B16 cells were stained with DDAO-SE (Invitrogen) and incubated for 3hrs in the absence or presence of γδ T-cells (at indicated effector:target ratios). Washed and EDTA-detached tumour cells were stained with Annexin-V (eBioscience) and analyzed by flow cytometry.

Chemokine protein array

Tumours were isolated at day 14 from C57BL/6 or *Tcrd*$^{-/-}$ mice. Post-centrifugation cell pellets were homogenized in lysis buffer containing protease inhibitors. Total protein was quantified by the Bradford method, and 100mg were incubated with membranes of the Quantibody Chemokine Mouse Array (RayBiotech Inc.) and developed by chemiluminescence according to the manufacturer´s protocol. Chemokine concentrations were measured (based on internal controls) using UnScanit software.

Ca^{2+} mobilization assay

Intracellular calcium concentrations ($[Ca^{2+}]_i$) were measured using the FLIPR Calcium Plus Assay kit on FlexStation II (Molecular Devices) fluorometric microplate reader, according to the manufacturer´s instructions. Briefly, pre-plated γδ T-cells ($2x10^5$/well) were exposed to CCL2 (0.1-10 nM), fluorescence intensity ratios at 485/525 nm ($\lambda_{ex}/\lambda_{em}$) were recorded, and transferred from SoftMax Pro to SigmaPlot software for construction of concentration-response graphs.

In vitro Chemotaxis assay

Lymphocyte chemotaxis was determined using HTS Transwell-a 96 well permeable supports (Corning). Plain RPMI medium or with rCCL2 (Peprotech), or tumour extracts were added to the bottom wells of the chamber. In the indicated wells, anti-CCL2 antibody was added. The top wells were loaded with $5x10^4$ FACS-sorted γδ T-cells. After 3h incubation at 37ºC, filters were removed and cells that migrated to the lower chamber were counted. The chemotaxis index was calculated as the ratio of cells migrating towards the tumour extracts or rCCL2 and cells randomly migrating across the membrane.

Real-time quantitative PCR

Total RNA was extracted and reverse-transcribed, and qPCR was performed as previously described (Correia Blood 2011). Primers were designed using Primer3v.0.4.0, and sequences are available upon request.

Analysis of human γδ T-cells

Peripheral blood lymphocytes were isolated as described (Correia Blood 2011), and Vδ1 and Vδ2 T-cell subsets were either freshly-analysed or sorted for *in vitro* cultures. Cells were activated for 7 days (in RPMI medium) with 4mg/mL phytohemagglutinin (PHA) (Sigma) or 10 nM of 4-hydroxy-3-methyl-but-2-enyl pyrophosphate (HMB-PP; Echelon Biosciences), in the presence of 100U/mL interleukin 2 (IL-2). CCR2 surface expressions was assessed by flow cytometry using the CCR2-PerCPCy5.5 mAb (Biolegend).

Bioinformatic analysis

Microarray data was obtained from previously published studies using Human Exon 1.0 ST platform and available in GEO database for: breast cancer (GSE33692); colorectal cancer (GSE24551); gastric cancer (GSE27342); liver cancer (GSE12941); lung adenocarcinoma (GSE12236); oral squamous cell carcinoma (GSE25099); ovary cancer (GSE29156); primary and metastatic prostate cancer (GSE21034). Data quality was assessed using arrayQualityMetrics BioConductor package (http://www.bioconductor.org/packages/2.1/bioc/html/arrayQualityMetrics.html). Determination of significant deregulation was determined by AltAnalyze application [303] using moderated T-test with Benjamin-Hochberg p-value correction (indicated when significant as (*p <0.05).

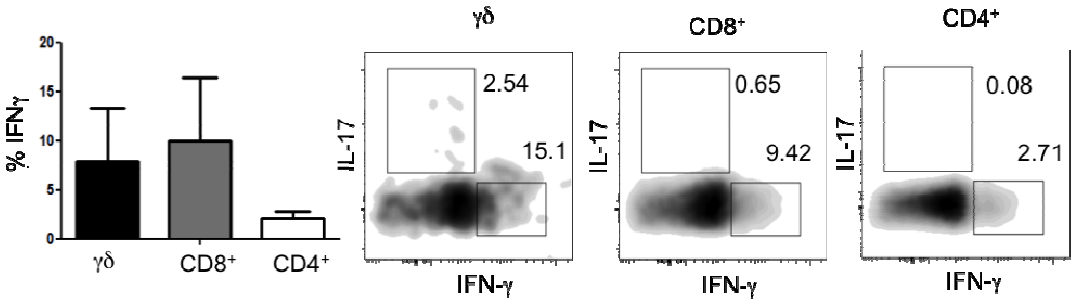
Statistics

Statistical significance of differences between populations was assessed using Student´s t-test and is indicated when significant as *p<0.05; **p<0.01; ***p<0.001.

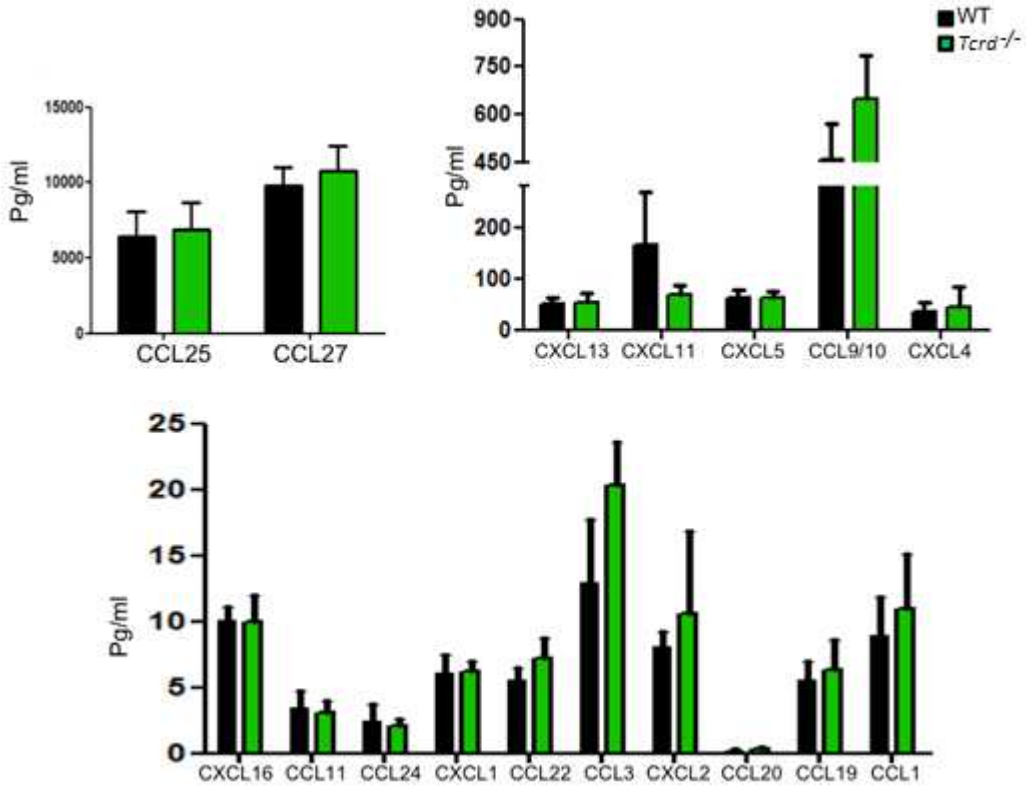
Study approval

All experiments were performed in compliance with the relevant laws and institutional guidelines and have been approved by the local Ethics Committees.

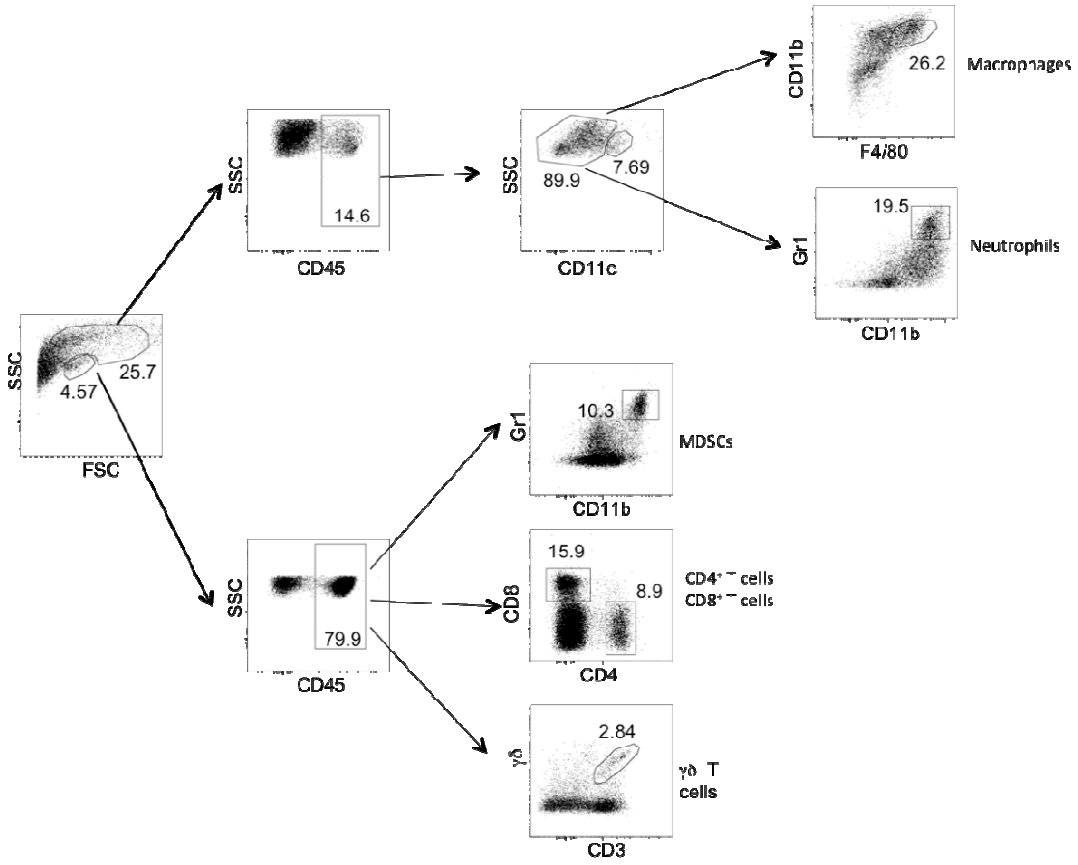
Supplemental figures



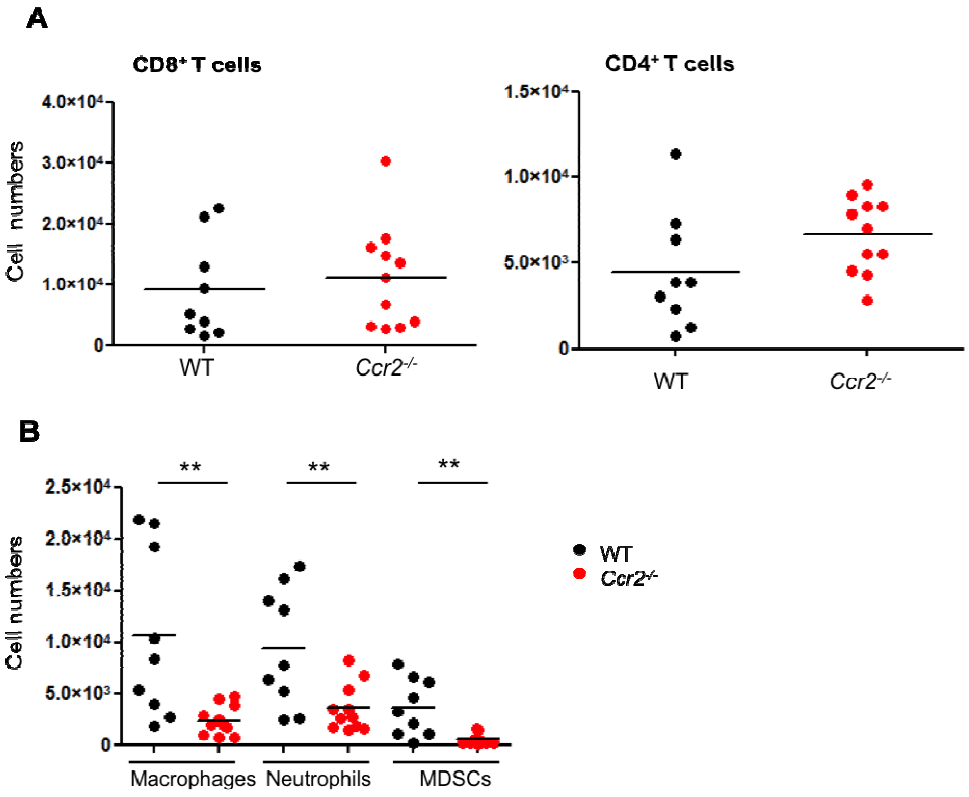
Supplemental Figure 1 - IFN–γ production by T-cell subsets from tumour-draining lymph nodes. Total γδ, $CD8^+$ or $CD4^+$ T-cells were FACS-sorted from tumour-draining lymph nodes, 9 days after injection of $5x10^4$ B16-F0 melanoma cells into C57Bl/6 mice. Cells were stained intracellularly for IFN-γ and IL-17 as previously described[155]. Graph shows percentage of IFN-γ^+ cells per subset (n=5)



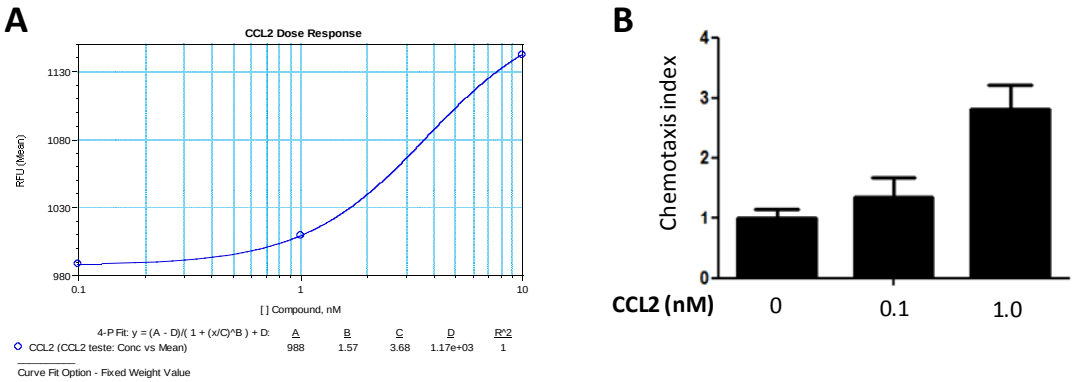
Supplemental Figure 2 - Chemokine concentrations in protein extracts from day 14-tumours from WT or *Tcrd*$^{-/-}$ mice, as measured by Quantibody Chemokine Mouse Array. Bars indicate SD (n=3; *p<0.05; **p<0.01).



Supplemental Figure 3 - Gating strategies for identification of tumour-infiltrating leukocyte subsets by flow cytometry. The corresponding fluorescently-labeled antibodies are listed in the Methods. FSC, forward scatter; SSC, side scatter; MDSCs, myeloid-derived suppressor cells.



Supplemental Figure 4 - Leukocyte infiltration in tumours from WT or *Ccr2*$^{-/-}$ mice. C57Bl/6 WT or *Ccr2*$^{-/-}$ mice were injected s.c. with 5x10^4 B16-F0 cells and sacrificed after 14 days for analysis of tumour infiltrates. Graphs represent numbers of tumour-infiltrating CD8$^+$ and CD4$^+$ T-cells **(A)** or macrophages, neutrophils and MDSCs **(B)** (as defined in Supplemental Fig. 3). Each dot represents one tumour/ one mouse. Statistical differences are noted as **p<0.01.



Supplemental Figure 5 -- *In vitro* responses of human γδ T-cells to recombinant CCL2. γδ T-cells were expanded *in vitro* from human peripheral blood mononuclear cells as previously described (ref. 16). **(A)** Calcium influx in response to increasing concentrations of rhCCL2. RFU, relative fluorescence units. **(B)** Transwell migration in the presence of the indicated concentrations of rhCCL2. Bars indicate SD (n=3).

CHAPTER IV:

Promotion of tumour growth by γδ T cells

Contribution of IL-17-producing γδ T cells for tumour progression

4.1 Abstract

Recent studies suggest that murine γδ T cells are important innate IL-17 producers with implications for infection and autoimmunity. On the other hand, the secretion of large amounts of IFN-γ by γδ T cells has long been described and linked to their anti-tumour function. In this study we directly compared two murine tumour models in which γδ T cells seem to play opposite roles. We observed that γδ T cell-deficient mice showed decreased tumour burden in the ID8 ovarian carcinoma model and increased tumour burden in the B16 melanoma model, when compared with wild-type mice. We analysed cytokine secretion by tumour-infiltrating γδ T cells in both tumour models and observed a selective expansion of IL-17 producing-γδ T cells in the ID8 tumour model but not in the B16 tumour model. Moreover, γδ T cells were the main source of IL-17 in the ID8 model, which has been previously shown to depend on IL-17 for tumour growth. We therefore hypothesise that γδ T cells promote ID8 tumour progression through the production of IL-17.

4.2 Introduction

γδ T cells have long been described as potent anti-tumour effectors, both by directly killing transformed cells and/or by producing cytokines, such as IFN-γ and TNF-α, that orchestrate a Th1 polarized response. The non-redundant role of murine γδ T cells in tumour immunosurveillance was demonstrated in several studies where γδ T cell-deficient mice were shown to be more susceptible to several regimens of cutaneous carcinogenesis[8, 249, 252], as well as in models of transplantable tumour cell lines[8, 248] (including our data from chapter III).

Nevertheless, this view was recently challenged, when IL-17 production by γδ T cells was suggested to promote tumour growth by stimulation of vascular endothelial growth factor (VEGF)[42]. By contrast, two studies proposed that IL-17-producing γδ T cells are necessary for therapeutic efficacy of chemotherapy[43, 44] and BCG treatment of bladder cancer[266].

In fact, the contribution of the IL-17 pathway to tumour development is currently controversial , as discordant effects have been described for this cytokine in several murine tumour models[304]. Most of the studies that addressed the role of IL-17 in the tumour context, relied on Th17 cells, but other cell types, like γδ T cells, NKT cells, $CD8^+$ T cells and NKT cells can also produce IL-17[305].

Many functions of IL-17 in the tumour microenvironment contribute to tumour progression. Apart from a direct effect on the tumour cells by promoting their proliferation and survival (via STAT3 in tumour cells that express the IL-17R), the major pro-tumour role of IL-17 in inflammation-associated cancer relies on its proangiogenic property toward surrounding endothelial cells and fibroblasts. By acting on stromal cells and fibroblasts, IL-17 induces a wide range of angiogenic

mediators, including VEGF[306] that markedly promote inflammation and tumour progression.

The tumour-inhibitory effects of IL-17 have also been described in a number of reports. For example, Th17-polarized cells seem to me more effective than Th1 cells in eliminating large established tumours[35]. In a similar study, $CD8^+$ T cells were skewed to secrete IL-17 in Th17 polarising conditions and mediated efficient tumour destruction when adoptively transferred into tumour bearing mice[307]. Interestingly, in both studies, the response was highly dependent on IFN-γ, as these cells were found to convert in vivo into IFN-γ producers. Another recent study attributed the anti-tumour properties of IL-17 derived from Th17 cells, to the induction of Th1 type chemokines, CXCL9 and CXCL10, by tumour cells, which potentially attract effector T cells into tumour lesions[96].

Besides Th17 cells, other leukocyte subsets have been shown to induce tumour progression by inhibiting effector T cells. The best described suppressive population are T regulatory cells, while myeloid cells, such as macrophages and neutrophils, have also been shown to polarize towards a suppressive phenotype. Recently, myeloid-derived suppressor cells (MDSCs) were discovered as a heterogeneous population of myeloid precursors characterized by their strong capacity to inhibit both innate and acquired immunity[63] particularly T cell responses[64]. Murine MDSCs can be identified by the expression of Gr1 (includes Ly6C and Ly6G, macrophage and neutrophil markers, respectively) and CD11b (characteristic of macrophages). MDSCs use a diversity of mechanisms to suppress T cell function, including the uptake of arginine and cysteine (essential amino acid for T cell activation) and the nitration of the TCR[63].

The possibility of improving anti-tumour immune responses by targeting MDSCs has been explored in pre-clinical models[63 66]. Inhibition of MDSC and Treg function within B16 melanomas using blocking antibodies to CTLA-4 (Ipilimumab already in clinical use in late-stage melanoma) and to PD-1, reduced tumour development and increased mouse survival[67].

In sum, various leukocyte subsets can display either tumour-promoting or tumour-inhibitory properties. Given the contradictory reports for γδ T cells, in this study we set out to explore their potential pro- *versus* anti- tumour roles in distinct *in vivo* tumour models.

