Universidade de Lisboa

Faculdade de Medicina

The impact of CD6 targeting in T cell function and immunopathology

Raquel Filipa Reis de Freitas

Orientador: Professor Doutor Luís Ricardo Simões da Silva Graça

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Biomédicas, especialidade em Imunologia

2019

Universidade de Lisboa

Faculdade de Medicina de Lisboa

The impact of CD6 targeting in T cell function and immunopathology

Raquel Filipa Reis de Freitas

Orientador: Professor Doutor Luís Graça

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Biomédicas, especialidade em Imunologia

Júri:

Presidente:

- Doutor João Eurico Cortez Cabral da Fonseca, Professor Catedrático e Vice-Presidente do Conselho Científico da Faculdade de Medicina da Universidade de Lisboa.

Vogais:

- Doutor Alexandre Valentim Xavier Mourão do Carmo, Investigador Principal do Instituto de Biologia Molecular e Celular do Porto;
- Doutora Helena Isabel Martins Soares, Investigadora Principal do Centro de Estudos de Doenças Crónicas da Universidade Nova de Lisboa;
- Doutora Íris Maria Ferreira Caramalho, Especialista de reconhecido Mérito e Competência, Investigadora do Instituto Gulbenkian de Ciência;
- Doutor Luís Ricardo Simões da Silva Graça, Professor Associado com Agregação da Faculdade de Medicina da Universidade de Lisboa (Orientador);
- Doutor Bruno Miguel de Carvalho e Silva Santos, Professor Associado com Agregação da Faculdade de Medicina da Universidade de Lisboa.

Instituição Financiadora: Fundação para a Ciência e Tecnologia (SFRH/BD/52230/2013)

2019

Todas as afirmações efetuadas no presente documento são da exclusiva responsabilidade do seu autor, não cabendo qualquer responsabilidade à Faculdade de Medicina de Lisboa pelos conteúdos nele apresentados

Para a minha avó Maria com um coração demasiado grande para apenas dois netos. Estarás sempre comigo😊.

Lutar&Acreditar

Raquel Freitas 😊

“São meus discípulos, se alguns tenho, os que estão contra mim; porque esses guardam no fundo da alma a força que verdadeiramente me anima e que mais desejaria transmitir-lhes: a de não se conformarem”

Agostinho da Silva 😊

Table of Contents

Acknowledgements

Quero agradecer aos meus dois pirilampos 😊 Sílvia e Andreia, que quando eu não via mais o caminho, o iluminaram para eu o conseguir acabar de percorrer, gosto muito de vocês e obrigada por tudoooo!!!

Sílvia, obrigada pela tua orientação sempre me defendeste quando eu precisava e trazias-me á terra quando também era necessário, estiveste lá sempre que chamei por ti!!! 😊

Andreia, obrigada por me levares para pensamentos positivos e me distraíres do que não importava 😊, e ás tuas meninas duas luzesinhas.

Obrigada 😊 Raquelinha minha homónima que também estiveste sempre lá para mim, ensinavas me truques tinhas paciência para mim e ajudavas me no que podias.

Obrigada Filipinha, Catarina, Afonso e Ricardo e claro a nossa cubana favorita 😊 Yaqueline.

Obrigada Saumya 😊 por teres salvo o Chita e me teres ajudado a projectar o meu CD6zinho lol num modelo 3D lolololol

Obrigada ao meu orientador de tese o professor Luís Graça.

Obrigada ao meu Marquinho por me aturar quando chegava a casa com pouca paciência ou o fazia ir-me buscar ao laboratório de madrugada lol.

Obrigada ao meu Avô 😊 por me chatear para despachar de uma vez por todas, esta tese lolololol e à minha Tia 😊, às minhas Primas e á nova aquisição a Matilde, aos meus Pais e ao meu Mano mais o pirilampo que está para chegar, à minha avó e à minha estrelinha do céu que tomou conta de mim lá de cima a minha avó Maria 😊.

Obrigada aos Mochos Nocturnos lolololololol, Eduardo, Diana e André, muito importantes na manutenção da minha sanidade mental.

Obrigada à Carina, ao Afonsinho, á Bigui, Maggie e Ricardo a malta das caminhadas 😊, à Trindade e á Teresa do Yoga que apareceram na minha vida mesmo na hora certa.

Um grande obrigada á AMIRA, a associação de animais que faço parte e me permitiu relembrar o que me motiva na ciência, a vontade de contribuir e ajudar 😊

E aos meus pikachus 😊😊😊😊o meu Kiko a minha rochinha peluda, à nova aquisição a minha Estrelinha e ao Farber, Doroteia e Carolina 😊 que me aturaram mais que ninguém durante a escrita da tese.

Quero agradecer ao prof. Luís Graça por me ter aceite na sua equipa como estudante de doutoramento.

Um grande obrigada 😊 a todas as pessoas que cruzaram o meu caminho durante este doutoramento e fizeram o que poderam para me ajudar nesta aventura 😊