4.3 Results

4.3.1 γδ T cells play opposite roles in tumour progression in two distinct tumour models

It has been demonstrated that γδ T cells prevent tumour progression in several murine tumour models[8, 248, 252]. However, recent findings suggest that γδ T cells may also have pro-tumourigenic properties[42]. To address these apparently

contradictory behaviours of murine γδ T cells, we compared survival of WT mice and TCRδ-deficient mice using two different murine transplantable tumour cell lines, B16 melanoma and ID8 ovarian carcinoma. In agreement with our study from chapter III and, we expectedly observed that TCRδ-deficient mice show decreased survival compared with WT mice when challenged with the B16 melanoma tumour cell line (Fig.1). Surprisingly, we observed the opposite effect when mice were transplanted with the ID8 tumour cell line (Fig.1), that is, TCRδ-deficient mice showed increased survival compared with WT mice. Of note, to exclude differences due to the route of administration, both cell lines were transplanted intra-periteonally (B16 cells are usually injected sub-cutaneously and ID8 are usually injected intra-periteonally).

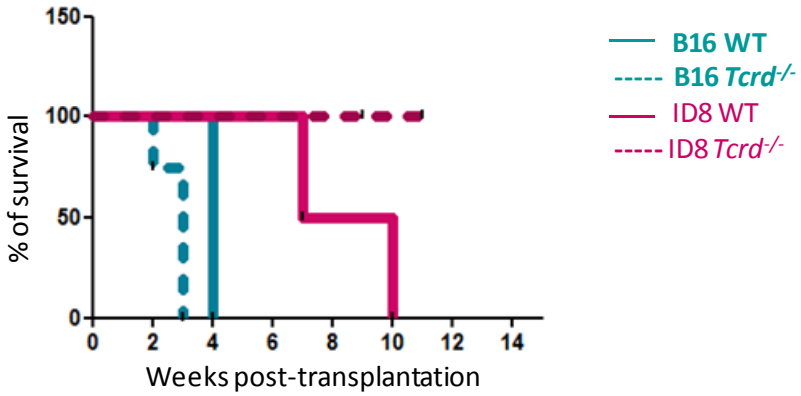


Figure 1 – TCRδ-deficient mice show decreased survival upon B16 tumour challenge and increased survival upon ID8 tumour challenge. Wild-type B6 mice (n=5) and *Tcrd*$^{-/-}$mice (n=5) were transplanted intraperitonealy with 5x10^{4} B16 luciferase (luc) cells or 5x10^{6} ID8 luc cells, and mice survival was determined over 12 weeks. Data presented in this figure are representative of at least three independent experiments with consistent results.

4.3.2 Distinct γδ T cell functions in ID8 and B16 models are not accounted by cytotoxicity of IFN-γ production

We first hypothesised that the opposite results we observed in mouse survival were due to different susceptibilities of B16 and ID8 tumour cells to γδ–mediated cytotoxicity. However, we found that *in vitro*-activated γδ T cells killed B16 and ID8 tumour cell lines with similar efficiencies (Fig.2A and 2B). We then examined the impact of tumour challenge on the accumulation of γδ T cells in the peritoneal cavity, and observed that in both tumour models, there was an increase in total γδ T cell numbers (Fig. 3), although not statistically significant in the B16 tumour bearing mice. These results suggested that differential accumulation of peritoneal γδ T cells could not *per se* explain the opposite survival patterns we observed for B16 and ID8 tumour models in *Tcrd*$^{-/-}$ mice *versus* WT mice.

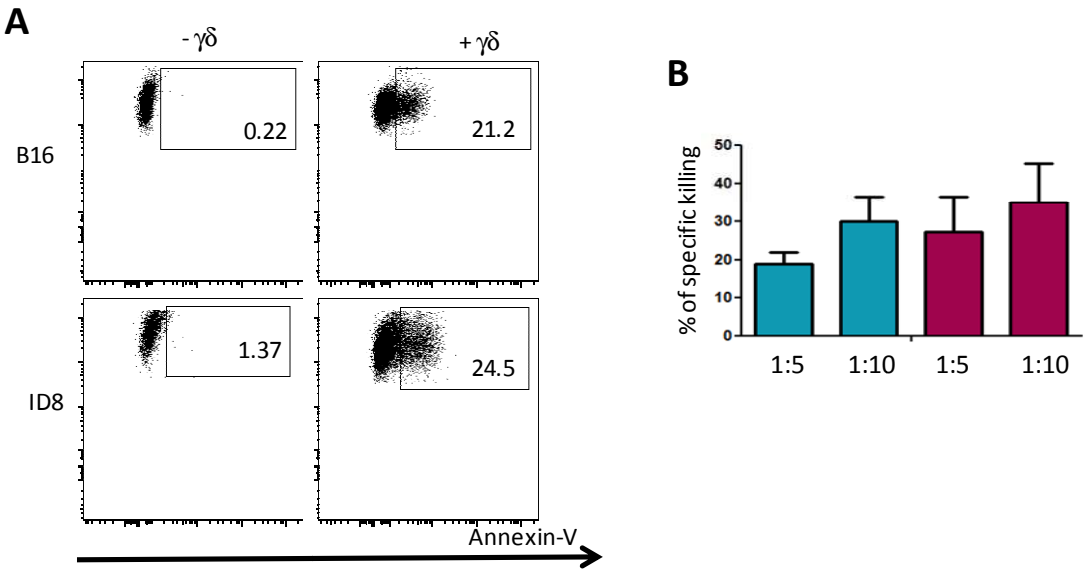


Figure 2 – Activated γδ T cells show equivalent *in vitro* cytotoxicity toward B16 and ID8 tumour cell lines. γδ T cells were sorted from naïve spleen of wild-type mice an activated *in vitro* with α-CD3 for 48hours. γδ T cells were incubated with DDAO-SE-labelled tumour cells B16 (blue) and ID8 (magenta) and after three hours the culture was stained for Annexin-V for apoptosis analysis.

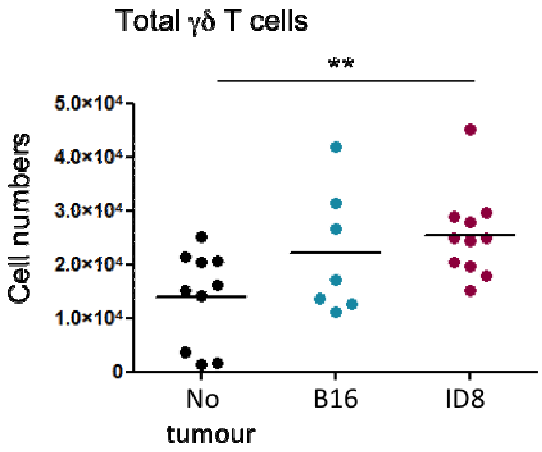


Figure 3 – Peritoneal γδ T cells expand upon B16 and ID8 tumour transplantation. Wild-type B6 mice (n=10) were injected intraperitonealy with PBS (no tumour), 5x104 B16 luc cells (blue) or 5x106 ID8 luc cells (magenta), and peritoneal exudates were analysed after six days by flow-cytometry. Data presented in this figure are representative of at least three independent experiments with consistent results. Error bars represent SD (n=3, **p<0.01).

The anti-tumour properties of IFN-γ-producing γδ T cells are well described for several tumour models, including the B16 model[248]. We therefore hypothesised

that differential IFN–γ production could explain the different phenotypes in ID8 *versus* B16 models. We compared the number of IFN-γ producing γδ T cells in the peritoneal cavity of naïve mice, B16 tumour bearing mice and ID8 tumour bearing mice. Briefly, we collected peritoneal exudates at several time points after tumour transplantation and performed intracellular cytokine staining for IFN-γ.

At day three post-transplantation, for the B16 tumour model, we observed similar numbers of IFN-γ producing γδ T cells as in healthy mice (Fig.4). Also IFN-γ MIF levels were similar (data not shown). Interestingly, at one week post-transplantation, there was a reduction on the number of IFN-γ^+ γδ T cells (Fig.4), which was accompanied of decreased survival of B16 tumour bearing mice (Fig.1).

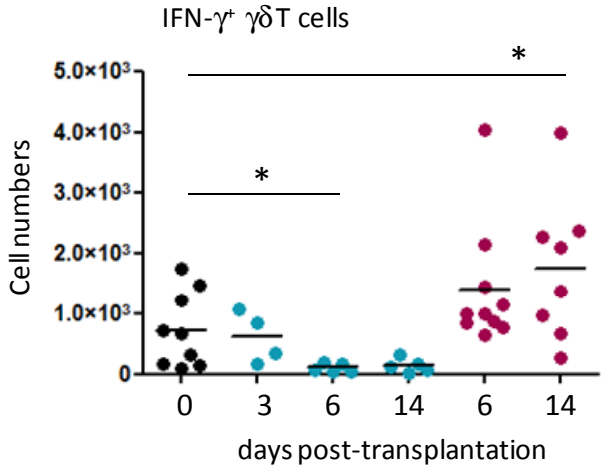


Figure 4 - IFN-γ+ γδ T cells accumulate in the peritoneal cavity of ID8 tumour bearing mice. Wild-type B6 mice (n=10) were injected intraperitonealy with PBS (day 0), $5x10^4$ B16 luc cells (blue) or $5x10^6$ ID8 luc cells (magenta), and peritoneal exudates were analyzed several days pos-tumour transplantation by flow-cytometry. Data presented in this figure are representative of at least three independent experiments with consistent results. Error bars represent SD (n=3, *p<0.05).

Most strikingly, and by contrast to the B16 model, we observed an accumulation of IFNγ^+ γδ T cells along tumour progression in the ID8 model (Fig 4.). This accumulation was maintained for at least until ten weeks post-transplantation (data not shown). Thus, contrary to our expectation, there was higher accumulation of IFN-γ^+ γδ T cells in the ID8 model than in the B16 model. This suggests that the distinct phenotypes of *Tcrd*$^{-/-}$ mice in the two models cannot be explained by differential IFN-γ production.

Finally, because murine γδ T cells need CD27 signals to efficiently produce IFN–γ[155], we compared tumour progression in WT and *Cd27*$^{-/-}$ mice using an *in vivo* bioluminescence assay. Due to the fast tumour progression in the B16 tumour model, we could not assess tumour burden in this model. Nevertheless, in the ID8 tumour model we observed a tendency for higher tumour burden in *Cd27*$^{-/-}$ mice compared with WT mice (Fig. 5A and 5B). Although not significant, this data suggests

that IFN-γ production by γδ T cells in the ID8 tumour model may also play a role, as described for the B16 model using the subcutaneous route[248].

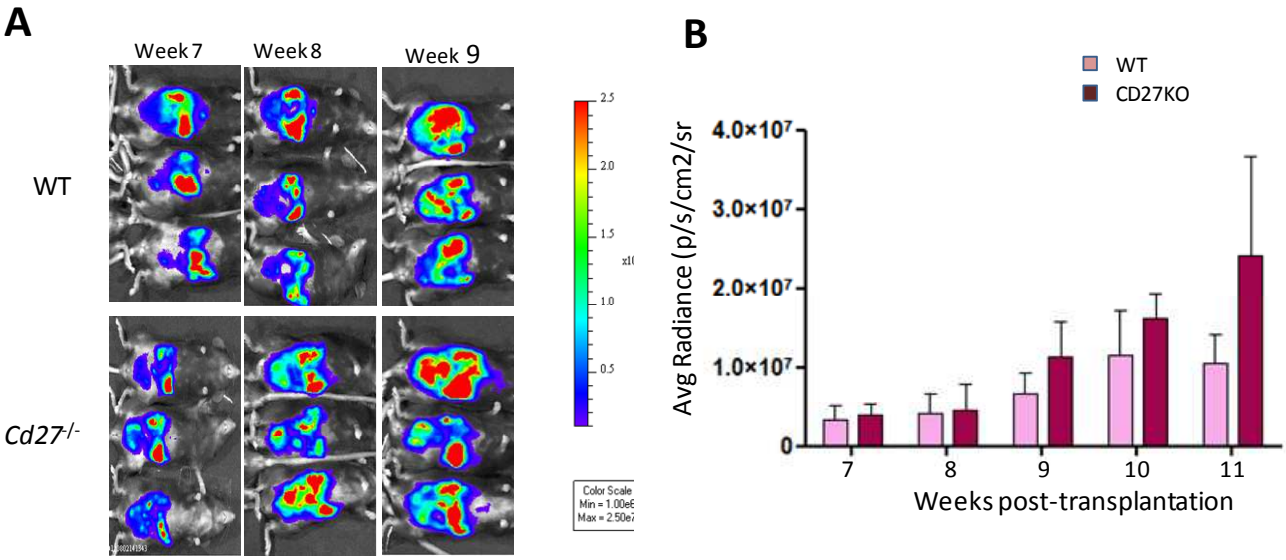


Figure 5 - ID8 tumour growth in CD27-deficient mice. Wild-type B6 mice and *Cd27*$^{-/-}$ mice (n=6) were transplanted intraperitonealy with 5x10^6 ID8 luc cells and tumour burden was assessed weekly by luminescence.

4.3.3 Accumulation of suppressive MDSCs during B16 tumour progression

After one week of B16 tumour challenge we observed an increase of total γδ T cell numbers (Fig.2) but a decrease of both percentage (data not shown) and cell numbers of IFNγ$^+$ γδ T cells compared with healthy mice (Fig.4). Interestingly, our myeloid markers (CD11b and GR1) which define the recently identified myeloid-derives suppressor cells (MDSCs), revealed their absence in healthy control mice, whereas they strongly accumulate in B16 tumour bearing mice (along with tumour progression (Fig. 6A). Moreover, MDSCs were not present (or present at very low numbers) in the peritoneal cavity of ID8 tumour bearing mice (at least at two weeks post-transplantation).

After observing the accumulation of MDSCs in the peritoneal cavity of B16 tumour bearing mice, we examined the functionality of these cells by performing an *in vitro* suppression assay. We sorted MDSCs from the peritoneal cavity of B16 tumour bearing mice (two weeks post-transplantation) and incubated them with γδ T cells for three days and assessed γδ T cell proliferation by thymidine incorporation. We observed a significant decreased proliferation of γδ T cells (but not CD4$^+$ T cells) when in presence of MDSCs (Fig. 6B), which suggests that the decrease numbers of IFN-γ$^+$ γδ T cells could be in part explained by the suppression activity of MDSCs.

Further studies are needed to disclose the mechanism by which MDSCs interact and inhibit γδ T cells.

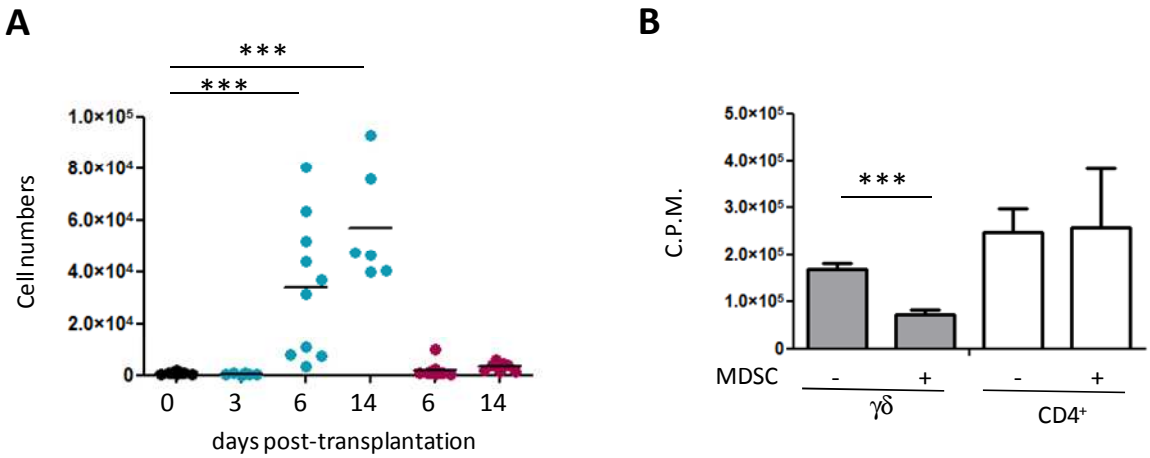


Figure 6 - MDSCs accumulate in the peritoneal cavity of B16 tumour bearing mice and inhibit γδ T cell proliferation *in vitro*. (A) Wild-type B6 mice (n=10) were injected intraperitonealy with PBS (day 0), $5x10^4$ B16 luc cells (blue) or $5x10^6$ ID8 luc cells (magenta), and peritoneal exudates were analyzed several days later by flow-cytometry. Data presented in this figure are representative of at least three independent experiments with consistent results. Error bars represent SD (**p<0.01). (B) MDSCs were sorted by flow-cytometry from the peritoneal cavity of B16 tumour bearing mice and incubated for 3 days with γδ T cells or $CD4^+$ T cells (sorted by flow cytometry from spleens of naïve mice). The cultures were then scored for thymidine incorporation. Error bars represent SD (*** p<0.001).

4.3.4 Accumulation of IL-17$^+$ producing γδ T cells in the peritoneal cavity of ID8 tumour bearing mice may promote tumour progression

While anti-tumour properties of IFN-γ producing cells are well described, the role of IL-17-producing cells in tumour immunosurveillance is quite controversial. Moreover γδ T cells have been described to produce IFN-γ and/or IL-17 in various tumour models[42, 248].

Critically in the ID8 tumour model, IL-17 was previously demonstrated to promote tumour progression[267]. In that work, Th17 cells were described as the major source of IL-17, but importantly, the presence of γδ T cells was not addressed. For this reason, we set out to compare the presence of IL-17$^+$ γδ T cells in healthy, B16 and ID8 tumour bearing mice. Interestingly, we observed IL-17$^+$ γδ T cell accumulation in the peritoneal cavity of ID8 tumour bearing mice but not in B16 tumour bearing mice (Fig. 7). Also, after gating in CD3$^+$IL-17$^+$ cells of the peritoneal exudates of ID8 tumour bearing mice, we noted that γδ T cells were the main T cell subset producing IL-17 (Fig. 8A). We also observed that γδ T cells presented the highest MFI for IL-17 of all CD45$^+$ lymphocytic gated T cells (Fig. 8B), indicative of higher IL-17 expression on a per cell basis. These data suggest that γδ T cells are an

important source of IL-17 in the ID8 tumour model, which is a likely mechanism[267] to explain their pro-tumour role in this (but not the B16) tumour model.

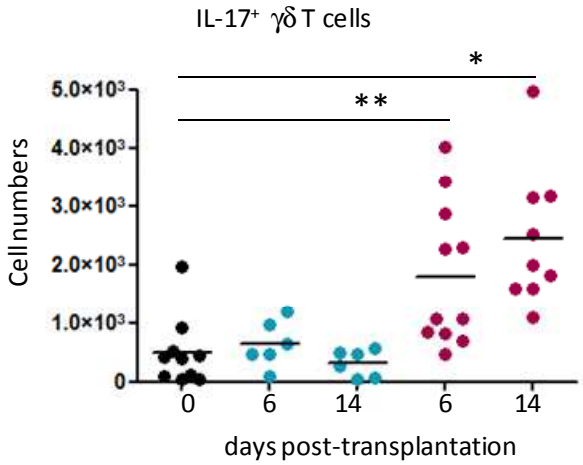


Figure 7 - IL-17$^+$ γδ T cells accumulate in the peritoneal cavity of ID8 tumour bearing mice. Wild-type B6 mice (n=10) were injected intraperitonealy with PBS (day 0), 5x10^4 B16 luc cells (blue) or 5x10^6 ID8 luc cells (magenta), and peritoneal exudates were analyzed several days pos-tumour transplantation by flow-cytometry. Data presented in this figure are representative of at least three independent experiments with consistent results. Error bars represent SD (*p<0.05; **p<0.01).

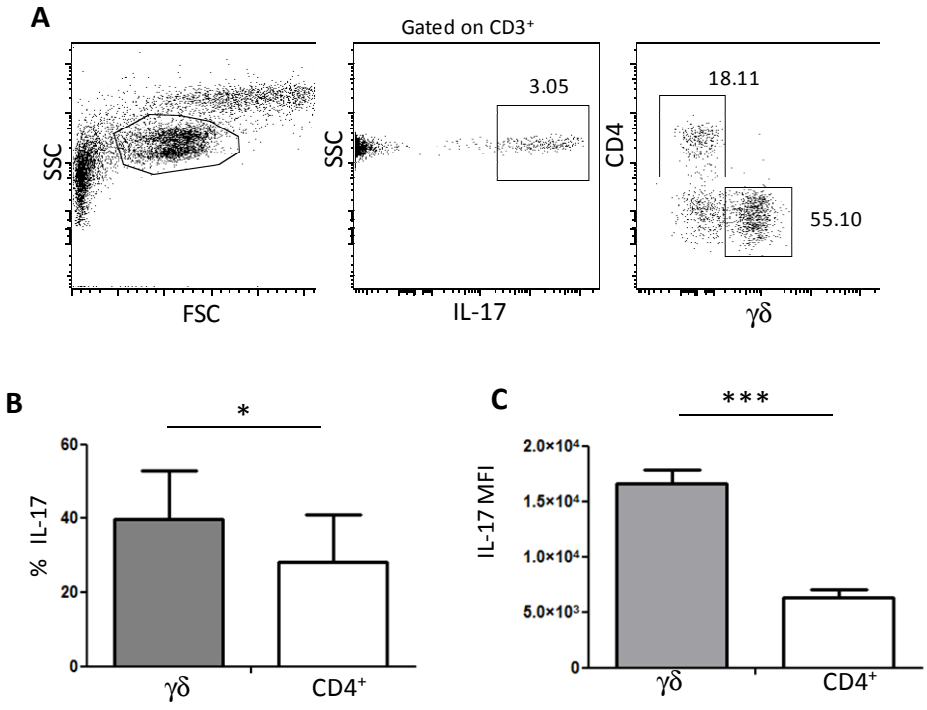


Figure 8 - γδ T cells are a major source of IL-17 in the peritoneal cavity of ID8 tumour bearing mice. Wild-type B6 mice (n=10) were transplanted intraperitonealy with 5x10^6 ID8 luc cells, and peritoneal exudates were analyzed fourteen days pos-tumour transplantation by flow-cytometry. **(A)** Flow cytometry of IL-17$^+$ T cell subsets based on intracellular IL-17 staining; **(B)** Percentage of IL-17$^+$ cells within

$CD3^+$ T cells **(C)** Mean fluorescence intensity (MFI) for IL-17 intracellular staining. Data presented in this figure are representative of at least three independent experiments with consistent results. Error bars represent SD (*p<0.05; ***p<0.001).

4.4 Discussion and future work

We found in this study that γδ T cells make strikingly different contributions to tumour progression in two different murine transplantable tumour models. Thus, *Tcrd*$^{-/-}$ mice showed increased survival (compared to WT controls) in the ID8 model (Fig. 1), but reduced survival (compared with WT mice) in the B16 model. This establishes a pro-tumour role of γδ T cells in the ID8 model and an anti-tumour role of γδ T cells in the B16 model. Of note, this was independent of the route of administration of the tumour cells, as the phenotypes were similar following intraperitoneal (i.p) or subcutaneous (s.c.) injections (Fig. 1 and data not shown).

In order to dissect the mechanism underlying this divergent behaviour of murine γδ T cells, we first addressed if B16 and ID8 tumour cells displayed distinct susceptibilities to γδ-mediated cytotoxicity. We performed in *vitro* cytotoxicity assays using γδ T cells previously activated with α-CD3. Interestingly, we found that B16 and ID8 tumour cells were similarly killed. We thus excluded the hypothesis that γδ T cells preferentially killed B16 tumour cells in comparison with ID8 tumour cells, as an explanation for the distinct *Tcrd*$^{-/-}$ phenotypes in the two models.

The mechanisms of anti-tumour functions assigned to γδ T cells have been extensively documented both in murine and human settings, and mostly include (besides cytotoxicity) production of IFN-γ and TNF-α[144]. We observed an accumulation of IFN-γ^+ γδ T cells in the ID8 model but not in the B16 model. Considering that IFN-γ was shown to be indispensable for the protective function of γδ T cell in the B16 model[248], we propose that steady-state provision of IFN-γ by peritoneal γδ T cells accounts for the protective effect of γδ T cells in the B16 model.

In order to understand the mechanism behind the decrease in IFN-γ^+ γδ T cells in the peritoneal cavity of B16 tumour bearing mice after one week, we analysed the presence of suppressor cells, namely MDSCs. Interestingly, we observed a dramatic increase in MDSCs numbers after one week of tumour transplantation onwards. Importantly, we did not observe accumulation of MDSCs in ID8 tumour bearing mice (at least at two weeks post-transplantation). To investigate if MDSCs could directly impact on γδ T cells, we performed *in vitro* suppression assay, and observed that in the presence of MDSC, γδ T cells significantly proliferate less. We thus propose that MDSC can be a tumour immune escape mechanism that can partially explain why γδ T cells cannot control B16 tumour progression beyond one week of tumour challenge.

Considering IFN-γ as the hallmark cytokine for immunosurveillance, we would not expect an accumulation of IFN-γ^+ γδ T cells in the ID8 tumour bearing mice compared with WT mice, where the net effect of γδ T cells is pro-tumour. We thus hypothesize that other mechanisms may dominate over IFN–γ accumulation

As it was previously shown that IL-17 promotes tumour progression in the ID8 model, we hypothesized that peritoneal γδ T cells could be an important source

of IL-17 in this tumour model. Charles and colleagues[267] demonstrated that Th17 cells were a source of IL-17 in ID8 tumour bearing mice, but they did not analyse the contribution of γδ T cells. Interestingly, we observed that IL-17-producing γδ T cells expanded significantly in the peritoneal cavity of ID8 tumour bearing mice (compared with healthy mice), thus becoming the major T cell source of IL-17 in this model.

The analysis of cytokine production by peritoneal γδ T cells in both tumour models revealed that, both effector subtypes (IL-17-producers and IFN-γ-producers) accumulated in the ID8 model, where the net effect of γδ T cells is pro-tumour. Thus, we believe that the pro-tumour function of IL-17^{+} γδ T cells dominates over IFN-γ^{+} γδ T cells in the ID8 model. Interestingly, a report from Wang and colleagues[37] showed that growth of B16 and MB49 (bladder carcinoma cell line) tumours was reduced in *Il-17*$^{-/-}$ mice but accelerated in *Ifng*$^{-/-}$ mice. In addition, IL-17/IFN-γ double knockout mice were resistant to tumour cell growth similarly to *Il-17*$^{-/-}$, suggesting a dominant role of IL-17 in tumour progression. In this study, the authors suggested that Th17 cells were the predominant source of IL-17, but they did not evaluate the contribution of γδ T cells.

To definitely test if IL-17 is the molecular determinant of the pro-tumour function of γδ cells in the ID8 model, we plan to compare ID8 tumour progression upon IL-17 antibody blockade in WT versus TCRδ$^{-/-}$ mice. Moreover, we aim to generate double knockout mice *Tcrd*$^{-/-}$ *Il*-17$^{-/-}$ mice and compare tumour progression with single knockout mice.

Most studies dealing with IL-17 producing γδ T cells (including ours) have been performed in mice, and it is therefore unclear whether such functional features also apply to human γδ T cells[308]. A recent paper by Dieli and colleagues, showed an increased percentage of IL-17-producing Vγ9Vδ2 T cells in the peripheral blood and cerebrospinal fluid in children with bacterial meningitis[309]. While this data suggest a significant contribution of IL-17^{+} γδ T cells in an infection context, it will be important to investigate the contribution of IL-17 production by human γδ T cells in a tumour setting.

While it is evident that murine γδ T cells can produce IFN-γ or IL-17, the factors that determine which cytokines are produce in each tumour context are not clear. We propose that the tumour microenvironment comprises a complex cytokine network that may determine the functional properties of tumour-infiltrating lymphocytes, namely γδ T cells. The dissection of these components of the tumour microenvironment will be a focus of future research.

4.5 Material and methods

Mice

C57BL/6 (wild-type) mice were obtained by Charles River. *Tcrd*$^{-/-}$ mice were obtained from The Jackson Laboratory. Female mice, aged from 6 to 10 weeks, were used for all experiments. All experiments had at least five mice per group and were repeated 2-3 times.

In vitro killing assay

γδ T cells were sorted by fluorescence-activated cell sorting (FACS) from pooled spleens and lymph nodes of C57BL/6 mice. They were activated for 48h with plate-bound αCD3. B16 and ID8 cells were stained with DDAO-SE (Invitrogen) and plated in round-bottom 96 well plates the day before the assay. Tumour cells were incubated for 3h in the absence or presence of γδ T cells. γδ T cells were washed and tumour cells were detached with EDTA and stained for Annexin V (eBioscience). The percentage of DDAO-SE positive, apoptotic cells (AnnexinV^{+}) was determined by flow cytometry.

Tumour challenge

B16 murine melanoma cells and ID8 ovarian carcinoma cells (stably expressing firefly luciferase) were maintained in Dulbecco´s modified Eagle´s medium supplemented with fetal calf serum and penicillin-streptomycin at 37ºC in 5% CO_2. For tumour challenge, 5x10^4 B16 luc cells or 5x10^6 ID8 luc cells (in 200µl of PBS) were injected intraperitonealy (i.p.). Tumour burden was assessed weakly by *in vivo* bioluminesce imaging (IVIS, Caliper Lifesciences) upon intra-peritoneal injection of luciferin. Photon signals were quantified with LivingImage software (Caliper Lifesciences).

Peritoneal exudates leukocyte analysis

Peritoneal exudates were collected by injecting 5ml of PBS in the peritoneal cavity of mice. After red-blood cell lysis, samples were washed in FACS buffer (PBS 2% FBS) and stained for 20min at 4ºC with the relevant antibodies. Surface antibodies used were: γδTCR PE, CD3 V500, CD4 FITC, CD45 APCCy, IL-17 APC, IFN-γ PeCy7, CD11b APC, Gr1 PE (eBioscience). For measurements of intracellular cytokines, cells were re-stimulated for 4h with PMA and Ionomycin in the presence of 1ug/ml Brefeldin A (Sigma). Cells were then fixed and permeabilized using the FoxP3 kit (according to manufactures guidelines) and analysed by flow cytometry on a FACS Fortessa (BD Pharmigen).

CHAPTER V:

General discussion and future perspectives

5.1 General discussion

In this thesis, we have characterized three key aspects of tumour immunology associated with γδ T cells, namely: tumour infiltration mechanisms, tumour cell recognition, and putative pro-tumourigenic properties. We believe our findings make an important contribution to basic and pre-clinical knowledge on γδ T cell response to tumours.

5.1.1 Tumour cell recognition by γδ T cells

Most γδ T cells have a phenotype of spontaneous activation with a fast turnover rate *in vivo*[310] and quickly expand in the first few days after tumour challenge. They are recruited to tumour transplantation sites earlier than αβ T cells, where they provide an early source of IFN-γ[248]. These remarkable anti-tumour properties of γδ T cells have been explored in several clinical trials based on their stimulation by the administration of small non-peptidic phosphoantigens or bisphosphonates in combination with rIL-2[290].

Despite the enrolment of γδ T cells in tumour clinical trials, very few tumour-associated antigens have been identified, and this constitutes the greatest enigma in γδ T cell biology. In chapter 2 we set out to investigate the molecular requirements for γδ T cell recognition of haematological tumour cells.

Interestingly we observed that while NKG2D signaling was necessary for tumour cell recognition by Vγ9Vδ2 T cells, TCR signaling was not. This constitutes a provocative finding, as for conventional T cells ($CD4^+$ and $CD8^+$ T cells), TCR specificity is coupled to target cell recognition. Nevertheless, for γδ T cells, conflicting results have been reported concerning the roles of TCRγδ and NKG2D in recognition of tumour cell targets. In haematological tumours, TCR was shown to be important for the killing of Daudi cells[311], but not Molt-4 or K562 cell lines[311, 312] (consistent with our data). Concerning epithelial tumours, namely metastatic renal cell[313], colorectal[261] and hepatocellular[314] carcinomas, both NKG2D and TCRγδ were shown to be involved in tumour cell recognition.

Some studies that propose strong dependency on TCR stimulation, claim that Vγ9Vδ2 T cells recognize tumour targets through the interaction of TCRγδ with endogenous phosphoantigens produced by tumour cells, while NK receptor signals contribute only to fine tune the cell activation threshold[171, 203, 282, 283, 315]. Moreover, the recognition of Daudi and K562 tumour cell lines by Vγ9Vδ2 T cells, correlated with IPP production[183] and F1-ATPase/ApoA-I expression[177] on target cells. By contrast, it was shown that Vγ9Vδ2 T cells can be directly activated by NKG2D (MICA fusion protein) without the need of TCRγδ stimulation[201].

Our study and others from our lab[172, 240] suggests that Vγ9Vδ2 T cell-mediated immunosurveillance of haematologic tumours is a two-step process, where effector lymphocyte activation, proliferation and cytokine production is achieved through TCRγδ stimulation, but tumour cell recognition is "NK-like", predominantly mediated by NKG2D and/or other NK receptors. Considering a haematopoietic immunotherapy scenario, this feature of Vγ9Vδ2 T cells may constitute an advantage over conventional T cells, as the chances of encountering a specific antigen by conventional T cells are putatively lower than the chances of encountering a stress-inducible molecule by NKG2D.

The independency of TCR for tumour target cell recognition was also observed for the Nkp30$^+$ Vδ1$^+$ subset, in which both NKG2D and natural cytotoxicity receptors (NCRs), but not TCR Vδ1, were involved in tumour cell recognition[240]. Provocatively, for human cytotoxic T lymphocytes (CTLs), some reports also postulated that NKG2D can mediate cytolysis independently of TCR engagement, when CTLs are exposed to IL-15 or high doses of IL-2[204, 205].

The observation that tumour target recognition was NKG2D-dependent led us to investigate the expression of NKG2D ligands on the surface of haematological tumour cell lines. We observed that ULBP1 expression clearly segregated with susceptible lines, that is, it was highly expressed in all susceptible cell lines but moderately expressed or absent in resistant cells. Through a series of loss- and gain-of-function assays, we showed that ULBP1 expression determines leukaemia and lymphoma recognition by Vγ9Vδ2 T cells. Notably, we presented the first physiologic evidence for lymphocyte requirement of NKG2D-ligand expression in tumours, since previous studies[199, 212, 222, 288] were based on ectopic expression of the corresponding ligands.

Our findings have important implications for immunotherapy of haematological malignancies, since it may be possible to manipulate ULBP1 expression in tumours *in vivo*, for example through administration of some conventional cancer drugs. Thus, bortezumib (an approved drug for the treatment of plasma cell myeloma) treatment of head and neck squamous cell carcinoma (HNSCC) cell lines, dramatically upregulated ULBP1 protein expression at the cell surface[289]. 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[316]. Also, DNA-damaging agents and DNA synthesis inhibitors may upregulated ULBP1, as genomic instability is frequently associated with expression of stress-molecules[208].

Our findings further suggest that ULBP1 can be used as a biomarker for patient selection on clinical trials of haematological tumours exploring Vγ9Vδ2 T cells. We hypothesize that ULBP1 expression in patient cancer cells will be a predictor of the efficiency of these therapies, as cytotoxicity mediated by Vγ9Vδ2 T cells will presumably depend on ULBP1 expression. Considering the variable percentage of partial remission in patients that underwent γδ T cell-based treatments, it is tempting to speculate that those results may reflect the

heterogeneous expression on ULBP1 in tumour cells. In fact, ULBP family members have been shown to have predictive value, as their expression has been correlated with improved survival in cancer patients[222] and have been shown to be an independent prognostic parameter in breast cancer patients[227].

NKG2D ligand upregulation occurs during the cell transformation process, as these proteins are induced upon stress. Nonetheless, not all tumour cells express NKG2D ligands, as evidenced in our expression data of NKG2D ligands in lymphoma and leukaemia samples. This could be viewed as an immune evasion mechanism employed by tumour cells in order to avoid immune recognition, and thus sustain tumour growth. Several strategies employed by tumours have been proposed to induce the downmodulation of NKG2D ligands, including: the production of immunomodulatory cytokines, such as TGF-β[232, 317 318 195] and decreased tumour surface expression by shedding of the extracellular domain by metalloproteases[319, 320]. Moreover expression of NKG2D ligands can be actively regulated by microRNAs, and tumour cells also have been shown to manipulate this system via miRNA overexpression[321].

Some tumours exploit an opposite strategy to manipulate NKG2D mediated signaling, and instead induce high levels of NKG2D ligands[322]. The sustained expression of NK2D ligands may lead to the downmodulation of the NKG2D receptor, and thus compromise tumour cell recognition. For example, the high expression of ULBP2 in ovarian cancer patients inversely correlated with disease survival[323]. Also, soluble forms of NKG2D ligands, that are shedded from tumour cell surface by proteolytic cleavage[319] or by incorporation into exossomes[218], may downmodulate NKG2D expression. Oppenheim and coworkers also suggested that constitutive engagement of NKG2D impaired NK cell function beyond its downmodulation and subsequent inability to engage its ligands on tumour cells[324]. Similar observations were described for the murine intraepithelial subset (Vγ5Vδ1)[252].

In this study we demonstrate the importance of ULBP1 for the recognition of haematological tumours by Vγ9Vδ2 T cells. This notwithstanding, we probably cannot extrapolate these findings to other cancer types. Further studies will be required to address this question. Indeed, some studies indicate that other NKG2D ligands may play a major role in other types of tumours. For example, ectopic expression of ULBP4 was recently shown to trigger Vγ9Vδ2 T cell cytotoxicity against ovarian and colon carcinomas[178]. Interestingly, in this study the authors propose that ULBP4 binds both to TCRγδ and to NKG2D. Tissue-associated $V\delta1^+$ T cells were also found to respond to NKG2D ligands, namely MICA/B and ULBP3[279].

NKG2D is an unusual receptor because it binds to multiple ligands that have limited sequence homology. This ligand variety opens the question for the need of redundancy in NKG2D-mediated sense of transformation. Because transcription of the several ligands is independent of each other, different ligand expression may reflect a different form of stress sensing. Also, ligands engage NKG2D with different affinities, suggesting that they may not be functionally equivalent. Moreover, the redundancy may broaden the spectrum of recognizable tumour targets. Still, we

(and others) have observed a large proportion of resistant cell lines and patient samples in our studies. This could mean that NKG2D ligands are not involved in tumour cell recognition or that they might be downregulated, as a form of potential immune escape mechanisms. Additionally, other receptors can be involved in tumour cell recognition.

In fact, other receptors (than TCRγδ and NKG2D) have been shown to participate in tumour recognition by γδ T cells, namely DNAM-1 for Vγ9Vδ2 T cells[243] and NKp30 for Vδ1$^+$ T cells[240]. DNAX accessory molecule-1 (DNAM-1) was shown to be expressed by Vγ9Vδ2 T cells, and its ligands, Nectin-like-5 and Nectin-2 were present on most hepatocellular carcinoma (HCC) cell lines[243]. mAb-mediated blocking experiments demonstrated that cytotoxicity against HCC cells, as well as IFN-γ production by γδ T cells, were dependent on DNAM-1. Finally, they showed by combined mAb-mediated blockade experiments, that DNAM-1 and NKG2D could cooperate in the cell lysis of HCC[243]. Although Vδ1 T cells do not express the natural cytotoxicity receptor NKp30 constitutively, it can be upregulated by AKT-depended signals provided synergistically by γc cytokines (IL-2 or IL-15) and TCR stimulation[240]. NKp30 expression on Vδ1 T cells endowed them with enhanced cytotoxic functions, which allowed recognition of lymphoid leukaemias considered resistant for Vγ9Vδ2 T cells[240].

Because many of the indentified TCRγδ ligands are non-self molecules expressed by microorganisms[325], it is possible that the Vγ9Vδ2 TCR was evolutionary selected to detect microbial molecules or metabolites, while the sensing/recognition of “self” and “self stressed” is mostly mediated by NK receptors. Indeed, although the gene signature of resting and early activated Vγ9Vδ2 γδ T-cells is closer to CD4$^+$ T-cells, that of established and activated Vγ9Vδ2 γδ T cells is mostly similar to gene signature of NK cells [326]. This, together with the fact that human NK cells and γδ T-cells share many activating/inhibitory receptors, asks for a more careful comparative study of NK and γδ T cell biology in order to understand their functions and mechanisms in different cancer settings.

In sum, our current working model includes two stages for γδ T-cell activation and tumour cell recognition. First, γδ T cells are activated by TCRγδ ligands, which are mostly unknown (but are somehow linked to phosphoantigens in the case of Vγ9Vδ2 T cells), in the presence of IL-2. This endows γδ T cells with potent cytolytic (and cytokine-secreting) function, but requires a subsequent phase of target identification, namely for discrimination between tumour and healthy cells. We propose this is mainly achieved through activating NK receptors that bind proteins that selectively accumulate on the surface of tumour cells. We believe the integration of these two phases in the conception of γδ T cell-based immunotherapy protocols will be key for their clinical success.