Abbreviation list

AD- autoimmune diseases

ADCC- antibody-dependent cell-mediated cytotoxicity

AHR- aryl hydrocarbon-receptor

AID - activation-induced cytidine deaminase

AIRE- autoimmune regulator genes

ALCAM- activated leukocyte cell adhesion molecule

ALL- acute lymphoblastic leukemia

AP-1 – activator protein 1

APCs- antigen presenting cells

ASCL2 - achaete-scute complex homolog 2

ATP- adenosine thriphosphate

BBB- blood brain barrier

BCRs- B cell receptors

BM- bone marrow

BMDC- bone marrow derived dendritic cells

C57Bl/6- C57 black 6

CDR- complementarity determining regions

CFA- complete Freund's Adjuvant

CIA- collagen-induced arthritis

CK2- casein kinase II

CLL- chronic lymphocytic leukemia

CMJ - corticomedullary junction

CNS- central nervous system

CSR - class switch recombination

CTLA4- cytotoxic T-lymphocyte-associated protein 4

CTV- CellTrace violet

CXCL- C-X-C motif ligand

CXCR- C-X-C motif receptor

DAMPs- damage associated molecular patterns

DBA-1- Dilute Brown Non-Agouti 1

DCs- dendritic cells

DLN- draining lymph node

DN- double negative

DNA- deoxyribonucleic acid

DP- double positive

DTH- delayed-type hypersensitivity

EAE- experimental autoimmune encephalomyelitis

EMA- European medicines agency

FCS- Fetal calf serum

FDA- food and drug administration

Foxp3- forkhead box p3

Gads- Grb2-related adaptor downstream of Shc

GITR- glucocorticoid-induced TNFR-related protein

GM-CSF - granulocyte-macrophage colony-stimulating factor

GWAS- genome-wide association studies

HSC- hematopoietic stem cells

i.p.- intraperitoneal

i.v.- intravenous

IBD- Inflammatory bowel disease

ICOS- inducible costimulatory receptor

ICOSL- inducible costimulatory ligand

IEL- intraepithelial lymphocyte

IFN-γ – interferon gamma

Ig- immunoglobulin

IL- interleukin

ILCs- innate lymphoid cells

iPBMCs- irradiated peripheral blood mononuclear cells

IPEX- immune dysregulation, polyendocrinopathy, enteropathy, x-linked

IS- immunological synapse

LAT- linker for activation of T cells

LPS- lipopolysaccharide

LSCL- lymphosarcoma cell leukemia

mAb- monoclonal antibody

MAC- membrane attack complex

MAPK- mitogen-activated protein kinases

MFI- median fluorescence intensity

MHC- major histocompatibility complex

MLR- mixed lymphocyte reaction

MOG- myelin oligodendrocyte glycoprotein

MS- multiple Sclerosis

mTECs- medullary thymic epithelial cells

mTOR- mammalian target of rapamycin

NF-κβ - nuclear factor-κβ

NK- natural killer cells

NKT- natural killer T cells

NLR – NOD-like receptor

NO- nitric oxide

NOD- nucleotide-binding oligomerization domain-like receptors

OVA- ovalbumin

PAMPs- pathogen associated molecular patterns

PBMCs- peripheral blood mononuclear cells

PD1- programmed cell death protein1

PI3K- phosphatidylinositol-4,5-bisphosphate 3-kinase

PKC- protein kinase C

PMA- phorbol myristate acetate

PRRs- pattern recognition receptor

PTEN- phosphatase and tensin homolog

RA- rheumathoid arthritis

RAG- recombination-activating genes

ROR- RAR-related orphan receptor

ROS- reactive oxygen species

s.c.- subcutaneous

SAg- superantigen

Satb1- SATB homeobox 1

SCZ- subcapsular zone

SEA- staphylococcal enterotoxin A

SEB- staphylococcal enterotoxin B

SEE- staphylococcal enterotoxin E

SLP76- Src homology 2 (SH2) domain-containing leukocyte protein of 76 kD

SNP- single nucleotide polymorphisms

SO- superoxide

SOCS- suppressors of cytokine signaling

SRCR- scavenger receptor cysteine-rich

SRSF1- serine and arginine Rich Splicing Factor 1

STAT- signal transducer and activator of transcription

TCRs- T cell receptors

TdT- terminal deoxynucleotidyl transferase

TECs- thymic epithelial cells

Teff- T effector cells

Tfh- T follicular helper cells

TGF-β - transforming growth factor beta 1

Th- T helper cells

TLRs- Toll-like receptor

TNF- tumour necrosis factor

Tregs- T regulatory cells

TSAd- T cell-specific adapter

TSPs - thymic seeding progenitors

TSST-1 -toxic shock syndrome toxin 1

tTregs- thymic Tregs

Sumário

Nas últimas décadas, tem se vindo a verificar um aumento crescente no uso de anticorpos monoclonais (mAb) sendo que actualmente são a classe de agentes terapêuticos mais usada, representando uma indústria de milhões com já 30 mAb aprovados para terapêuticas em humanos e mais ainda sob avaliação clínica.

As suas principais vantagens em comparação com outras terapêuticas consistem num elevado grau de especificidade e também flexibilidade que se usadas contra alvos na sinapse imunológica irão realçar as suas propriedades imuno-moduladoras.

O CD6 uma glicoproteína transmembranar da sinapse imunológica, importante para estabilidade da apresentação de antigénio, maturação da sinapse imunológica e uma proliferação das células T óptima, foi reavivado enquanto alvo terapêutico aquando da criação de um novo anticorpo monoclonal não-depletante em meados dos anos 90. Apesar de ter sido estudado extensivamente ao longo dos anos, compreender a sua dinâmica tem sido bastante difícil sobretudo devido à existência de resultados paradoxais. Contudo uma coisa é certa, o seu papel nas patologias autoimunes, como é o caso da esclerose múltipla, artrite reumatoide e psoríase.

Um exemplo de quão paradoxal o papel do CD6 é, é o caso em que se por um lado em modelos animais de esclerose múltipla ou psoríase a sua ausência (KO) resulta em atenuação ou protecção da doença, por outro lado num modelo de artrite a sua ausência torna a doença ainda pior.

Para compreender melhor a razão de tal comportamento paradoxal, decidimos tirar partido deste novo anticorpo não depletante, anti-CD6 d1, desenvolvido em Cuba pelos nossos colaboradores, e tentar então descobrir como é que o “targeting” do CD6 pode afectar as propriedades funcionais das células T e como é que isso se relaciona com as imuno-patologias aqui estudadas. Aqui mostro como é que ambos os anticorpos, ratinho e humano, contra o CD6 vão fazer exatamente isso.

Primeiro investigámos como é que fazendo “targeting” do CD6 com o nosso anticorpo iria afectar o normal desenvolvimento de um modelo animal de esclerose

múltipla, para tal usámos o modelo de EAE, um modelo já bem estabelecido no nosso laboratório.

O tratamento com o nosso anti-CD6 foi intrigante, uma vez que os resultados obtidos estavam fortemente relacionados com as doses administradas. Ou seja, enquanto uma dose baixa era protectora, já uma dose alta ou era equivalente ao controlo ou acentuava a doença ainda mais. Contudo estes resultados em ratinho apesar de inesperados, estão em concordância com relatórios de ensaios clínicos em artrite reumatoide, onde também os efeitos protetores mais persistentes no tempo, advêm da dose testada mais baixa. Para compreender os mecanismos por detrás destas observações, investigámos como é que o "targeting" de CD6 estaria a afetar a normal especialização funcional das células T (polarização). E de acordo com os nossos resultados *in-vitro*, verificámos uma vez mais um efeito dependente da dose, enquanto doses crescentes de anti-CD6 d1 comprometiam a polarização das células T reguladoras, pelo outro lado eram mais favoráveis à polarização de células Th1.

De forma a tentar excluir um potencial efeito de impedimento estérico, resultante do elevado tamanho de um anticorpo IgG, usámos CD166 solúvel (ligando CD6 d3) como forma de quebrar as interações entre o CD6 nas células T com o CD166 (ALCAM) nas células apresentadoras de antigénio. Contudo o uso do CD166 solúvel não foi capaz de mimetizar o efeito do nosso anti-CD6 d1, sugerindo um efeito independente de impedimento estérico.

Mais ainda, fazer "targeting" do CD6 com o nosso anticorpo, sugere um efeito mais direcionado para a transdução de sinal propriamente dita, porque estas propriedades imuno-moduladoras só são detetáveis se condições de activação fisiológicas forem usadas. Para reforçar o seu impacto a um nível mais específico, temos o facto de que nem a proliferação ou sobrevivência foram afetadas significativamente. Na verdade, o uso de condições supra-fisiológicas, como activação por anti-CD3/anti-CD28 resulta na perda de quaisquer efeitos a nível de polarização.

Seguindo o racional de um efeito dependente de dose aquando do "targeting" de CD6, decidimos explorar o seu potencial terapêutico noutros modelos de doença

com uma cinética de acção oposta às doenças autoimunes. Investigámos então, se num modelo de cancro da mama doses altas de anti-CD6, dadas com diferentes estratégias resultariam no total desaparecimento do tumor ou num crescimento mais reduzido. Os nossos resultados confirmaram então que uma dose alta de anti-CD6 d1, ainda que administrada de forma cumulativa, resulta num abrandamento do crescimento tumoral. Contudo a forma como o "targeting" de CD6 especificamente afeta as células T $CD4^+$ permanece inconclusiva, visto não haver significância estatística. Ainda assim, os dados parecem sugerir um impacto negativo ao nível das células T reguladoras $CD25^+Foxp3^+$, especificamente as que infiltram os tumores. Outra observação a considerar, foi um aumento ao nível da expressão de IL-17 por estas mesmas Tregs infiltrantes, expressão esta descrita como associada com caminhos de activação de MAPKinases previamente também eles associados com activação de CD6.

Sob estas mesmas condições os nossos dados sugerem ainda um impacto negativo por parte do anti-CD6 d1, ao nível da activação propriamente dita como mostrado pelos níveis de MFI de CD25, uma associação previamente estudada em células humanas por outro laboratório.

Como forma de validar o nosso anti-CD6 d1 de ratinho enquanto substituto de estudo adequado para o Itolizumab (human anti-CD6 d1), tivemos então de investigar também como é que o próprio Itolizumab afectava as propriedades funcionais das células T $CD4^+$ *in-vitro.* E tal como esperado, quando tratadas com ant-CD6 d1, as células humanas também mostraram um impacto dependente da dose em que doses crescentes impedium a polarização de Tregs enquanto por outro lado favoreciam as Th1s. Contudo, ao contrário do observado em ratinhos, e apesar de não afetar a sobrevivência, o "targeting" do CD6 afectou ligeiramente a proliferação em células humanas. Além disso, os dados das células humanas sugeriram também um efeito independente do impedimento estérico e dependente de condições de activação as mais fisiológicas possíveis de forma a que o impacto do anti-CD6 d1 pudesse ser percetível.

Estratégias de activação como anti-CD3/anti-CD28 ou mistura de SAgs e APCs, precisamente porque não permitem que haja um impacto do anti-CD6 d1 ao nível

das céulas T $CD4^+$ ajudam a perceber quais as vias de sinalização em que o CD6 está de facto envolvido.

Em suma, os nossos dados mostram um efeito dependente de dose ao nível da polarização das células T quando “targeted” com anti-CD6 d1, efeito esse que consiste numa menor polarização de Tregs à medida que se aumenta a dose enquanto que ao mesmo tempo a polarização de Th1 é favorecida, observável tanto em células de ratinho como humanas.

Uma explicação potencial para estas observações poderia ser a relação existente entre níveis de ativação e sensibilidade de polarização, isto é, níveis diferentes de ativação causados pelo anti-CD6 podem resultar em fenótipos de polarização específicos.