5.1.2 Recruitment of γδ tumour-infiltrating lymphocytes

In chapter IV we demonstrated that γδ T cell recruitment to tumours *in vivo* requires CCR2/CCL2 chemokine signaling. The dependency on the CCR2/CCL2 chemokine axis for γδ T cell migration has been previously shown by Penido and colleagues specifically for lung infiltration during infection[295] and allergic inflammation[294]. This notwithstanding, our data is pioneer in suggesting a role for CCR2/CCL2 in the recruitment of murine γδ T cells in a tumour context. Strikingly, we observed that B16 tumours were significantly bigger in *Ccr2*$^{-/-}$ than in WT mice. These data suggested a dominance of CCR2/CCL2-dependent anti-tumour functions (mediated by γδ T cells), over the well-established CCR2/CCL2-dependent tumour-promoting activities of myeloid cells.

CCL2 is found in the majority of solid cancers and its role in tumour progression is both prominent and multifaceted[291]. Apart from a minor direct role in tumour cells themselves, CCL2 has received attention concerning its involvement in the recruitment of tumour-infiltrating leukocytes. CCL2 is one of the main determinants of tumour-infiltrating macrophages (TAMs) and tailors macrophage differentiation toward the protumourigenic M2-phenotype[97]. Mice injected with B16 melanoma cells transfected with a shRNA against CCL2 exhibited reduced malignant pleural effusions and enhance survival, together with reduced number of macrophages and blood monocytes compared with control shRNA transfectants[327]. Moreover, CCL2 recruits monocytes to pulmonary metastatic sites of murine breast cancer. As a consequence, infiltrated monocytes promote extravasation of tumour cells, as a necessary step for metastasis, in a process that requires monocyte-derived VEGF[296]. CCL2 is also involved in the recruitment of MDSCs, in various types of mouse cancers, including Lewis lung carcinoma, methA sarcoma, melanoma and lymphoma[98]. CCR2-expressing MDSC accumulate in melanomas where they hampered CD8$^+$ T cell entry to the tumour site, thus limiting the efficacy of cancer immunotherapy[300].

Although historically the CCR2/CCL2 axis has been implicated in tumour progression, some recent reports show that CCL2 allows the infiltration of typical anti-tumour lymphocyte substes, such has Th1 cells, CD8$^+$T cells and NK cells. For example, B16 tumour growth was increased in *Ccl2*$^{-/-}$ mice, with tumour progression being associated with decreased CD4$^+$ and CD8$^+$ T cells and NK cells[328]. Also, CCL2 transduction of hepatocellular tumour cells retards tumour growth *in vivo* by activating NK cell activity[329]. CCL2 has also been to show to have a preponderant role in the context of cancer immunotherapy adoptive transfer protocols. Human cytotoxic lymphocytes (CTLs) adoptively transferred into nude mice with human melanoma xenografts that do not express CCL2, showed reduced T cell homing to the tumour[301]. Moreover, adoptive transfer of Th17 CD4$^+$ T cells prevented B16 lung metastasis regression. Th17 cells stimulated the expression of CCL2 and CCL20 in the lung microenvironment promoting the recruitment of various leukocyte subsets (DCs and CD4$^+$ and CD8$^+$ T cells)[330].

Several factors may account for these contradictory effects of the CCR2/CCL2 axis in the tumour context. First, the levels of CCL2 can affect the overall tumour growth. For example, Nesbit and colleagues showed that the biological effects of tumour-derived CCL2 are biphasic, depending on the level of secretion. Low level of

CCL2 secretion resulted in modest monocyte infiltration, which in turn promoted tumour progression; whereas high CCL2 secretion was associated with massive monocyte/ macrophage infiltration into the tumour mass, leading to its destruction[331]. In fact, we should take into account that many studies rely in the ectopic expression of CCL2, which generally results in overexpression, while other studies rely on the "natural" expression by tumour cells or cells of the microenvironment. Second, the nature of tumour-infiltrating leukocytes can be heterogeneous and context dependent. For example, macrophages can show dual phenotypes, M1 and M2, and thus, depending on the infiltrating phenotype, CCL2 recruitment of macrophages can have an anti- or pro-tumour role. This type of dichotomy for the role of chemokines is tightly linked to the tumour microenvironment. There will be different outcomes depending on whether the leukocytes that are recruited are capable of tumour cell killing, or whether they, for example, promote tumour metastasis through the release of factors that facilitate the intravasation of tumour cells into the vascular system. Thirdly, CCL2 can suffer post-translational modifications *in situ*, thus altering its function. The pioneer work by Viola and colleagues demonstrated that reactive nitrogen species (RNS) presented intratumoraly induce CCL2 nitration that hinders CCL2-mediated $CD8^+$ T cell infiltration[102]. This finding demonstrates that although CCL2 is present at the tumour site its function is abrogated.

Considering the dichotomy of the inflammatory CCR2/CCL2 axis in tumour progression, our results suggest a novel anti-tumour role trough the recruitment of γδ T cells. Our B16 melanoma *in vivo* data demonstrated that both γδ T cells and myeloid cells (macrophages, neutrophils and MDSCs) were less abundant in tumour lesions from *Ccr2*$^{-/-}$ mice. Because *Ccr2*$^{-/-}$ mice showed bigger tumours than WT mice, two possibilities can explain the phenotype we observed: the reduction on γδ T cell numbers has a greater impact on tumour growth than thereduction of myeloid cells; or, bearing in mind the dual phenotype macrophages (M1 and M2) and neutrophils (N1 and N2) can display, myeloid cells infiltrating B16 lesions may belong to the anti-tumour phenotype M1 and N1. We did not assess this experimentally, so we can only speculate about it. In any case, MDSCs were never reported to play a anti-tumour role, so their concomitant reduction with γδ T cells, suggests that γδ T cell reduction dominates over MDSC reduction, resulting in increased tumour burden.

Interestingly, we did not observed significant changes in $CD8^+$ or $CD4^+$ tumour-infiltrating T cells. Because a dependency on CCL2 has been described for $CD8^+$ T cell recruitment, this discrepancy may reflect differences in the tumour microenvironment between studies. Moreover, the chemokine system is highly redundant, and thus in the B16 melanoma model, conventional T cells may use other chemokines to infiltrate tumour lesions.

Given the role of CCL2 in the tumour microenvironment, antibodies or antagonists to the CCL2/CCR2 axis have been investigated. Administration of anti-CCL2 antibody to prostate tumour bearing mice resulted in decrease tumour burden and bone resorption, concomitant with lower CCL2-induced VEGF levels[332]. Also, anti-CCL2 antibody administration reduced recruitment of inflammatory monocytes to lungs and inhibited the spreading of breast cancer cells to the lung niche, resulting

in survival prolongation of tumour-bearing mice[296]. Moreover, anti-CCL2 antibodies can also synergyze with cancer therapy agents; tumour regression was induced when anti-CCL2 was administered in combination with docetaxel for the treatment of mice suffering from prostate cancer[333]; and when anti-CCL2/CCL12 was administered along with vaccination, tumour size was reduced in subcutaneous tumours[334].

The results from the pre-clinical data with anti-CCL2 antibodies have prompted clinical trials with blocking agents for both CCL2 and CCR2. The anti-CCL2 antibody, CNTO 888, underwent a phase I trial for patients with solid tumours, and no dose-limiting toxic effects in 21 patients were observed[100]. Additionally, the anti-CCR2 antibody MLN1292 is undergoing a Phase II trial in patients with bone metastases[100]. Considering that we propose a novel anti-tumour function to the CCR2/CCL2 inflammatory axis (through the recruitment of γδ T cells), we believe that caution should be taken in the future clinical trials based on blocking agents either for the CCR2 receptor or for the CCL2 ligand.

Human peripheral blood γδ T cells contain two major subsets that express either the Vδ1 (5-30%) or Vδ2 (60-95%) chains. While the Vδ2 subset has been largely explored in cancer immunotherapy clinical trials, the therapeutic potential of Vδ1 population is poorly defined. This is probably due to the fact that Vδ9Vδ2 comprise the major population of peripheral blood-γδ T cells and due to the availability of clinical grade agonists ("phosphoantigens") for the Vγ9Vδ2 receptor. Nonetheless, the usage of the Vδ2 subset for cancer immunotherapy is disadvantageous (comparing to the Vδ1 subset) in that they have been reported to poorly infiltrate tumours[335]. Unlike their counterparts, Vδ1 T cells seem to selectively accumulate in various human epithelial tumours (lung, kidney and colon carcinoma) and lymphomas[161, 256, 264]. Consistent with this, we interestingly observed that Vδ1 cells expressed the CCR2 receptor constitutively (within fresh PBMCs and after activation with PHA and IL-2) and migrated to CCL2 *in vitro*, while the Vδ2 subset does not seem to rely on the CCR2/CCL2 axis for migration. On the other hand, Vδ1 T cells were shown to express different chemokine receptors, namely CCR4 and CCR8, in lymphoma patients[336]. Also, systemic intraperitoneal treatment with $V\delta2^-$ T cell clones delayed growth of xenograft HT29 subcutaneous tumours in a CCR3-dependent manner[337].

Finally, we investigated how CCL2 levels were regulated in human malignancies. Genome-wide trancriptomic data denoted that while in some tumours CCL2 was upregulated (squamous cell carcinoma, breast cancer and primary prostate cancer) when compared to matched healthy tissues; in other tumours, CCL2 was downregulated (metastatic prostate cancer, liver and lung cancer). It is interesting to speculate that downmodulation of CCL2 may be an evasion-mechanism selected in tumours to impair Vδ1 infiltration.

The levels of CCL2 within several tumours have been correlated with prognostic value for cancer patients. In breast[338, 339], prostate carcinoma[340] and colon carcinoma[341, 342] CCL2 is negatively associated with prognosis; whereas in pancreatic cancer[343], CCL2 levels are associated with positive prognosis. Importantly,

these studies proposed that CCL2 levels are correlated with the infiltration of tumour-associated macrophages. Correlation of CCL2 levels with infiltration of other leukocytes was not addressed in the former studies. It is important to bear in mind that another level of complexity is added if considering that the activity of chemokines is controlled not only through the regulation of their expression but also through silent decoy receptors and by posttranslational modifications, including proteolytic processing, glycosilation, deamination, citrullination of the chemokines themselves[344] and nitrosylation[102].

5.1.3 Promotion of tumour growth by γδ T cells

Our results in chapter IV showed that distinct anti- versus pro- tumour contributions of γδ-TILs in the B16 and ID8 models (respectively) associated with a sticking accumulation of IL-17-producing γδ T cells in the latter model (ID8). It was previously shown that IL-17 plays a critical role in ID8 tumour progression[267]. However, the authors of this study attributed IL-17 production to $CD4^+$ Th17 cells and did not address the contribution of γδ T cells. In line with our data, IL-17-producing γδ-TILs were shown to promote tumour growth by promoting angiogenesis[40, 42]. Such IL-17^+ γδ TILs exhibited low levels of perforin and were stimulated through TCR, NKG2D and receptors for IL-23, IL-6 and TGF-β to produce IL-17 and cyclooxygenase (COX-2) in tumour beds[42].

By contrast, it has been recently reported that IL-17-producing γδ T cells contribute to the efficacy of anticancer therapies. After anthracycline treatment, γδ T cells infiltrated tumour beads and produced IL-17. In these studies several preclinical models were analysed and both γδ T cells and CTLs, as well as IL-17 and IFN-γ, seemed to be essential for the therapeutic success of anthracyclines[43, 44]. Moreover, a beneficial role of IL-17-producing γδ T cells in cancer is supported by a study showing that IL-17^+ γδ T cells were indispensable for the efficacy of *Mycobacterium bovis* bacillus Calmette-Guerin (BCG) against bladder cancer by recruiting neutrophils[266]. These divergent effects of $IL17^+$ γδ T cells may reflect the widely reported contradictory role of IL-17 in the tumour microenvironment, as this cytokine has been both described as a pro- and anti-tumour factor[40].

Many functions of IL-17 in the tumour microenvironment seem to contribute to tumour progression. Apart from a minor direct effect on the proliferation and survival of tumour cells (as not all tumour cells express the IL-17 receptor and respond to IL-17), the major pro-tumour function of IL-17 in inflammation-associated cancer seems to rely on its proangiogenic property on surrounding endothelial cells and fibroblasts[304]. By acting on stromal cells and fibroblasts, IL-17 induces a wide range of angiogenic mediators[306, 345] including VEGF, which markedly promotes inflammatory and tumour angiogenesis. The contribution of IL-17 as a pro-tumourigenic factor has been demonstrated in murine tumour models. For example, tumour growth of several origins (prostate, lymphoma and melanoma) was decreased in IL-17 receptor-deficient mice[346]. Also, *Il17*$^{-/-}$ mice were resistant to

spontaneous intestinal tumourigenesis[39], and showed reduced melanoma and bladder carcinoma tumour progression[37].

By contrast, a sizeable number of reports have described potent anti-tumour functions for IL-17 and IL-17 producing subsets. For example, tumour growth and lung metastasis were enhanced in *il17*$^{-/-}$ mice[34] and the adoptive transfer of IL-17-producing CD4^{+} [33, 35] and CD8^{+} [347] T cells was found to lead to tumour regression in experimental models. The reasons for the severe discrepancies between reports that argue for anti *versus* pro- tumour activities of IL-17 are still unclear and merit further investigation. One interesting possibility is that cells that initially only produce IL-17 may co-produce or even convert into IFN-γ producers[35, 36]. Interestingly, members of our group and collaborators have recently observed IL-17^{+} IFN-γ^{+} (double producers) γδ T cells specifically ate late stages of ID8 development (unpublished data).

We observed that ID8 tumour bearing mice accumulated not only IL-17-producing γδ T cells, but also IFN-γ-producing γδ T cells. Considering that ID8 tumour growth is enhanced in WT mice compared with *Tcrd*$^{-/-}$ mice, we suggest that the pro-tumour IL-17 effects dominate over the anti-tumour IFN-γ effects. This hypothesis is supported by two studies suggesting a minor role of IFN-γ in IL-17 mediated tumour promotion. One study reported that B16 and MB49 (bladder carcinoma) tumours were reduced in *Il-17*$^{-/-}$ mice but accelerated in *Ifng*$^{-/-}$ mice, while the double knockout, *Il-17*$^{-/-}$*Ifng* $^{-/-}$ mice, were resistant to tumour cell growth similarly to *Il17*$^{-/-}$ mice. Another study showed that development of tumours was inhibited in *Il17R*$^{-/-}$ mice; a defect in IFN-γ receptor increased tumour growth, whereas tumour growth was inhibited in mice that were deficient in both IL-17 receptor and IFN-γ receptor (*Il17R*$^{-/-}$*/Ifngr*$^{-/-}$) compared with wild-type animals[346].

To definitely test if IL-17 is the molecular determinant of the pro-tumour function of γδ cells in the ID8 model, we plan to compare ID8 tumour progression upon IL-17 blockade in WT versus *Tcrd*$^{-/-}$ mice. Moreover, we aim to generate double knockout mice *Tcrd*$^{-/-}$*/Il17*$^{-/-}$ mice and compare tumour progression with single knockout mice.

If we confirm that IL-17^{+} γδ T cells are the tumour promoting factor in the ID8 tumour model, it would be interesting to address what determines IL-17^{+} γδ T cell expansion or recruitment in this tumour model. By contrast with CD4^{+} T cells that can be polarized on the periphery depending on the cytokine environment, γδ T cells are committed in the thymus to either produce IFN-γ or IL-17[155, 348]. Seminal work from our laboratory further demonstrated that these subsets can be distinguished and isolated based on the tumour necrosis factor (TNF) receptor super family member CD27, which is expressed by IFN-γ secreting γδ T cells but not in their IL-17-producing counterparts[155]. The determination of the functional properties of γδ T cells seems to be influenced by the strength of the TCR and/or CD27 signaling in the thymus. Thus, the engagement of TCR and CD27 were shown to promote an IFN-γ secreting program whereas the lack of such interactions resulted in IL-17 production[144, 348, 349].

The cytokine requirements for the expansion of each subset have been addressed in other contexts than tumour microenvironment. For example, in a

model of autoimmunity, the requirements for IL-17 production by γδ T cells seem to include IL-23 and IL-1β[350]. This study showed that γδ T cells expressed IL-23R and stimulation with IL-1 and IL-23 promoted RORγt, IL-17A, IL-17F, IL-21, and IL22 expression by γδ T cells in the absence of TCR engagement. IL-23 and IL-1b are also critical for the expansion of IL-17$^+$ γδ T cells in response to microbial products[351]. For the IFN-γ producing CD27$^+$ γδ T cells, CD27 costimulation synergizes with the TCR to provide survival and proliferative signals that determine their expansion upon viral or parasitic infection *in vivo*[351].

Wakita´s work[42] suggested that IL17$^+$ γδ T cells differentiated in tumour lesions *in situ.* Although the authors present *in vitro* TGF-β, IL-6 or IL-23 antibody blockade data that argue for this, it is possible that different rules apply *in vivo*. Indeed, the proportion of IL17$^+$ γδ T cells can differ significantly between *ex vivo* and *in vitro* settings (particularly upon TGF-β treatment)[155]. The physiological triggers of IL-17$^+$ γδ T cell expansion within the tumour microenvironment thus remain to be identified. Furthermore, the interactions between IL17$^+$ γδ T cells and other T cell populations, such as regulatory cells (Tregs), which are known to suppress IL17$^+$ γδ T cell activity[352], need to be dissected in tumour bearing mice.

Although IL-17 and Th17 cells have been shown to accumulate in various human tumours, the relevance of IL-17 production by human γδ T cells remains unknown. Of note, the major γδ T cell subset present in peripheral blood, Vγ9Vδ2, is highly polarized towards IFN-γ production early in neonatal development and in adults[246, 353] as well as upon *in vitro* activation with physiological Vγ9Vδ2 TCR agonists[172].

Two initial studies proposed the accumulation of IL-17$^+$ human γδ T cells in the context of infection[354, 355]. However, in these studies, IL-17$^+$ γδ T cells accounted for less than 2% of total γδ peripheral blood lymphocytes, and thus their impact on clinical outcome remains elusive. More convincing work by Dieli and colleagues[309], showed an increased percentage of IL-17-producing Vγ9Vδ2 T cells in the peripheral blood and cerebrospinal fluid (>70% of Vγ9Vδ2 T cells were IL-17$^+$) in children with bacterial meningitis. While this data suggest a significant contribution of IL-17$^+$ Vγ9Vδ2 T cells in infection contexts, it will be important to investigate IL-17 production by γδ T cells in a tumour setting. Moreover, it will be important to examine IL-17 production in subsets in human γδ T cells other than the Vγ9Vδ2 subset. This is particularly relevant for Vδ1 T cells, which are enriched in tissues, and preferentially infiltrate tumours and are thus more prone to interact with solid tumour cells.

5.2 Future perspectives

Many challenges remain ahead to clarify the roles of γδ T cells in tumour immunology and to successfully modulate their activity in the clinic. To fully understand γδ T cell activation, it will be very important to determine how exactly

phosphoantigens interact with Vγ9Vδ2 TCR. One important recent study showed that intracellular accumulation of phosphoantigens is associated with membrane reorganization of CD277 molecules (BTN3A), which in turn leads to Vγ9Vδ2 T cell activation[356]. Moreover, the authors also described agonist and blocking CD277-specific antibodies that could be used for immunotherapeutic modulation of Vγ9Vδ2 T-cell responses toward tumour cells.

Furthermore, the anergy of repeatedly challenged phosphoantigens-treated Vγ9Vδ2 T cells reported *in vitro* and in clinical trials[268, 270, 273] constitutes a serious obstacle to phosphoantigen-based therapies. Moreover, because γδ T cells express a wide panel of inhibitory and activation receptors that directly impact on their activation state and function, it will be vital to unravel the full repertoire of tumour antigens and counter-receptors involved in γδ T cell recognition. Although many receptors have been described, we still lack a dynamic picture of the tumour-induced γδ T cell activation, and a deep understanding of how they elicit γδ T cell activation and what is the interplay between numerous signaling cascades induced upon sequential or concomitant receptor engagement[357].

As with all therapies, patient selection is of vital importance and requires the identification of biomarkers that may predict clinical outcome. Building on our work concerning molecular determinants of tumour susceptibility, we believe that pre-selection of patient will increase the success of γδ T cell-based clinical trials. Thus, leukaemia or lymphoma patients which tumours express ULBP1; or ovarian epithelial carcinoma or colonic carcinoma patients which tumours express ULBP4, presumably will benefit most from Vγ9Vδ2 T cell therapy (chapter II [178]). Also, additional work from our lab has identified a panel of 10 genes encoding cell surface proteins that segregated with "susceptible" *versus* "resistant" haematological tumours[286].

The anti-tumour properties of adoptively transferred γδ T cells can also be improved during *in vitro* expansion. This could be achieved, for example through addition of IL-15 (which may increase cytolytic properties and tumour reactivity of γδ T cells trough upregulation of NKG2D signaling), or IFN-α (which may increase TNF-related, apoptosis-inducing, ligand-dependent killing of tumour cells). Moreover, transduction of γδ T cells with tumour-specific TCRs, or chimeric tumour-specific immunoglobulin receptors (for example CD19) will enlarge the tumour cell recognition pattern of γδ T cells. Recent work from our lab demonstrated that the recognition of tumour targets by the Vδ1 T cell subset can be enhanced by their induced expression of NKp30[240].