Summary

In the last few decades, monoclonal antibodies have become one of the most widely used classes of therapeutic agents, representing a billion-dollar industry with more than 30 monoclonal antibodies approved for human therapeutics and many others under clinical evaluation.[1]

Their main advantages regarding other therapeutic agents consist of high specificity and high flexibility, which applied against targets involved in immune synapse will enhance its immunomodulatory therapeutic benefits. CD6 an immune synapse transmembrane glycoprotein, important for the stability of antigen presentation, maturation of immunological synapse and optimal T-cell proliferation, has been revived as a therapeutic target since the creation of a nondepleting anti-CD6 mAb in the early '90s. Despite CD6 has been extensively studied, understanding its biology has been difficult due to paradoxical results. Still, one thing is for sure, which is its role in autoimmune pathologies, as it is the case of Multiple Sclerosis, Rheumatoid Arthritis, and Psoriasis. And an example of CD6 paradoxical impact is, while in experimental autoimmune encephalomyelitis (EAE) and imiquimod-induced psoriasis CD6-deficient mice show disease protection or attenuation, in collagen-induced arthritis (CIA) the absence of CD6 made it even worst.

So, we have decided to take advantage of this new non-depleting mAb against CD6 d1, developed by our collaborators in Cuba and try to understand how targeting CD6 would impact T cell functional properties and how it would interfere in immune pathologies. Here, I show how both murine and human antibodies targeting CD6 domain 1 influenced exactly that.

First, we have investigated how targeting CD6 with our mAb would affect the normal development of a mouse model of MS, to do so we used a well-established EAE model, which had already been used in the lab.

Treatment with anti-CD6 was intriguing, since the outcome was heavily related to the dose being used, meaning while a low dose was protective, high doses showed a level of disease severity equivalent or even worse than the control group. However, our mice results do resemble the reports on RA clinical trials, where lower doses were the ones giving longer-term responses. To uncover the mechanisms behind it, we investigated how CD6 targeting was affecting CD4+ T cell functional specialization. And accordingly, to our in-vitro results, we verified that once more, in a dose-dependent manner, while increasing doses were

compromising Tregs polarization, in the case of Th1's it was favoring it. To try to exclude a possible steric hindrance effect due to the mAb size, we used a soluble CD166 (CD6 d3 ligand) as a means to disrupt T cell's CD6 interactions with APC's CD166. However, this did not mimic CD6 targeting with our anti-CD6 d1 mAb, suggesting a steric hindrance independent effect. More, targeting CD6 with our mAb suggests a direct effect over signaling itself, since its modulatory properties are only detectable if under activating physiologic conditions. Under supra-physiologic stimulation like with anti-CD3/anti-CD28, the impact over polarization is lost.

We expected the impact of anti-CD6 d1 to be a fine-tuned one since no major alterations were seen on either T cell survival or proliferation.

Following the rationale of this dose-dependent effect caused by CD6 targeting, we decided to explore its therapeutic potential on other disease models with opposite kinetics to autoimmune diseases. So, we investigated if in a model of breast cancer, high doses of anti-CD6, given under different delivery strategies, would result in total tumor clearance or reduced tumor growth. Our results ended up showing a reduced growth tumor ability if given in in-situ cumulative doses. However, the way CD6 targeting specifically impacted the CD4+ T cell population was not very conclusive due to the lack of statistical significance. But once more the data suggested a negative impact over $CD25^{+}Foxp3^{+}$ regulatory T cells, specifically tumor-infiltrating ones. Another observation was a potential increase of IL-17 expression by these very same infiltrating Tregs which has been associated with MAPK activation pathways also associated with CD6 activation. Under these same conditions our data also suggests, a negative impact of CD6 targeting over $CD4^{+}$ T cell activation as measured by CD25 MFI levels, a relation previously reported on human cells by literature.

To validate the mice anti-CD6 d1 mab as an adequate proxy of itolizumab, we have also investigated how Itolizumab would impact $CD4^{+}$ T cell's functional properties in-vitro. And as expected, when treated with anti-CD6 d1, human cells also displayed a dose-dependent negative impact over Treg polarization while on the other side favoring Th1's. But contrary to mice, and despite no impact on survival, targeting CD6 did significantly impact. Besides that, human data also suggested a steric hindrance independent effect and dependence on physiological activation conditions so that an impact on T-cell functional properties could be perceived. Activation strategies like anti-CD3/anti-CD28 or SAg mix and APCs, precisely because did not allow an impact of anti-CD6 d1 on $CD4^{+}$ T cells, shed some light into which signaling pathways anti-CD6 d1 was affecting.

Overall our data show a dose-dependent impact of anti-CD6 d1 over T cell functional specialization, meaning while increasingly high doses reduce T cell's polarization ability towards Tregs also favors Th1 induction, something true for both murine and human cells. A potential explanation for such observations is the relation between activation levels and polarization sensitivity, so different activation levels caused by CD6 targeting might favor specific polarization phenotypes.

Our data highlights the importance of dosage and how the same drug might be beneficial for different disease conditions.

Introduction

INTRODUCTION

1. The Immune System

Immunity derived from the Latin word immunis means free or untouched and is one of the most complex systems comparable only to the nervous system.

It is essentially a network of molecules, cells, tissues, and organs specialized in keeping the organisms in a state of equilibrium to avoid disease.

This equilibrium requires neutralization of pathogens like bacteria, virus, parasites and fungi, recognition and neutralization of harmful environmental substances and action against damaged or altered cells from the own body.

Any alteration in this equilibrium between pro and anti-inflammatory immune responses results in disease.[1]

We can divide the immune system in innate and adaptive immunity, but we must remember they depend on each other.

To be accurate the first line of defense against infection is anatomical and physiological barriers followed immediately by an innate response, the oldest form of immunity throughout evolution. This readiness happens because of its non-specificity towards antigen, however, adaptive immunity takes longer but is highly specific for each infection and upon re-exposure becomes faster and stronger.

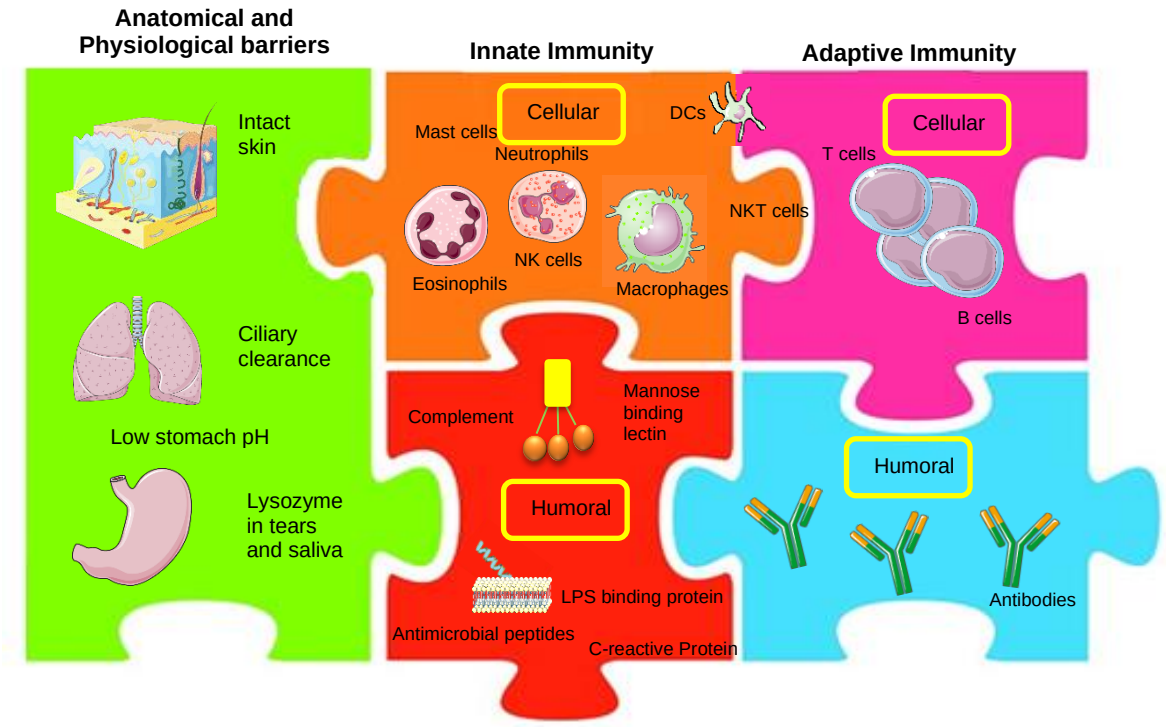


Fig.1- Interconnectivities of Human Immune System

As adapted from Turvey et al 2010 The human microbial defense can be divided into three arms: (i) anatomical and physiological barriers; (ii) innate immunity; and (iii) adaptive immunity. With some elements making the connection between the arms. [2]

1.1. Innate Immunity

When anatomical and physiological barriers like intact skin, mucociliary clearance, low stomach pH and bacteriolytic enzymes in secretions fail, the innate immunity gets immediately triggered and an inflammatory response begins. [3]

The triggers are damage-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs). The first, DAMPs, are molecules upregulated and released during cell lysis and tissue damage either in sterile or

infectious inflammation. The seconds, PAMPs, are highly conserved microbial components essential for survival and virulence of pathogens.

These triggers activate cells when recognized by pattern recognition receptors (PRRs), which are germline-encoded and although this lack of flexibility is a disadvantage it is overcome by its essentiality to pathogen survival and a ready to go response.

But there is also the opposite strategy where innate immune cells must recognize specific molecules expressed only by healthy cells to inhibit their activation.

This independence of genetic recombination and developmental phases buys time for the adaptive immunity to get triggered and ready.

PRRs are divided into several classes like the nucleotide oligomerization domain (NOD)-like receptors (NLR), Toll-like receptors (TLRs), C-type lectin receptors (CLRs) scavenger receptors and cytosolic DNA sensors between others. They are found either at the cell surface, cytoplasm or endosomes which allows them to detect both internal and external threats.[2]

At the cell surface where they detect microbial cell-wall components like LPS (TLR 4), we find TLR 1, 2, 4, 5 and 6. In endosomes where they recognize microbial nucleic-acids like ds-RNAs (TLR3), we find TLRs 3, 7, 8 and 9. But TLRs also recognize DAMPs from the host, like heat shock proteins (TLR2-4) and Chromatin-IgG complexes (TLR9). [4,5]

Triggering PRRs activates transcription factors like NF-ƙβ, AP-1, and IRFs, this will start pro-inflammatory cytokines and chemokines production, presentation of co-stimulatory signals (contact-dependent or independent) and finally cell recruitment to the site of injury with activation of the adaptive immunity.

The elements involved in this initial phase are from the humoral and cellular origin. Between the humoral components, we have the Complement, LPS-binding, and C-reactive proteins as well as other pentraxins, collectins and anti-microbial peptides like defensins. Regarding the cellular components they can be of hematopoietic and non-hematopoietic origin. [2]

The complement system (C1-C9) it's a liaison between inflammatory triggers and other immune responses like antibodies and phagocytic cells. This is set in

motion by a coordinated enzyme cascade resulting in danger clearance, through pathogen recognition, opsonization, and lysis.

Lysis occurs through a structure called membrane attack complex (MAC) that essentially introduces pores on pathogen cell walls and kills them.