The *in vivo* efficacy of γδ T cell based-immunotherapies can also be improved by using combinatorial regimens with immunotherapy and chemotherapy. For example, prior patient´s lymphodepletion (similarly to the protocols applied before bone marrow transplantation) may sustain γδ T cell proliferation and survival after adoptive transfer protocols. Moreover, along with the studies in mice[43, 44], γδ T cells seem to be beneficial after chemotherapy induced tumour cell death.

Finally, our work demonstrated that despite the promise of γδ T cells for cancer immunotherapy, these cells can, in certain conditions have pro-tumour

functions. This is probably related to γδ T cell L-17 production and consequently increased angiogenesis. Moreover, Vδ1-TIL infiltration was correlated with breast cancer patients poor survival (and with the infiltration of Tregs)[119]. Therefore, the identification of markers of anti-*versus* pro-tumour Vδ1 T cells need to be addressed.

. We hope the work developed in this PhD thesis, which identified new mechanisms and potential biomarkers, may help in the design of new γδ T cell-based therapy protocols for patients affected by cancer.

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APPENDIX

Appendix- Peer-reviewed articles associated with this thesis:

Acoording to Decreto-Lei 388/70, art. 8º, parágrafo 2, the results presented in this thesis are published or submitted in the following articles:

1. Correia DV*, d'Orey F*, Cardoso BA, **Lança T**, Grosso AR, deBarros A, martins LR, Barata JT, Silva-Santos B. **Highly active microbial phosphoantigen induces rapid yet sustained MEK-Erk- and PI-3K/Akt mediated signal transduction in anti-tumour 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 JT, Gomes da Silva M, Silva-Santos B**. Identification of a panel of ten surface protein antigens associated with immunotargeting of leukaemias and lymphomas by peripheral blood γδ T cells.** Haematologica. **2010**; 95(8):1397-404. *co-first authors

3. **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 leukaemia/lymphoma susceptibility to γδ T cell-cytotoxicity.** Blood. **2010**; 115(12): 2407-11

4. **Lança T**, Silva-Santos B. **The split nature of tumour-infiltrating leukocytes: implications for cancer surveillance and immunotherapy**. OncoImmunology. **2012**; 1(5): 717-725

5. **Lança T**, Costa MF, Gonçalves-Sousa, Grosso AR, Penido, Silva-Santos B. **Protective role for CCR2/CCL2 through recruitment of tumour-infiltrating γδ T-lymphocytes.** *Submitted*

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PLoS one

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

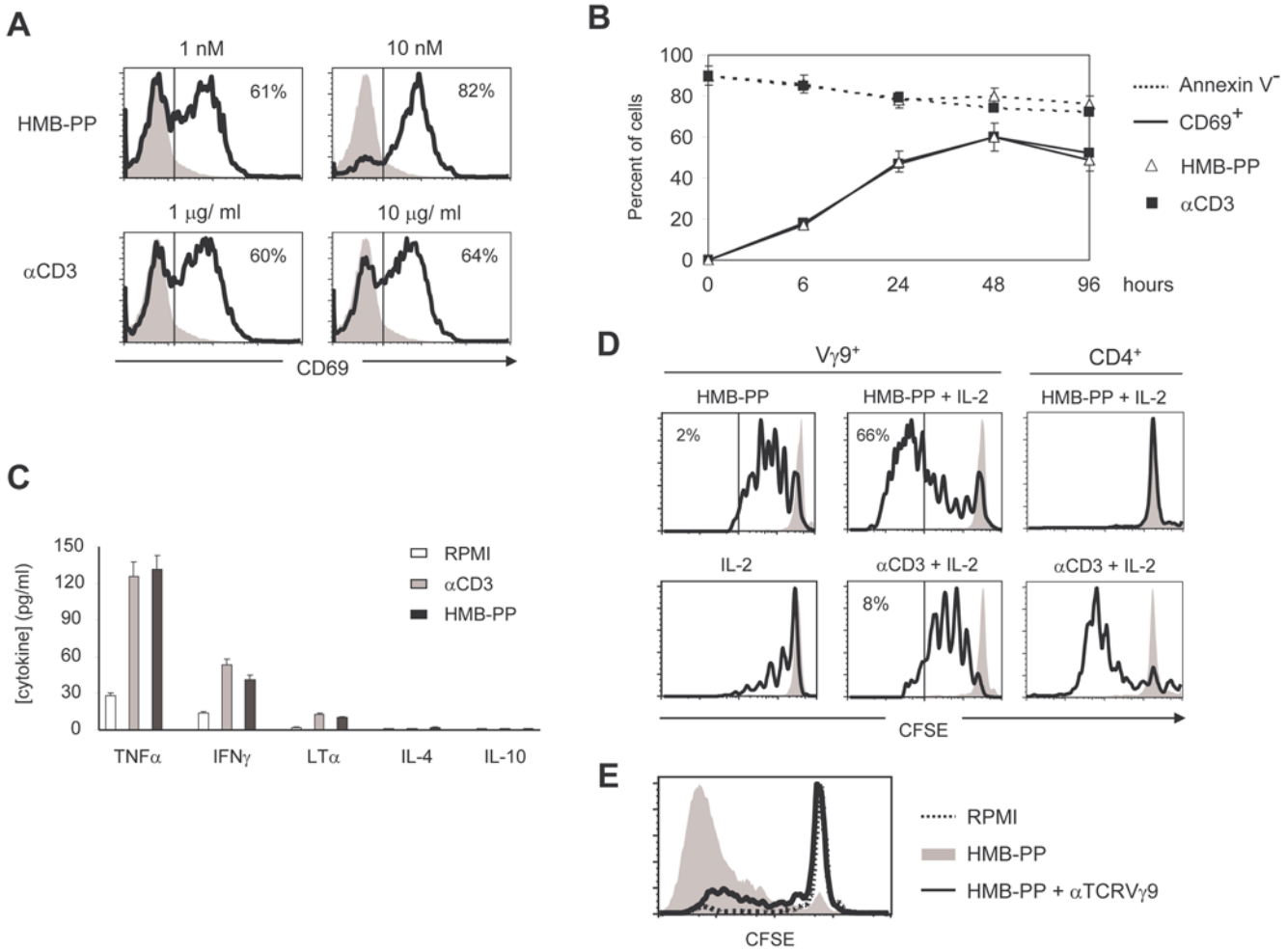

Figure 1. Nanomolar HMB-PP replicates saturating TCR/CD3 ligation for Vγ9⁺ T-cell activation. (**A**) Flow cytometry analysis for the expression of the activation marker CD69 in MACS-sorted (97–98% purity) γδ PBL, stimulated for 48 hours with the indicated amounts of HMB-PP or anti-CD3 mAb (OKT3). Shaded 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

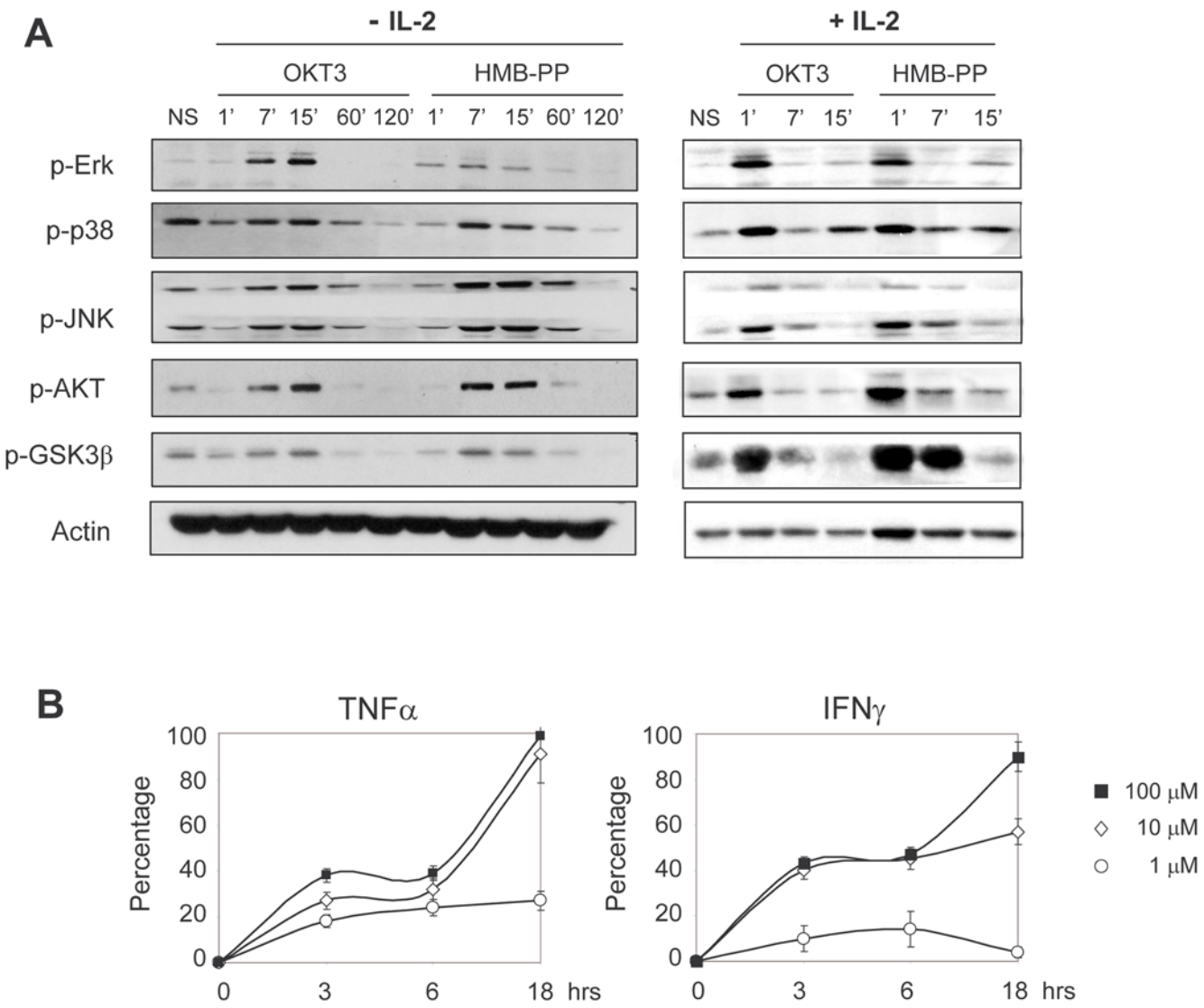

Figure 2. HMB-PP stimulation kinetically mimics Vγ9$^+$ TCR/CD3 signal transduction. (**A**) Phosphoimmunoblotting for kinases implicated in TCR signaling. MACS-sorted γδ PBL (of which 80–95% Vγ9$^+$) were incubated with OKT3 (1 μg/ml) or HMB-PP (1 nM), in the absence (*left panel*) or presence (*right 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,

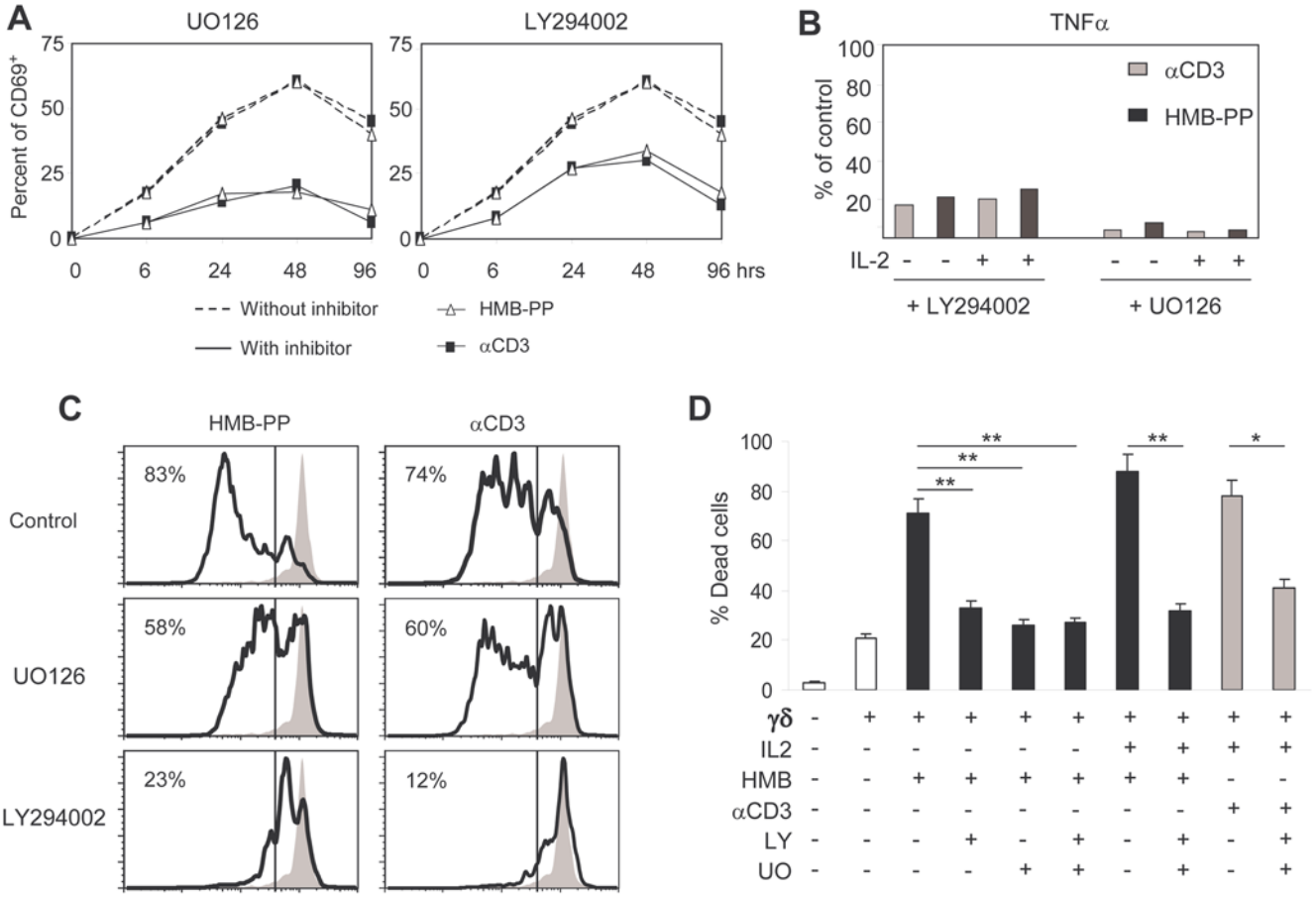

Figure 3. HMB-PP-mediated Vγ9$^+$ T-cell activation requires functional MEK/Erk and PI-3K/Akt signaling pathways. Effects of MEK/Erk inhibitor UO126 and PI-3K/Akt inhibitor LY294002 on the activation and function of MACS-sorted γδ PBL (of which 85–95% Vγ9$^+$). (**A**) Expression of activation marker 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 **$p<0.01$).
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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

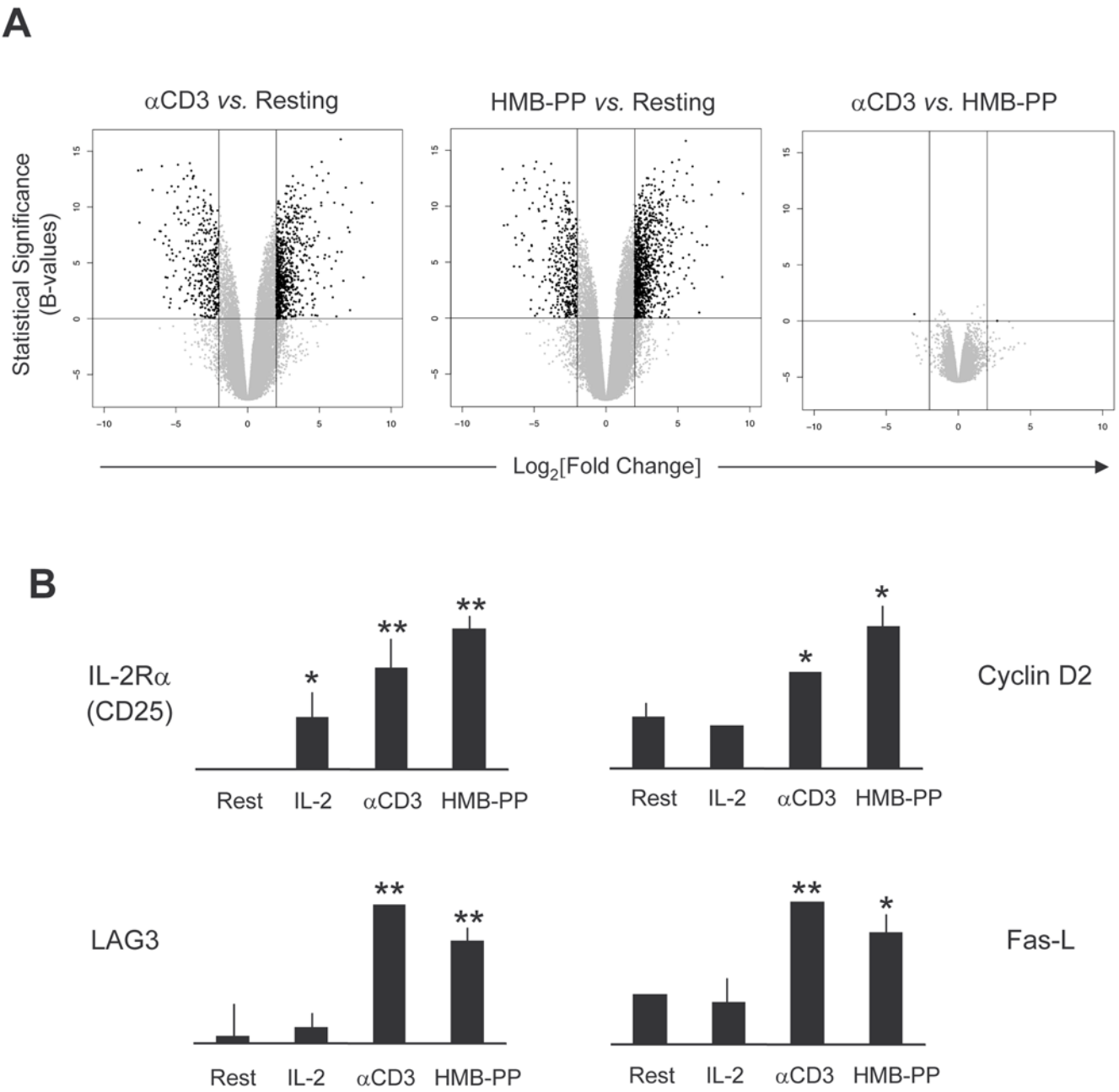

Figure 4. HMB-PP treatment reproduces the transcriptional alterations induced by TCR/CD3 ligation on γδ T-cells. (**A**) Volcano plots of DNA microarray comparisons between αCD3 (OKT3) mAb-treated, HMB-PP-treated and non-stimulated (''resting'') MACS-sorted γδ PBL (of which 85–95% $V\gamma9^+$). After 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 $**p<0.01$).
doi:10.1371/journal.pone.0005657.g004

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.
doi:10.1371/journal.pone.0005657.t001

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

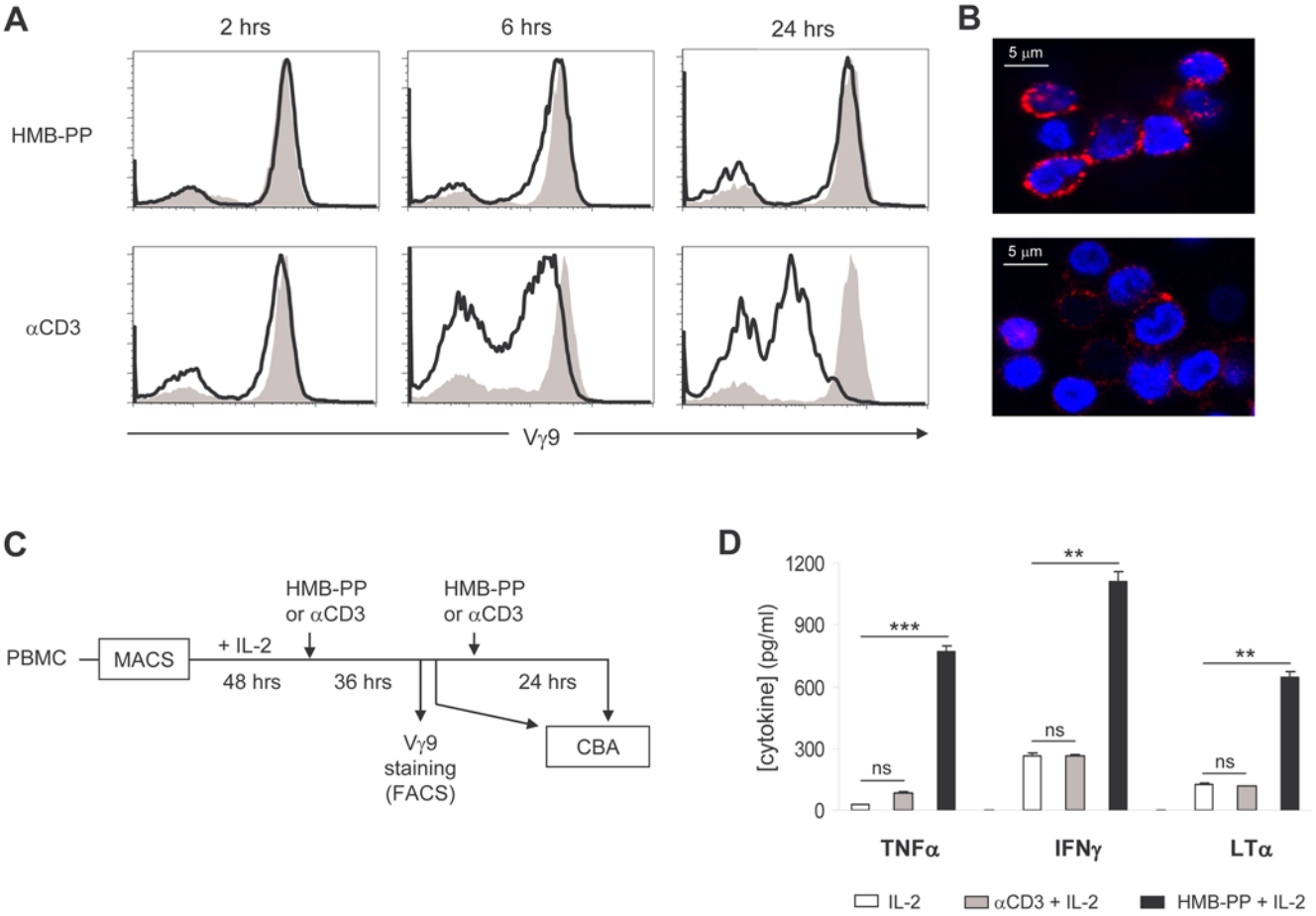

Figure 5. HMB-PP does not induce down-modulation of surface Vγ9$^+$ TCR and sustains cytokine production upon re-stimulation. (**A**) MACS-sorted γδ PBL (of which 80–90% Vγ9$^+$) were incubated for the indicated times with HMB-PP or OKT3, and stained with anti-Vγ9 mAb for flow cytometry analysis. Bold 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].