This is a highly regulated system, which requires activation of its precursors in a proper sequence to form enzymatic complexes which rapidly dissociate and return to inactivity.[6]

The hematopoietic group includes both myeloid cells (macrophages, dendritic cells-DCs, neutrophils, eosinophils, mast cells, and basophils) and innate lymphoid cells (ILCs). While the non-hematopoietic, also known as immune stroma includes fibroblasts, endothelial and epithelial cells essentially cells that form the architectural structure necessary for proper cell interactions and proper display of molecular cues needed for position growth and survival.

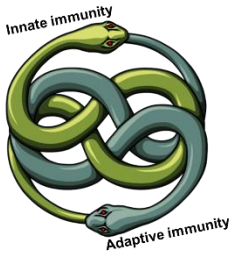
Macrophages and DCs reside in tissues while scavenging it to find signs of danger, once they get activated and initiate a pro-inflammatory response, blood circulating neutrophils are recruited to the tissue. All the three are highly phagocytic cells, but while macrophages and neutrophils are responsible for removal and disposal of pathogens, infected cells and immune complexes using strategies like nitric oxide (NO), superoxide (SO), reactive oxygen species (ROS), enzymes and pro-inflammatory cytokines with anti-microbial properties, DCs are responsible for the connection with the adaptive immune system.7 They bridge this connection by presenting the antigen to T cells, and although other cell types can also present it (macrophages and B cells) they are the professional antigen-presenting cells APCs.

When the pathogens are large parasites like helminths they cannot be phagocytized, so eosinophils come into action and kill them by releasing cytotoxic granules, cytokines, and lipid mediators. This will increase inflammation as well as tissue destruction, in fact, eosinophils are responsible for allergic diseases like asthma. [8]

Allergic diseases are also misdirected immune responses perpetrated by long-lived tissue-resident mast cells and short-lived blood circulating basophils which

releases inflammatory mediators like histamine and have anti-bacterial properties.[9]

But lymphoid cells are also involved in innate immunity, they are the ones with the germline-encoded ready to go antigen receptors. For more than 30 years ILCs were all about NK cells but recently, new populations arise, the ILC1, 2 and 3. They look like the innate version of T cells with both ILC1 and Th1 producing IFN-γ, lLC2 and Th2 producing lL-4, 5, 9 and 13, ILC3 and Th17 producing IL-17 and 22 and finally NK and $CD8^+$ T cells both being cytotoxic. However, they are much better pro-inflammatory and immunoregulatory cytokines producers, and this allows them to direct adaptive immunity in the best way to fight each specific threat. Their localization is also strategic, they are at sites of potential invasion and colonization by pathogens like barrier surfaces: skin, lung, intestine, some adipose tissues, and mucosa lymphoid tissues. [10,11]



Innate immunity and adaptive immunity are intrinsically connected. [12]

If on the one hand the adaptive immunity activation depends on antigen presentation, where DCs uptake the pathogen, migrate to draining lymph nodes, process it mature and present it to naïve T cells and also on the type of DC stimulus, crucial for T cell polarization, accordingly to the specific group of pathogens to be cleared. On the other hand, the classical activation of the complement system is dependent on antibodies to initiate the enzymatic cascade. Essentially, to function properly they need each other.[13]

1.2. Adaptive Immunity

Three words: specificity, adaptability, and memory. As its name states it adapts to each new threat by making use of genetic recombination to produce antigen-specific receptors that allow, specific, faster and stronger responses on subsequent exposures to the same threat.

Like with innate immunity, we can also divide adaptive immunity into humoral and cellular components.

Humoral components include antibodies that depend on B cell activation and cellular components include $CD8^+$ cytotoxic T cells which depend on T cell activation. However, we must keep in mind that $CD4^+$ T helper cells are also essential since they provide both B, T and innate cells help.

B cells only require help from T cells if the antigen is a protein, and we call it thymus-dependent immune responses otherwise they independently activate themselves.

Once again both innate and adaptive immunity is triggered by cell receptors in this case, we have the BCRs (B cell receptors) and TCRs (T cell receptors), which account for specificity since they are antigen specific. But contrary to innate immunity here they are a result of random somatic recombination as well as a somatic mutation in B cells' case from germline pools of DNA segments.

These processes called recombination and somatic hypermutation, allow as much as 10^8 and 10^{10} possible TCR and BCR combinations to cover all the pathogens that could ever be encountered in a lifetime as well as increased affinity/avidity

Another advantage of adaptive immunity besides specificity and flexibility is the memory, innate immunity does not have it, no matter how many times they encounter the threat.

The immunological memory is the ability to respond specifically, faster and stronger upon antigen re-exposure and both B and T cells can differentiate into memory cells during a first encounter with the antigen, the pillar of vaccination.

Again, like some innate cells, B and T cells also derived from hematopoietic stem cells (HSC) which branched into the lymphoid lineage and while B cells mature in the bone marrow, T cell progenitors must migrate into the thymus to do so.