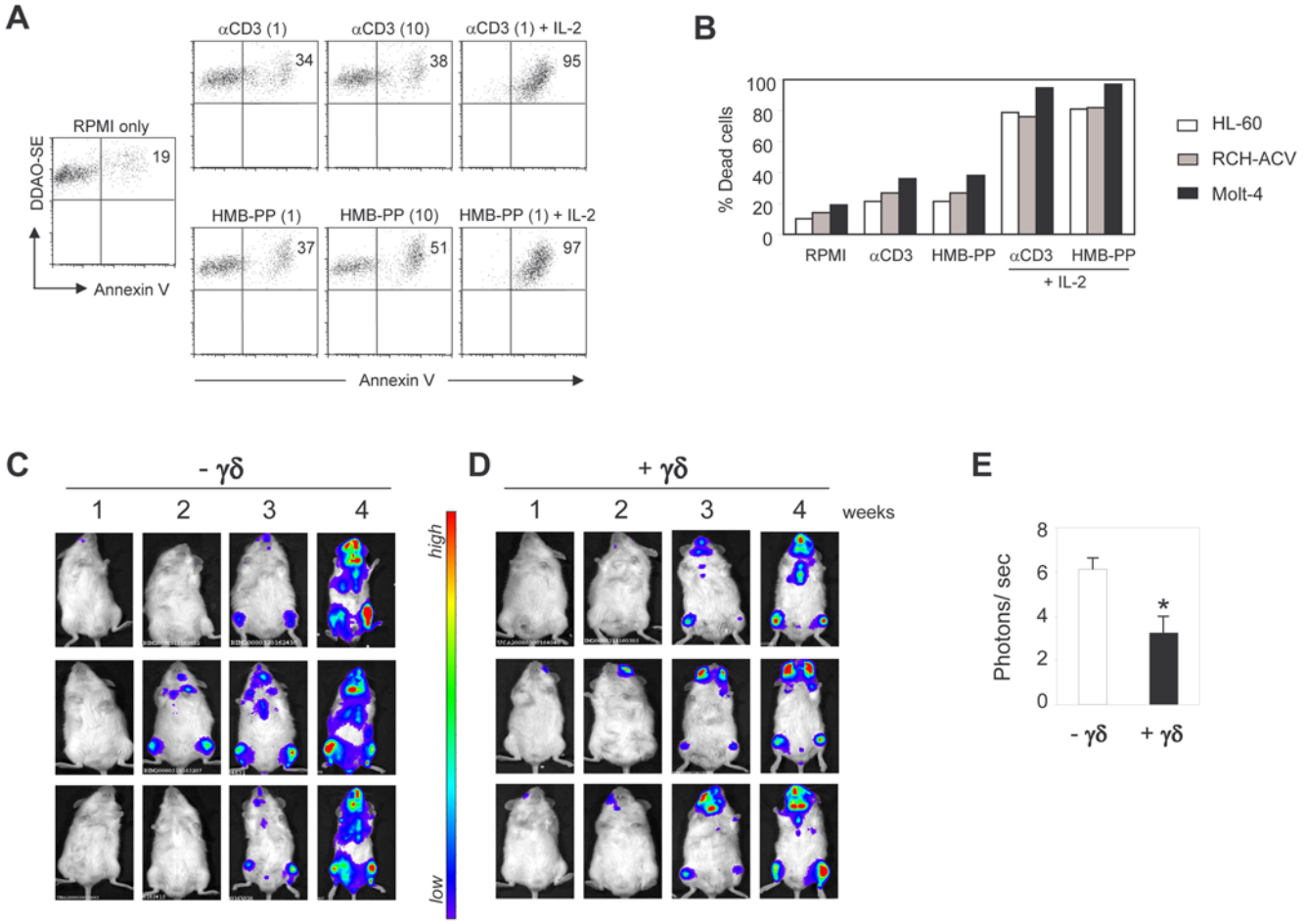

Figure 6. Leukemia cell killing by HMB-PP-activated γδ T-cells. (**A**) *In vitro* lysis of Molt-4 leukemia cells. MACS-sorted γδ PBL (of which 85–95% Vγ9$^+$) were pre-activated for 72 hours with 1 or 10 μg/ml αCD3 mAb (OKT3), or 1 or 10 nM HMB-PP in the absence of IL-2, and also combined at the lower 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.

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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

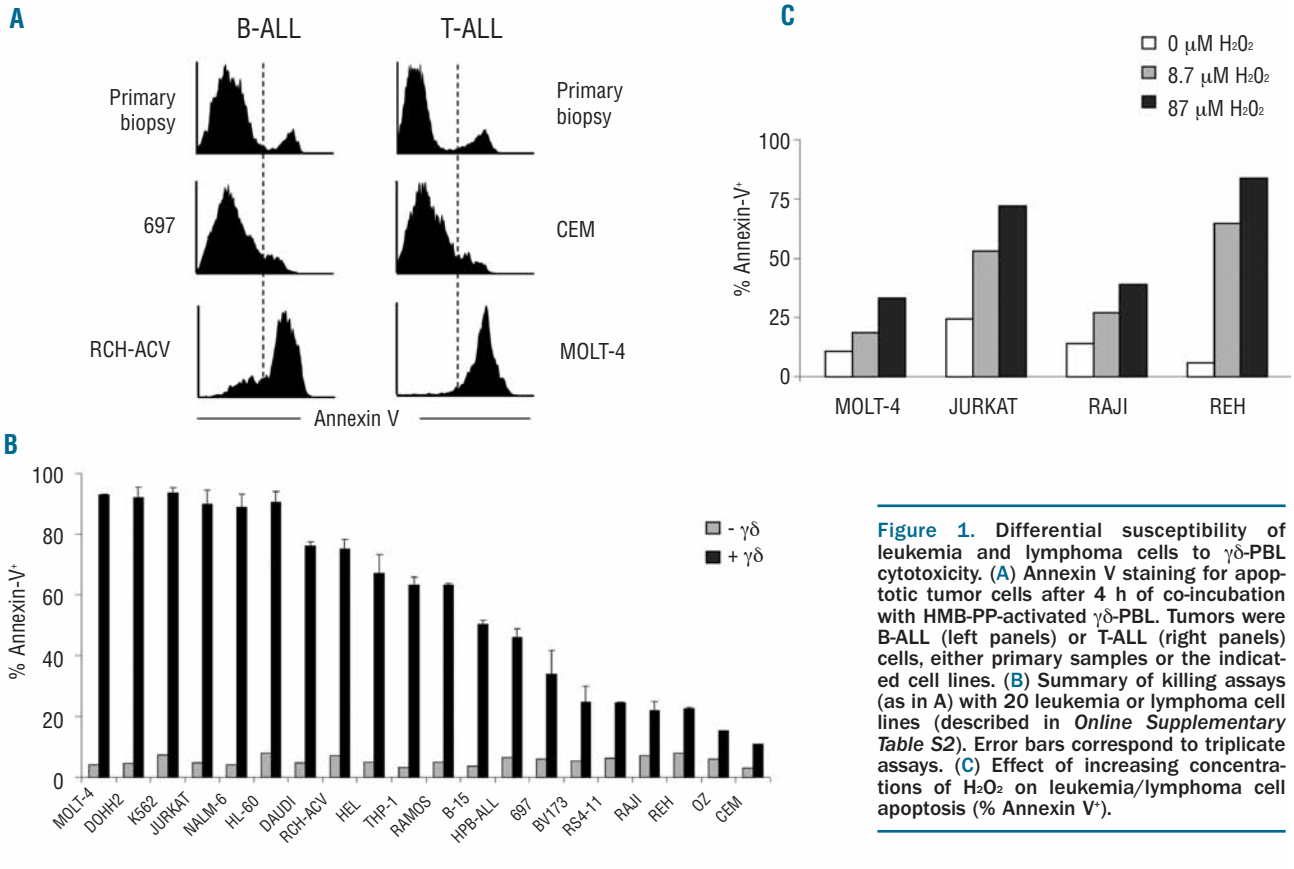

Figure 1. Differential susceptibility of leukemia and lymphoma cells to γδ-PBL cytotoxicity. (A) Annexin V staining for apoptotic tumor cells after 4 h of co-incubation with HMB-PP-activated γδ-PBL. Tumors were B-ALL (left panels) or T-ALL (right panels) cells, either primary samples or the indicated 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

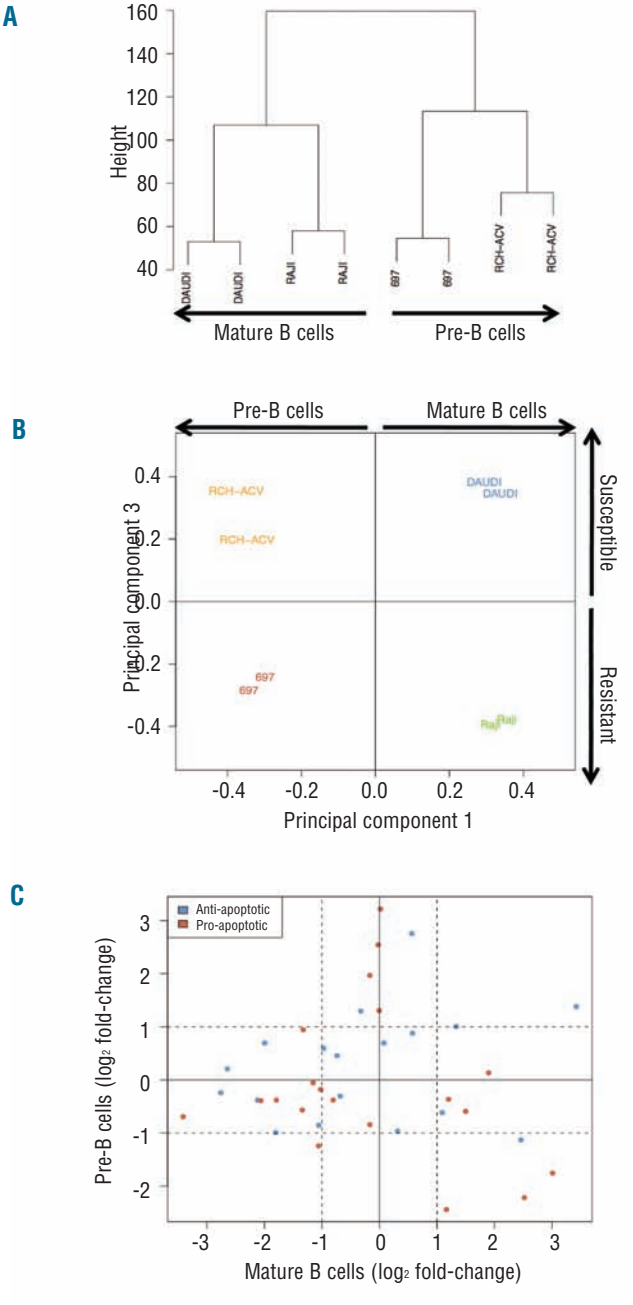

Figure 2. Comparison of gene expression in tumor cell lines susceptible or resistant to γδ-PBL cytotoxicity. Bioinformatics analyses of cDNA microarray comparisons between the Burkitt's lymphomas DAUDI and RAJI; and the B-ALL lines RCH-ACV and 697. (A) Unsupervised hierarchical clustering analysis. Samples 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

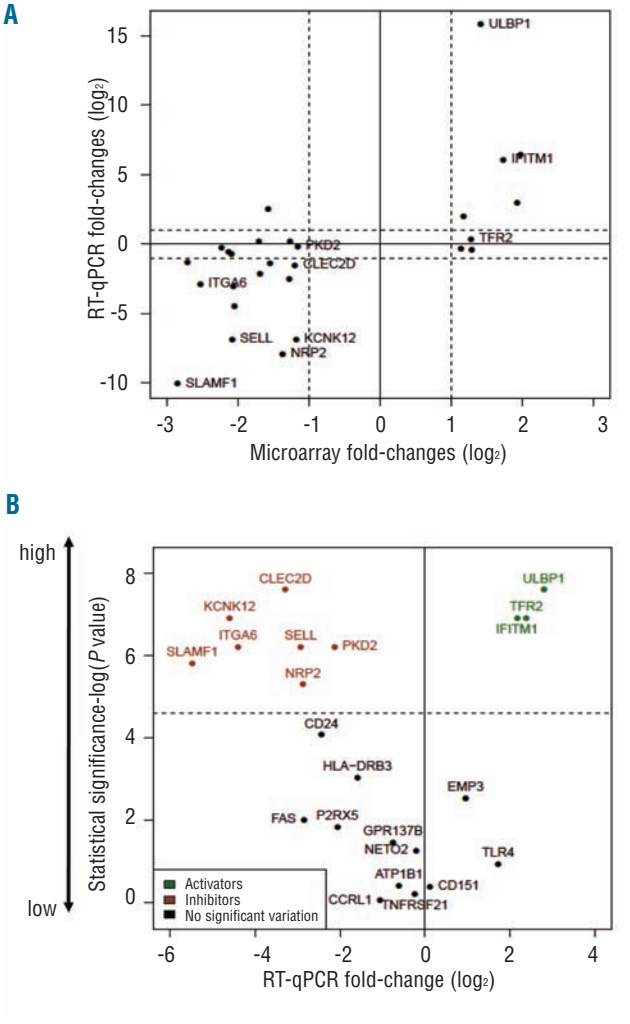

Figure 3. Variations in expression of genes encoding cell surface proteins that segregate between γδ-susceptible and γδ-resistant leukemia/lymphoma cell lines. (A) RT-qPCR validation of microarray results for the comparisons of Figure 2. The mRNA expression levels were normalized to GUSB and PSMB6 for 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

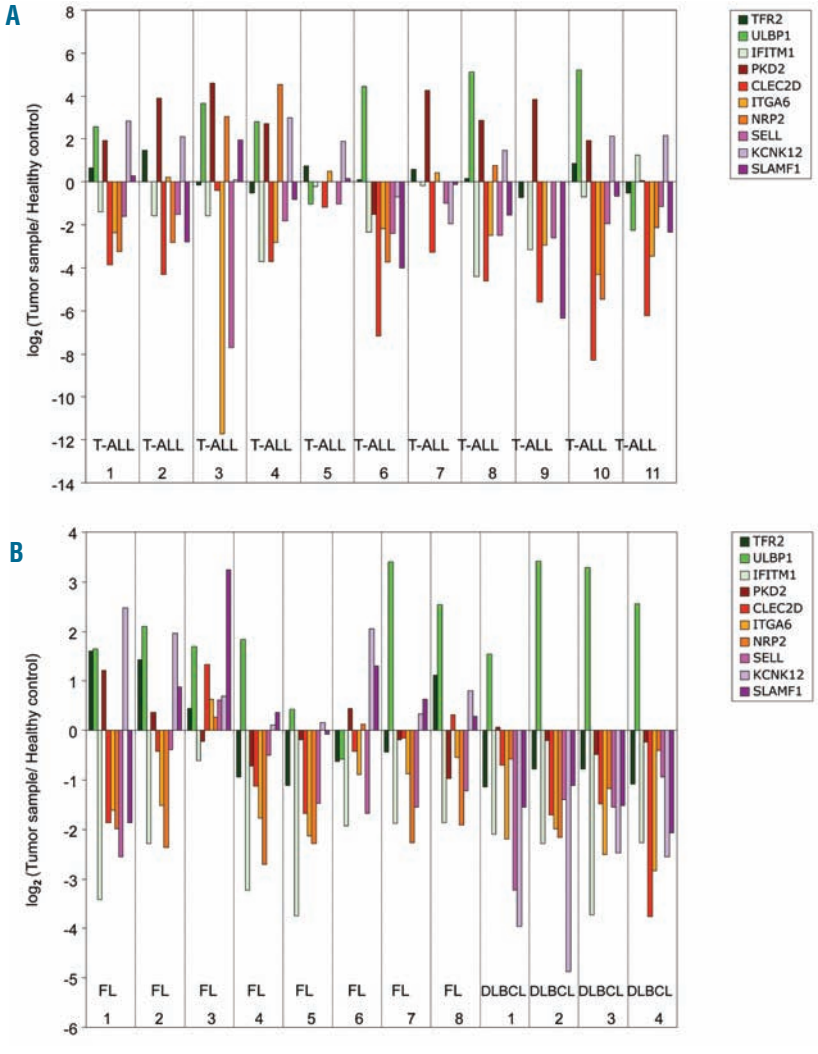

Figure 4. Quantification of mRNA expression levels of γδ-susceptibility markers in acute lymphoblastic leukemia and non-Hodgkin's lymphoma patients. (A) RT-qPCR analysis of mRNA expression in 11 T-cell acute lymphoblastic leukemia (T-ALL) samples, normalized to housekeeping genes (*GUSB* and PSMB6) and 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.

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The MHC class Ib protein ULBP1 is a nonredundant determinant of leukemia/lymphoma susceptibility to γδ 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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*A.Q.G. and B.S.-S. contributed equally to this study.

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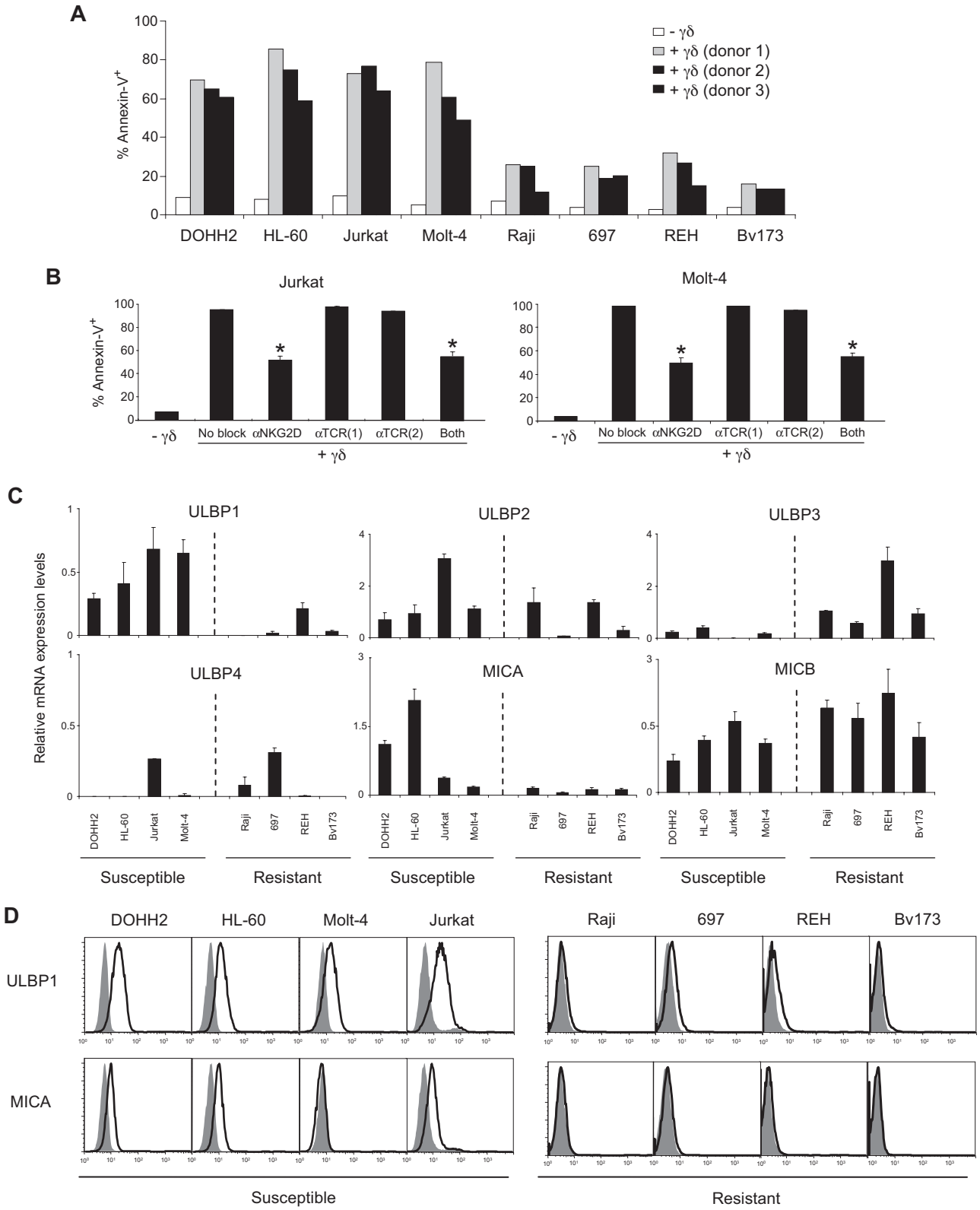

Figure 1. NKG2D mediates Vγ9Vδ2 T-cell recognition of hematopoietic tumors that endogenously overexpress ULBP1 and MICA. (A) Susceptibility of leukemia and lymphoma cell lines (described in supplemental Table 2) to HMB-PP–activated γδ-PBL (> 90% $V\gamma9^+$) cytotoxicity was assessed by coincubating 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.

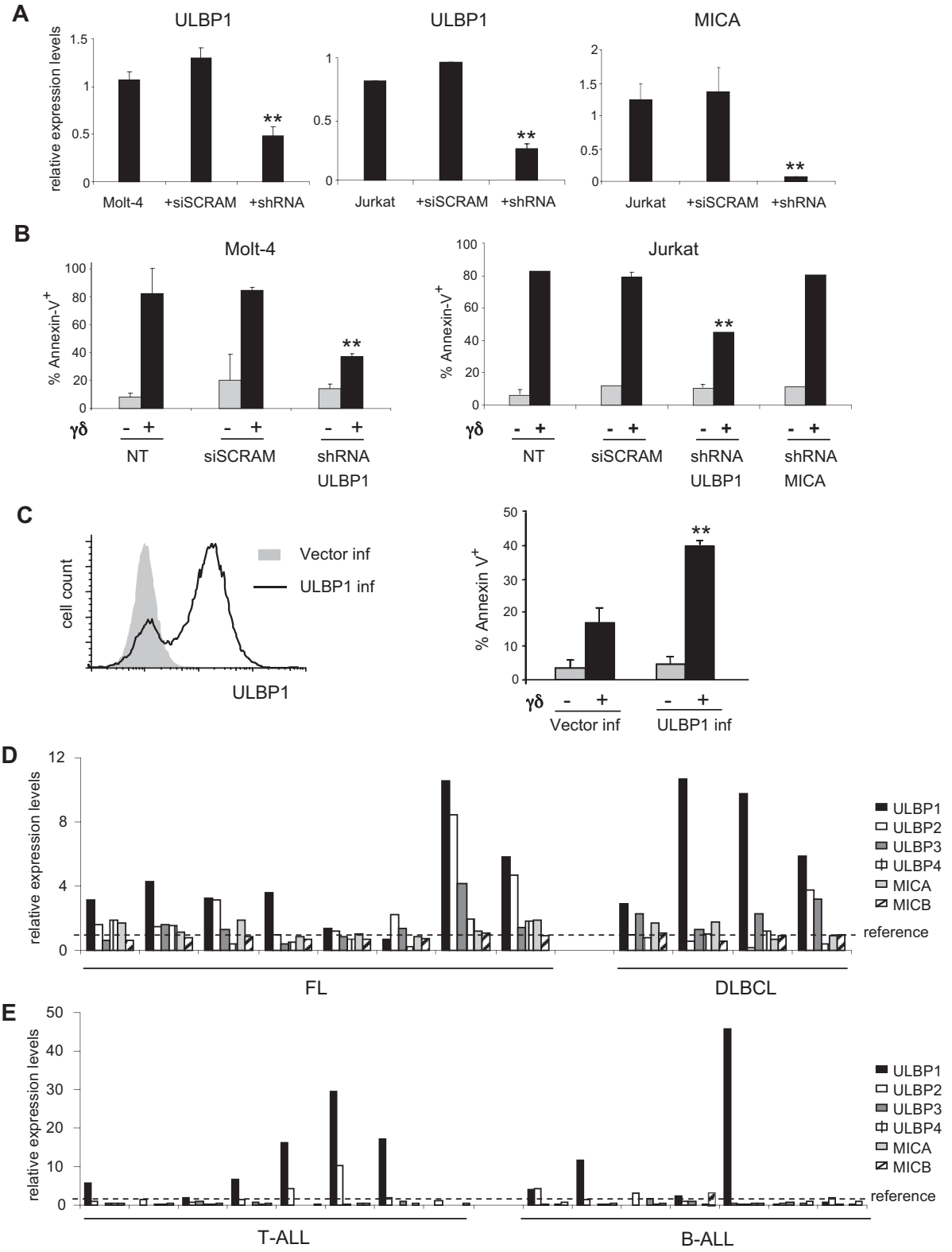

Figure 2 ULBP1 is required for Vγ9Vδ2 T-cell recognition of leukemia/lymphoma cells and displays a highly heterogeneous expression in cancer patients. (A) Lentiviral shRNA-mediated knockdown of ULBP1 and MICA in Molt-4 or Jurkat leukemia cells was confirmed by quantitative RT-PCR using GUSB and PSMB6 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.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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The split nature of tumor-infiltrating leukocytes
Implications for cancer surveillance and immunotherapy

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Abbreviations: TIL, tumor-infiltrating lymphocyte; Th, helper T cell; Treg, regulatory T cell; NK, natural killer cell; MDSC, myeloid-derived suppressor cell; IFN, interferon; IL, interleukin; VEGF, vascular endothelial growth factor; ACT, adoptive cell therapy

An important development in tumor immunology was the identification of highly diverse tumor-infiltrating leukocyte subsets that can play strikingly antagonistic functions. Namely, "anti-tumor" vs. "pro-tumor" roles have been suggested for Th1 and Th17 subsets of $CD4^+$ T cells, Type I or Type II NKT cells, M1 and M2 macrophages, or N1 and N2 neutrophils, respectively. While these findings are being validated in cancer patients, it is also clear that the balance between infiltrating $CD8^+$ cytotoxic and $Foxp3^+$ regulatory T cells has prognostic value. Here we review the pre-clinical and clinical data that have shaped our current understanding of tumor-infiltrating leukocytes.

Introduction

A fundamental principle of cancer immune surveillance is that tumors are infiltrated by leukocytes, particularly lymphocytes, capable of recognizing and targeting transformed cells, thus leading to their elimination before the tumor becomes clinically apparent. Moreover, the efficacy of immunotherapy against established tumors presumably depends on lymphocyte recruitment and effector function within the tumor bed. However, a major obstacle to anti-cancer therapy is the local immune suppression commonly found within the tumor microenvironment.[1] While earlier work had focused on tumor cell-derived factors that inhibit the local immune response, the past few years have demonstrated a dramatic contribution of leukocytes themselves to this "pro-tumor" environment. Recent reports have further clarified this paradoxical leukocyte behavior by identifying a very heterogeneous set of subpopulations, both of lymphoid and myeloid origin, that can play strikingly antagonistic roles within the tumors they co-infiltrate.

The prototypic anti-tumor function, displayed by various lymphocyte subsets (**Fig. 1**), is cytotoxicity via the perforin/granzyme system or, alternatively, by engaging death receptors (such as Fas). These properties are further promoted by interferon γ (IFNγ), the signature Th1 cytokine that is, in fact, secreted by multiple cell types (see below), often together with tumor necrosis factor (TNF). By contrast, cytokines such as TGFβ or IL-10 are highly immunosuppressive, and other secreted factors, like VEGF, directly promote angiogenesis and thus tumor growth (**Fig. 2**). The detailed characterization of gene expression and cytokine profiles in leukocyte populations isolated from tumor biopsies (or draining lymph nodes) has been instrumental in revealing the heterogeneity of tumor-infiltrating leukocytes, both of lymphoid and myeloid nature, which we will discuss in this review.

Many of the key studies on tumor-infiltrating leukocytes have been performed in mouse tumor models. Although they present several important limitations, including the artificial homogeneity and laboratory selection of tumor cell lines used in transplantable models, the lack of relevant physiology (including interactions between autologous tumors and immune cells) in xenograft models, and the commonly short span (2–4 weeks) of all these tumor development experiments, animal models provide a unique possibility of tracking and manipulating cancerigenesis in vivo.

This notwithstanding, it is obviously essential to validate all the findings from mouse tumor models in human cancer samples. Therefore, in this review we will discuss and summarize the most recent advances, both in the laboratory and in the clinic, in our understanding of the biology of tumor-infiltrating leukocytes. We will highlight their anti- or pro-tumor functions in mouse models, and how these translate (or not) into prognostic value in cancer patients.

The Traditional Players: NK, $CD8^+$ T and Th1 Cells

It has been known for three decades that NK cells and $CD8^+$ T lymphocytes, including those extracted from tumor biopsies, can efficiently kill transformed cells. Collectively, these killer lymphocytes recognize two important types of tumor antigens (among others): processed peptides presented by MHC Class Ia proteins via TCRαβ; and non-classical (Class Ib) MHC proteins via NKG2D.[2] The latter, which is expressed on NK, $CD8^+$ and also γδ T cells, has been recently shown to be a key genetic determinant of cancer immune surveillance.[3]

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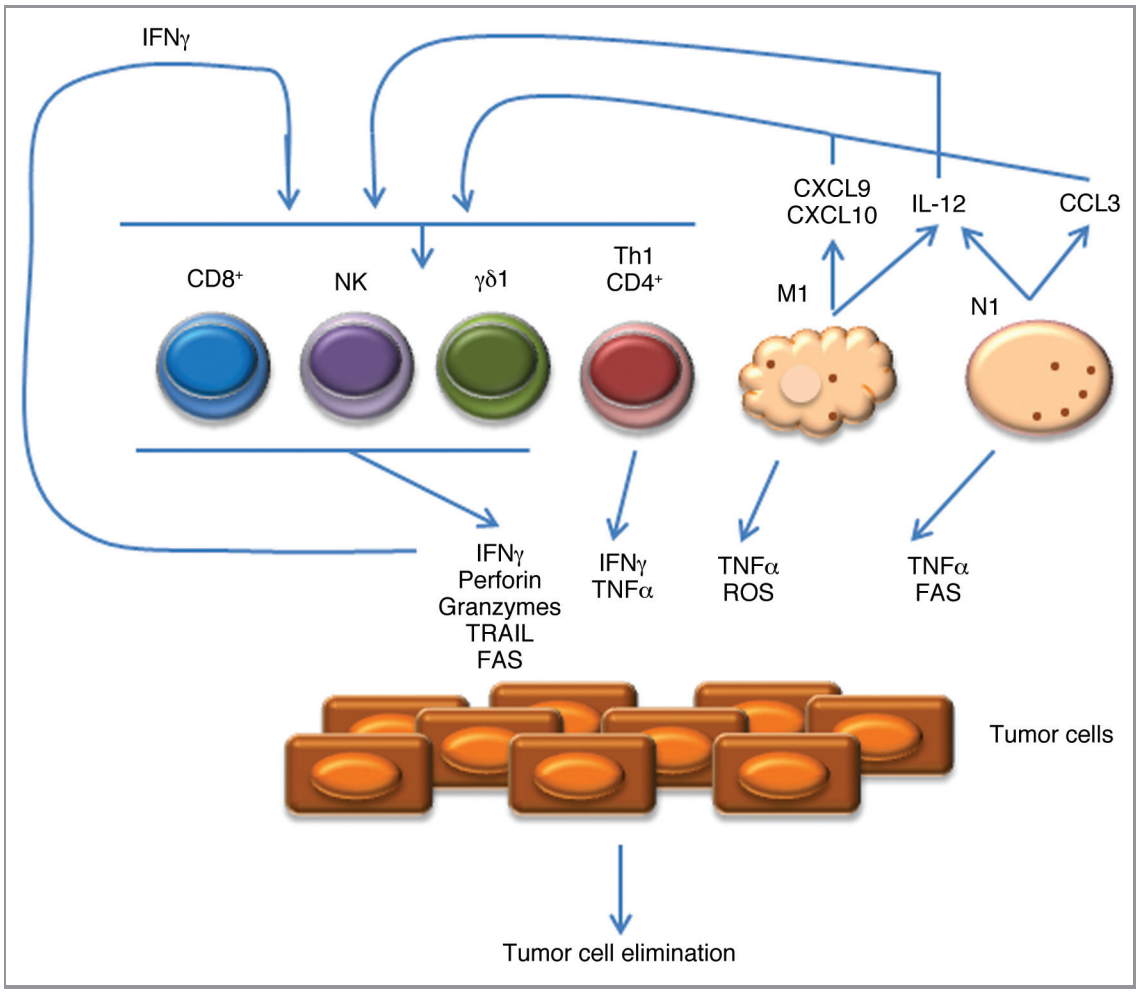

Figure 1. Anti-tumor infiltrating leukocytes and molecular mechanisms of action. Representation of the main anti-tumor lymphoid and myeloid cells. N1 and M1 refer to neutrophil and macrophage subsets, respectively. γδ1 and Th1 refer to IFNγ-producing γδ and CD4+ T cells, respectively. Depicted are also molecules produced by these leukocytes, including cytokines that impact on cell differentiation and expansion, and chemokines that control their recruitment/infiltration into tumors.

NK and CD8+ cells provide highly complementary anti-tumor strategies. Indeed, as demonstrated by the seminal work of Kärre and Kiessling, the downregulation of MHC class Ia, which is a common mechanism of evasion against CD8+ cells, renders tumors more susceptible to NK cell-mediated lysis. This "missing self" recognition by NK cells is based on a set of MHC class Ia-specific inhibitory receptors that include killer cell immunoglobulin-like receptors (KIRs) in humans, lectin-like Ly49 molecules in mice, and CD94/NKG2A heterodimers in both species; in fact, NK cells express a complex repertoire of inhibitory and activating receptors that calibrate this anti-tumor function, while ensuring self-tolerance.[4,5] In result, NK cells eliminate tumors that lack MHC class Ia expression; or that overexpress ligands for activating NK receptors like NKG2D or the natural cytotoxicity receptors NKp30, NKp44 and NKp46.[5] Furthermore, NK cells express high levels of low-affinity Fc receptor for IgG (CD16), which allows them to mediate antibody-dependent cell-mediated cytotoxicity (ADCC).[6]

NK cells have been described to infiltrate various types of tumors in the skin, lung, gut and kidney.[5] Recent data on human NK cells infiltrating highly aggressive non-small cell lung cancers (NSCLC) showed a profound alteration of their phenotype, with decreased ability to degranulate and to produce IFNγ, when compared with NK cells from distal lung tissues or blood from the same patients or from healthy donors.[7] This functional impairment of NK-TILs correlated with decreased expression of NKp30, NKp80, DNAM-1, CD16 and ILT2 receptors. Interestingly, among these, NKp30 has been shown to affect the prognosis of gastrointestinal stromal tumors through a specific pattern of alternative splicing.[8]

Various immunotherapeutic strategies have been proposed to tackle the common defects of NK cell activity in cancer patients:[5] activation of endogenous NK cells (with cytokines like IL-2, IL-15 and IL-18), NK-cell adoptive immunotherapy, NK-cell-based donor lymphocyte infusions and allogenic stem cell transplantation (SCT).[6] Although globally the objective responses

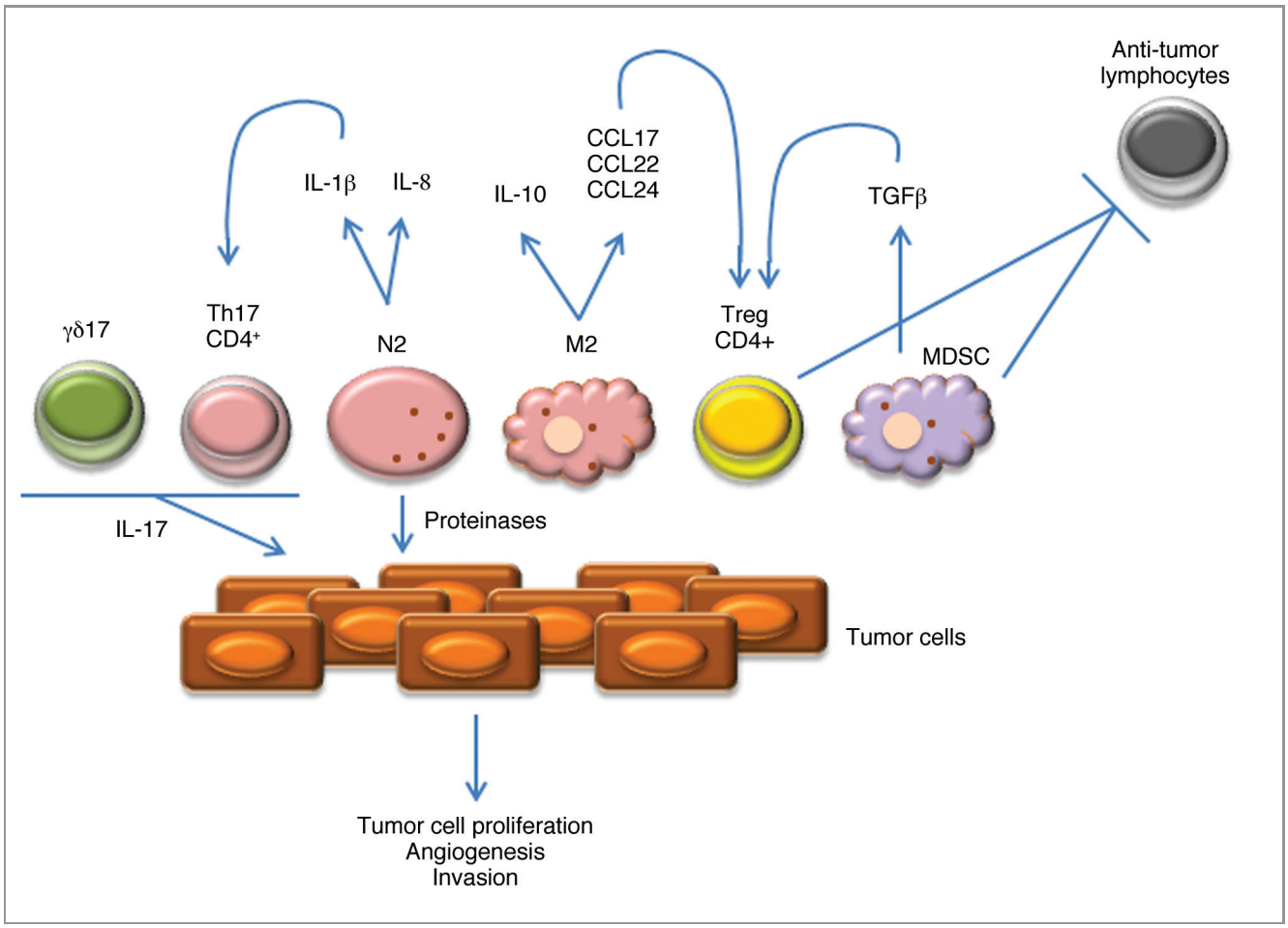

Figure 2. Pro-tumor infiltrating leukocytes and molecular mechanisms of action. Representation of the main pro-tumor lymphoid and myeloid cells. N2 and M2 refer to neutrophil and macrophage subsets, respectively. γδ17 and Th17 refer to IL-17-producing γδ and CD4+ T cells, respectively. Depicted are also molecules produced by these leukocytes, including cytokines that impact on cell differentiation and expansion, and chemokines that control their recruitment/infiltration into tumors.

have been disappointing, some data from allogenic and, more recently, haploidentical hematopoietic SCT have shown clinical (in the absence of adverse) effects mediated by NK cells.[5] This inspires further translational studies aimed at enhancing NK cell recruitment to tumors and their functional activity in situ.