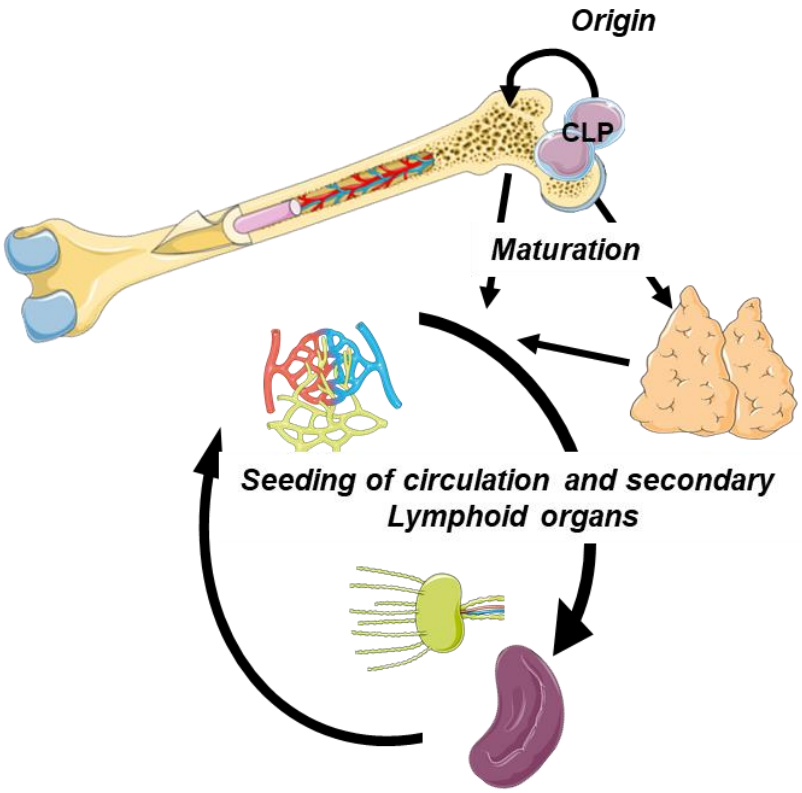


Fig2. Illustration of common lymphoid cell precursors' production and maturation at primary lymphoid organs and migration into circulation and secondary lymphoid organs.

Bone marrow (BM) and thymus constitute the primary lymphoid organs but is in the secondary lymphoid organs (spleen, lymph nodes, and mucosa-associated lymphoid tissues) where all the action takes place. Here B and T cells meet directly or are presented with the antigen to which they are specific.

While B cells are capable of directly recognize the antigen, T cells require the antigen to be presented within a major histocompatibility complex (MHC). There two types of MHCs the I and II, the MHCI is expressed by all nucleated and healthy cells and the MHCII is expressed only by APCs a group that includes DCs and B cells. Also, MHCI antigen presentation is specific of $CD8^+$ T cells, while MHCII of $CD4^+$ T cells. When all these conditions are reunited an adaptive immune response begins.

1.3. B cells

Back in the 1890s when diseases like diphtheria and tetanus were killing thousands of people a year, Behring and Kitasato discovered the importance of a group of circulating antitoxins, in the fight against them.[14]

Then, later in the twentieth century, Ehrlich came to propose that these anti-toxins (antibodies) were produced and released by certain cells due to antigen stimulation. [15] But it was only in the 1930s and late in the 1940s that both its physical nature and cellular source (B cells) were finally discovered. [16-18] However, it took until 1965 for B cells to be considered as an independent lymphocyte lineage. [19]

Like T cells, B cells also derive from hematopoietic stem cells in the bone marrow, where they will start their development that includes B cell receptor (BCRs) recombination and which is mediated by enzymes like RAG and TdT resulting in higher antigen receptor diversity. [20]

BCRs can be divided into two heavy (H-chain) and two light chains (L-chains) connected by disulfide bonds. But it will be their N- and C- terminal regions that will define them.

N-terminal regions of both H- and L- chains form the antigen-binding domain, accounting for antibody specificity. This domain is comprised of three hypervariable complementarity determining regions, CDR1-3, the targets of V(D)J recombination guarantying increased diversity of antigen specificity. [21,22]

By contrast, the C-terminal regions of both H- and L-chains are constant and define the antibody isotype, responsible for its function.

Naïve immature B cells, only express IgM and IgD isotypes, but when they migrate to secondary lymphoid organs and become activated, class switch recombination (CSR) is triggered. This process is also mediated by an enzyme called activation-induced cytidine deaminase (AID) and the outcome of switching will depend on environmental cues, like cytokines produced by T helper cells. Thus, if supported by Th2s they will produce IgG1 and IgE, if supported by Th1s, they will produce IgG2 but if they are in mucosal tissues, they will produce IgA. These five different isotypes are responsible for activating different types of immune cells specific for each situation while keeping the same specificity. [23-27]

To improve specificity and most of all adaptability B cells introduce random mutations into their Igs' CDR domains through a process of somatic hypermutation.

Random mutations will be followed by a process of affinity maturation selecting the B cell clone with the highest antibody affinity, this will ensure a proliferative advantage upon antigen recognition associated with increased survival and growth signals. [28]

Maturation of B cells will also result in memory, and these long-lived plasma cells upon antigen re-exposure are capable of rapidly react and secrete higher levels of antibodies for longer periods. This is the basis for vaccination however is also the reason why allergies and autoimmune diseases can perdure. [29]

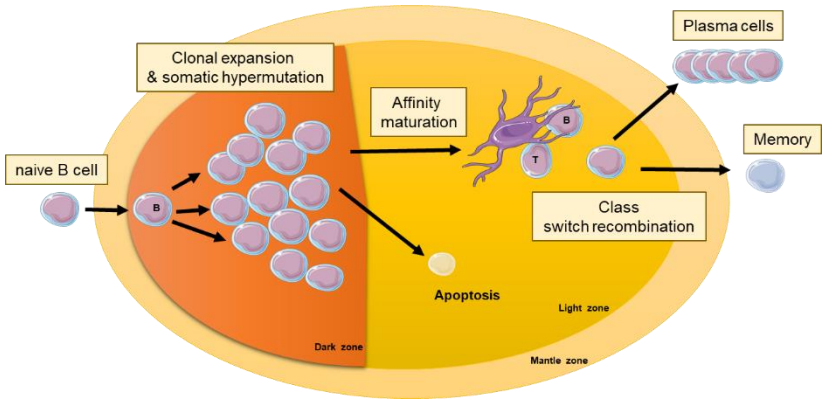


Fig.3 Illustration of a germinal center, where activated B cells proliferate, differentiate and go under several rounds of somatic hypermutation and affinity maturation of their antibody genes.