With regard to CD8+ T cell-based immunotherapy, many recent efforts have focused in activating and expanding CD8+ tumor-infiltrating lymphocytes (TILs) ex vivo and then re-infusing them into the cancer patient—adoptive cell therapy (ACT). ACT of CD8+ TILs into lymphodepleted metastatic melanoma patients has shown very high objective response rates, ranging from 50% up to 81%.[9] In fact, TIL-ACT (combined with high doses of IL-2) has mediated cancer regression in 49–72% of melanoma patients, and durable complete responses, beyond 3–7 y, are currently ongoing in 40% of the patients.[10]

In pre-clinical models, adoptively transferred naïve CD8+ cells were shown to infiltrate melanoma lesions, be activated in situ and differentiate into functional cytotoxic T lymphocytes (CTLs).[11] The naïve status of the infused population appeared to be an important parameter, as the differentiation stage of CTLs inversely correlated with their anti-tumor efficacy in vivo.[12] The enhanced anti-tumor function of naïve T cells was related to sustained effector cell development, prolonged cytokine production, and increased expansion in vivo.

Transduction of tumor antigen-specific TCRs[13] or chimeric antigen receptors (CARs)[14,15] represent exciting prospects to increase the efficacy of cytotoxic ACT. These strategies have thus far enabled cancer regression in patients with metastatic melanoma, synovial sarcoma, neuroblastoma and refractory lymphoma or leukemia.[10]

In addition to cytotoxicity, IFNγ secretion is a key anti-tumor function of CD8+ and NK cells, who share this property with various other lymphocyte populations, most notably "helper type 1" (Th1) CD4+ cells. These were first described 25 y ago in the context of the "Th1/ Th2" paradigm of immunity to infection, and since then clearly implicated in promoting anti-tumor responses: Th1 cells enhance the cytotoxic functions of NK and CD8+ cells, upregulate MHC Class I expression in tumor cells (a direct effect of IFNγ), and support CD8+ cell proliferation through the secretion of IL-2.[16] Moreover, Th1 cells condition

the antigen-presenting capacity of DCs and macrophages, thus shaping the CTL response. In fact, the combination of Th1 cell therapy with local radiation therapy augmented the generation of tumor-specific CTL at the tumor site and induced a complete regression of subcutaneous tumors.[17]

"New" Effector TILs: γδ T, NKT and Th17 Cells

The "Th1/Th2" paradigm for CD4+ T cell differentiation has been recently revised with the addition of Th17 cells, characterized by the production of interleukin-17 (IL-17). IL-17-deficient mice were shown to be more susceptible (than wild type animals) to tumor growth and lung metastasis.[18,19] Adoptive transfer studies from the Restifo lab showed that in vitro generated Th17 cells were more efficient at eradicating tumors than Th1 cells,[20] and this was recently associated with stem cell-like properties of Th17 cells.[21] Importantly, adoptively transferred Th17 cells gave rise in vivo to Th1-like effector cell progeny,[21] and IFNγ was actually necessary for the protective effects of adoptively transferred Th17 cells.[20] These data suggest that acquisition of Th1-like properties are required for an anti-tumor function by Th17 cells.

In stark contrast to the previous studies, IL-17-deficient mice presented reduced tumor growth in other models such as B16 melanoma and MB49 bladder carcinoma,[22] DMBA/TPA-induced skin carcinoma,[23] or in a spontaneous intestinal tumor model (driven by a mutation in the tumor suppressor gene APC).[24]

The pro-tumor functions of IL-17 have been tightly linked to angiogenesis: IL-17 has been shown to act on endothelial, stromal and tumor cells to induce the expression of pro-angiogenic factors like VEGF, Angiotensins, PGE2 and IL-8, and thus promote tumor vascularization.[25] The precise conditions that determine pro- vs. anti-tumor functions of Th17 TILs remain unclear and require further investigation.

Although Th17 cells are important providers of IL-17, this cytokine can be abundantly produced by other tumor-infiltrating leukocyte populations. Namely, murine γδ T cells can be the major source of IL-17, not only in homeostatic conditions,[26] but also upon infection or tumor challenge.[27,28] Like for Th17 cells, the role of IL-17 produced by γδ cells within the tumor microenvironment is controversial: it has been associated both with angiogenesis and promotion of tumor growth[25,27]; and with CD8+ T cell recruitment and the therapeutic effects of chemotherapy against several subcutaneous tumor lines.[28,29]

While the recently discovered ability of γδ cells to make IL-17 has attracted much attention, these lymphocytes were previously characterized as strong cytotoxic and IFNγ-producing cells, and thus prototypic anti-tumor mediators. Consistent with this, seminal work by Girardi and Hayday showed a decade ago that mice lacking γδ T cells were significantly more susceptible to chemically induced tumors.[30] This phenotype was subsequently extended to transplantable,[31] spontaneous[32] and transgenic[33] tumors.

In the murine B16 melanoma model, γδ T cells were shown to infiltrate tumor lesions already at day 3 post-transplantation and to provide a critically early source of IFNγ.[31] This contrasts with the above-mentioned findings on IL-17+ γδ-TILs.[27,28] A more detailed characterization of γδ-TILs is therefore required in a wider set of pre-clinical tumor models. This should take into account the two functional γδ T cell subsets recently identified on the basis of CD27 (and CCR6) expression: CD27+ γδ cells make IFNγ but no IL-17, whereas IL-17 production is restricted to CD27- γδ cells.[34]

γδ T cell-based clinical trials have thus far concentrated on the highly IFNγ-polarized (and cytotoxic) Vγ9Vδ2 subset that constitutes most of γδ cells circulating in the human peripheral blood. As these cells are specifically reactive to non-peptidic phosphoantigens, they can be selectively activated and expanded both in vitro (for ACT) and in vivo. In cancer patients, γδ T cell-based immunotherapy has thus far produced objective responses in the range of 10 to 33%.[35] Future research should also take into account the important roles played by NK receptors, including NKG2D[36] and NKp30,[37] in tumor cell recognition by Vγ9Vδ2 cells and by Vδ1 cells (which predominate in tissues).

NKT cells also employ NK receptors, as well as CD1d-restricted TCRs to recognize tumor targets. The vast majority of these T cells are canonical or invariant NKT (type I NKT) cells that possess a specific TCRα rearrangement (Vα14Jα18 in mice; Vα24Jα18 in humans), associated with Vβ chains of limited diversity. All the other NKT cells that are CD1d-restricted and do not express this invariant TCR are called Type II NKT cells.[38,39] Although CD1d-deficient mice showed increased susceptibility to MCA-induced sarcomas,[40] there is evidence of functional heterogeneity also within NKT cells: while Type I NKT cells seem to be protective, Type II NKT cells mostly suppress tumor immunity.[39,41]

In terms of cytokine production, activated NKT cells are potent providers of IFNγ and IL-4 (and, to lesser extent, of IL-17). In the B16 metastatic melanoma model, a dual role of NKT cells was linked to immune suppressive IL-4 production by the thymus-derived subpopulation; and protective IFNγ production by liver-derived Type I NKT cells.[42]

Based on the pre-clinical evidence for an anti-tumor role of type I NKT cells, and the availability of a specific TCR agonist, α-Gal-Cer, several clinical trials have attempted to activate endogenous iNKT cells, or—more promising given by relative rarity of NKT cells in humans—perform ACT with (ex vivo expanded) Type I NKT cells. However, the clinical effects of αGal-Cer or NKT ACT have been very limited,[39] thus illustrating the difficulty in translating findings from animal models of cancer into improved immunotherapies.

The Inflammatory Phagocytes: TAMs and TANs

Macrophages and neutrophils are important myeloid cells of the innate immune system and major drivers of inflammatory responses. Given the long-established association between cancer and inflammation, it is not surprising that tumor-associated macrophages (TAMs) and neutrophils (TANs) can have great impact on the course of tumor progression. While most studies have associated TAM and TAN infiltration with promotion of tumor cell growth, some other reports have proposed some

anti-tumor roles. Once again, these opposing behaviors may be explained by heterogeneous TAM and TAN phenotypes, with distinct intra-tumor dynamics in various models.

Mirroring Th1/ Th2 polarization of $CD4^+$ T cells, two distinct subsets of macrophages have been recognized: the "classical" activated (M1) macrophage phenotype and the "alternatively" activated (M2) macrophage phenotype.[43] IFNγ drives the polarization toward M1 macrophages, which are characterized by abundant production of TNF, IL-12 and IL-23, CXCL9 and CXCL10, reactive nitrogen and oxygen species; and by high expression of MHC class II and costimulatory molecules (making them efficient antigen-presenting cells).[44] Conversely, IL-4 polarizes macrophages toward the M2 phenotype, which is associated with low levels of IL-12 but high levels of IL-10, IL-1RA and IL-1 decoy receptor. M2 cells also produce CCL17, CCL22 and CCL24, which results in the recruitment of Tregs and Th2 cells, eosinophils and basophils.[44]

The balance between M1 and M2 phenotypes seems to be controlled by NFκB signaling. Thus, NFκB targeting switched macrophages from an M2 to an M1 phenotype and led to ovarian tumor regression in vivo.[45] Nonetheless, the most frequent TAM phenotype seems to be M2.[43] Consistent with this, TAM depletion was associated with improved anti-tumor immunity in models of metastatic breast, colon and non-small lung cancers.[46] The pro-tumor roles of M2 macrophages derive from various molecular mechanisms, including the production of the pro-angiogenic mediator semaphoring 4D[47] and the invasive proteases cathepsins B and S.[48]

In the case of neutrophils, besides secreting cytokines and chemokines (such as IL-1β, IL-8, and IL-12), they produce large amounts of proteinases that remodel the extracellular matrix and promote the release of pro-angiogenic VEGF, thus supporting tumor cell growth and invasiveness.[49] Particularly important neutrophil proteinases are elastase[50] and matrix metalloproteinases MMP-8 and MMP-9.[51]

Despite being widely accepted as pro-tumor mediators based on multiple pre-clinical and clinical studies,[49] a dual nature of tumor-infiltrating neutrophils has also been suggested recently.[52,53] Thus, anti-tumor N1 and pro-tumor N2 subsets were described and modulated within tumors by TGFβ[52] or IFNβ.[54] Consistent with such a complex neutrophil activity within the tumor microenvironment, the concentration of reactive oxygen species also seems to determine either pro-tumor (genotoxicity at modest concentrations) or anti-tumor (cytotoxicity at high concentrations) effects.[49] Consequently, the depletion of total neutrophils can lead to either reduced[52] or increased[55] tumor burden, further illustrating the globally paradoxical roles of tumor-infiltrating leukocytes.

Immunosuppressive Leukocytes: Treg and MDSCs

Myeloid-derived suppressor cells (MDSCs) represent a heterogeneous population of myeloid progenitors and precursors of macrophages, granulocytes and dendritic cells, which are better characterized by their strong capacity to inhibit both innate and acquired immunity[56] particularly T-cell responses.[57] Murine MDSCs can be identified by the expression of Gr1 (includes Ly6C and Ly6G, macrophage and neutrophil markers, respectively) and CD11b (characteristic of macrophages). In humans, MDSCs are characterized by a $CD11b^+$ $CD33^+$ $CD34^+$ $CD14^-$ $HLA\text{-}DR^-$ phenotype. Tumors produce various factors that promote MDSC expansion, such as IL-6, VEGF or GM-CSF, whereas they get further activated by local IFNγ, IL-1β or Toll-like receptor (TLR) signals.[57]

MDSCs use a diversity of mechanisms to suppress T-cell function, including the uptake of arginine and cysteine (essential amino acid for T cell activation) and the nitration of the TCR.[56] In addition, MDSCs have been recently shown to directly support tumor growth by promoting the epithelial-to-mesenchymal transition in melanocytes.[58]

The possibility of improving anti-tumor immune responses by targeting MDSCs has been explored in pre-clinical models. One of the chemical drugs that seem to be more effective for MDSC depletion was 5-fluorouracil (5-FU). In a model of thymoma EL4 cells transplanted subcutaneously, tumor-bearing mice treated with 5-FU showed reduced number of MDSC in tumor lesions. This associated with prolonged mouse survival and enhanced intratumoral $CD8^+$ T cell antigen-specific capacity to produce IFNγ.[59] Interestingly, combination therapy with an agent (cyclophosphamide, CTX) that reduces Tregs led to a synergistic protective effect. Consistent with this, another study showed that inhibition of MDSC and Treg function within B16 melanomas using blocking antibodies to CTLA-4 (already in clinical use—ipilimumab—in late-stage melanoma) and to PD-1 reduced tumor development and increased mouse survival.[60]

$Foxp3^+$ Tregs are well known to suppress the activation, proliferation and effector functions (such as cytokine production) of a wide range of immune cells, including αβ and γδ T cells, NK and NKT cells, B cells, macrophages and DCs. Suppressive functions displayed by Tregs include contact-dependent mechanisms, such as those that involve CTLA-4, PD-1 and GITR; and cytokine-mediated mechanisms such as TGFβ, IL-10 and IL-35.[61] TGFβ is particularly critical since, besides being strongly immunosuppressive, creates a potent positive feedback mechanism by instructing the differentiation of "inducible" Tregs.[1]

Experimental Treg depletion has been usually accomplished using anti-CD25 monoclonal antibodies, since there is a good correlation between CD25 and Foxp3 expression within $CD4^+$ T cells (although activated effector cells also upregulate CD25). Prophylactic Treg depletion in renal cell carcinoma and MCA carcinoma was shown to reduce tumor growth, with protection being dependent on $CD8^+$ and NK cells.[62,63]

While most studies have concentrated on the immunosuppressive function of Tregs, two recent reports have shown that they can also act by directly promoting tumor growth and dissemination. Thus, Treg TILs in ovarian cancer they secrete VEGF that promotes endothelial cell proliferation;[64] and in breast cancer they produce RANKL, which associates with lung metastasis.[65] Importantly, the latter study is one of many that demonstrates that Treg accumulation within tumors is a marker for poor clinical outcome.[66]

The Prognostic Value of Tumor-Infiltrating Leukocytes in the Clinic

Although a favorable association of high numbers of TILs in the primary tumors had been generally reported for decades in many human cancers, TILs had never reached the level of recognized prognostic marker (or proof for cancer immunosurveillance) probably due to their phenotypic and functional heterogeneity.[67] The recent observations that specific immune parameters have better prognostic value than standard staging systems, highlights the importance of the endogenous immune response in determining the clinical outcome. This may help to modify current classifications, and—most importantly—to identify the patients who would benefit the most from adjuvant immunotherapy.

Considering the data reviewed above, it is tempting to assume that good prognosis associates (for example) with $CD8^+$ and NK cells, whereas bad prognosis is linked to the accumulation of Tregs and MDSCs. Moreover, given the functional heterogeneity within many leukocyte populations, clearly distinct outcomes could be expected from Th1 vs. Th2 or Th17 $CD4^+$ subsets, M1 vs. M2 macrophages, N1 vs. N2 neutrophils. This level of refinement is obviously incompatible with traditional immunohistochemistry of cancer patient samples, thus requiring additional techniques like flow cytometry and molecular biology to provide an adequate characterization of tumor-infiltrating leukocytes. Furthermore, detailed imaging may also be important as to define the localization of TILs within the tumor mass. For example, in a pre-clinical model, $CD8^+$ T cells were recently shown to be trapped in the stroma and thus excluded from the core of tumor due to post-translational modifications (nitration) of the chemokine CCL2.[68] Of note, novel drugs that inhibited CCL2 nitration facilitated $CD8^+$ T cell infiltration and tumor regression.

The most comprehensive clinical studies correlating tumor-infiltrating leukocytes with disease outcome have been performed in colorectal cancer, where the general conclusion has been that disease free overall survival is positively associated with a coordinated Th1/ $CD8^+$ T cell infiltration[67] (**Table 1**). A similar result was reached for breast cancer;[69,70] and for hepatocellular carcinoma, where NK markers and the chemokines CCL2, CCL5 and CXCL10 were additional immune signatures predictive of patient survival (at early stages of the disease).[71]

By contrast, Treg infiltration has been generally associated with poor prognosis (**Table 2**). In ovarian carcinoma, melanoma, breast cancer, Hodgkin lymphoma and glioblastoma, the presence and frequency of Tregs correlated with tumor grade and with reduced patient survival.[66] These studies also highlighted the potential role for CCL17 and CCL22 (ligands for the chemokine receptor CCR4) in recruiting Tregs into tumors. The combined value of quantifying both $CD8^+$ and Treg TIL (antagonistic) subsets as prognostic of disease-free survival was demonstrated in hepatocellular carcinoma[72,73] and colorectal cancer.[74] However, in some cancer types, such as colorectal[75] and head and neck carcinomas,[76] Treg accumulation within tumors has been associated with favorable prognosis. This was suggested to be due to a dominant effect in suppressing infection-associated inflammation at mucosal interfaces.[77] Nonetheless, positive associations between survival and Treg numbers were also observed upon immunohistochemical analysis of biopsies from four types of lymphoma patients.[78,79]

Table 1. Tumor-infiltrating leukocytes associated with good prognosis for cancer patients

TIL	Cancer Type	References
$CD8^+$	colorectal cancer	74, 86, 87
	hepatocellular carcinoma	71, 72, 73
	esophageal carcinoma	84, 88, 89
	breast cancer	69
Th1 ($CD4^+$)	colorectal cancer	85
	hepatocellular carcinoma	71
	breast cancer	70
Th17 ($CD4^+$)	esophageal carcinoma	84
Tregs ($CD4^+$)	colorectal cancer	75
	head and neck carcinoma	76
	lymphoma	78, 79
γδ T cells	ovarian carcinoma	90
B cells	breast cancer	69
NK cells	esophageal carcinoma	84
	hepatocellular carcinoma	71

Whereas Th2 infiltration has been associated with poor prognosis in pancreatic cancer,[80] the role of Th17 TILs in human cancer is much more controversial. On one hand, Th17 cell infiltration has been correlated with poor prognosis in prostate cancer[81] and in hepatocellular carcinoma;[82] on the other, it has been associated with better overall survival in ovarian cancer[83] and in esophageal squamous cell carcinoma.[84] While the reasons for these discrepancies are unclear, it may be interesting to assess the co-production of IL-17 and IFNγ by Th17 cells, as well as their association with $CD8^+$ T cell recruitment.

Finally, a recent study attempted to integrate the prognostic values of Th1, Th2 and Th17 TILs by hierarchical clustering of signature gene transcripts in colorectal tumor specimens. The results showed that: the Th2 cluster did not correlate with prognosis; patients with high expression of the Th1 cluster had prolonged disease-free survival; and patients with high expression of Th17 cluster had poor prognosis.[85] In the future, we believe

Table 2. Tumor-infiltrating leucocytes associated with poor prognosis for cancer patients

TIL	Cancer Type	References
Th17 ($CD4^+$)	colorectal cancer	85
	hepatocellular carcinoma	82
	prostate cancer	81
Th2 ($CD4^+$)	pancreatic cancer	80
Tregs ($CD4^+$)	colorectal cancer	74
	hepatocellular carcinoma	72
	ovarian carcinoma	91
	breast cancer	92
MDSCs	esophageal, pancreatic and gastric	93
Macrophages	breast cancer	94
Neutrophils	renal cell carcinoma	95

that will be highly informative and important to collect similar data in many other cancer types.

Conclusions

The identification of highly diverse tumor-infiltrating leukocyte subsets and their distinct, sometimes antagonistic, functions in the tumor niche has been an important development in Oncoimmunology. This has allowed a better dissection and understanding of the interactions between immune components and tumor cells, not only in animal models but also in patients. We are now approaching an era where immune parameters will likely constitute some of the best prognostic markers for cancer progression/ regression. This will also allow a more insightful selection of patients to undergo immunotherapy as adjuvant treatment. Notwithstanding, many basic aspects of TIL biology still need to be clarified as to resolve key controversies in the field, such as the paradoxical behaviors of Th17 and γδ T cells (among others) in distinct cancer models/ types. Clinical studies must now routinely include in-depth population phenotyping and gene expression analysis in order to address the striking heterogeneity of the immune infiltrates. These future directions will be crucial to clinically promote an anti-tumor microenvironment and thus increase the success of cancer immunotherapy.

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