1.4. T cells

Like B cells, T cells also derive from bone marrow HSC precursors, but contrary to them they must migrate to the thymus to develop.

These thymic seeding progenitors (TSPs) [30,31], enter the thymus in reduced numbers and once they get in contact with thymic epithelium the journey begins.

The spatial and temporal organization is the key for proper T cell maturation. Thus, we can divide the thymus into four compartments: subcapsular zone, cortex, medulla and corticomedullary junction (CMJ). [32]

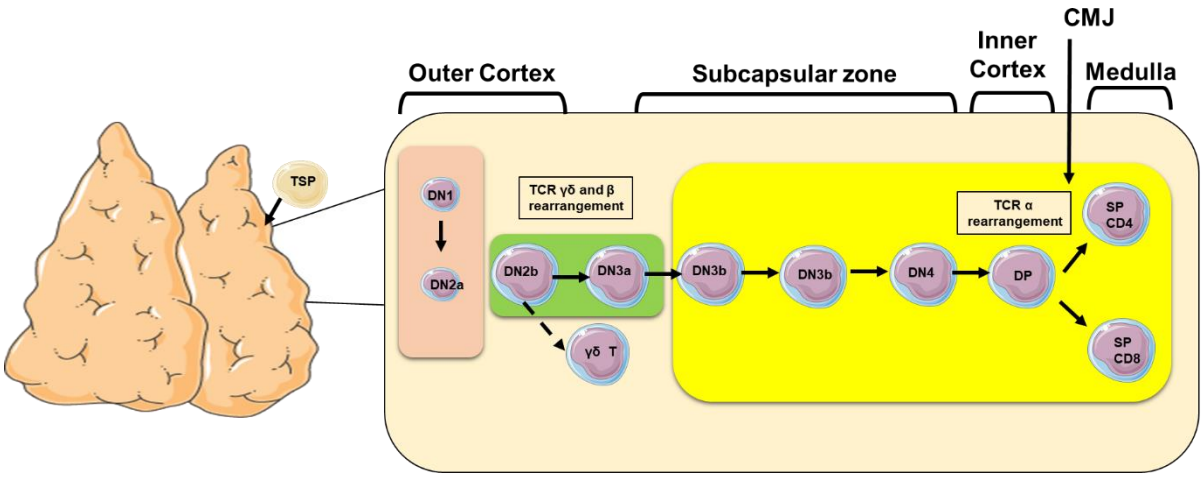


Fig. 4 Illustration of T cell development and maturation in the thymus.

First TSP enters the corticomedullary junction through postcapillary venules, where these early thymic precursors, also called double negative CD4- CD8 -(DN1) cells, proliferate and begin to differentiate. [32] After leaving the CMJ, DN1 cells migrate deeply into the cortex towards the subcapsular zone (SCZ). Here, DN1 will receive stimulatory signals from the stroma, mostly thymic epithelial cells (TECs) and fibroblasts.[32,33] At this stage (DN2) their fate becomes more restricted and gene rearrangement at the TCR gene loci begins with upregulation of pre-TCRα chain and *Rag1*.[34] When reaching DN3 stage, T cell lineage is already defined, either αβ or γδ.[35] T cells expressing αβ TCRs, require an additional checkpoint, a β-selection.

This checkpoint demands a fully functional pre-TCR, which is made of a rearranged TCRβ-chain, CD3 chains, and an invariant pre-TCRα chain. Together with Notch1, the cells can now receive signals for survival and metabolism.[36,37]

If successful, DN4 cells migrate back to the cortex until reach the medulla, along the way pre-TCR signaling allows for CD4 and CD8 expression, at this point Rag1 and 2 are re-expressed so that TCRα rearrangement can begin.[38]

Functional αβ TCRs will determine if the cell survives and its fate, by establishing the strength and specificity towards peptides in MHC ligands presented by cTECs,

DCs, and fibroblasts. This is Positive Selection, where only the TCRs with intermediate avidity for self-peptide-MHC complexes can thrive. [39]

After this second main checkpoint, DP thymocytes will commit either to CD4 or CD8 single-positive lineage (SP).

When finally, they reach the medulla as SP thymocytes they are again tested. In this third checkpoint (Negative Selection), the goal is to prevent autoreactivity so, thymocytes with high-affinity TCRs for self-peptides are instructed by mTECs, which can express tissue-specific antigens (encoded by the autoimmune regulator gene-AIRE) to commit apoptosis.[39,40]

Negative selection in the thymus is the first mechanism of tolerance also known as Central Tolerance, but this is not enough to constrain autoreactive T cells. Thus, the pool of mature T cells leaving the thymus towards the secondary lymphoid organs also include some immature self-reactive naïve cells.

All of us have autoreactive T cells, but not all of us have autoimmune diseases, in fact, a group of self-reactive T cells leaving the thymus and Foxp3+ take a part in maintaining peripheral tolerance.[41]

At the periphery, activation of naïve T cells requires interaction between the TCR/CD3 complex, and antigen presented at the MHC plus a second signal, co-stimulation.

Co-stimulation is an independent signaling pathway that synergizes with antigen-specific signals to allow lymphocyte activation otherwise they just become anergic.

Examples of co-stimulatory partnerships between T and APCs are CD28/CD80 or CD86; OX40: OX40L; ICOS/ICOSL and CD40L/CD40.

However, specific T cell responses are determined by a balance between not only, co-stimulation but also co-inhibition, examples of inhibitory receptors are CTLA4 and PD1.[42]

Besides the common αβ TCRs (90-95% T cells), also known as conventional T cells, there is also a group of unconventional T cells, γα T cells and NKT cells.[43]

The first to be associated with immunologic memory was the conventional T cells. These cells are characterized by their αβ TCRs plus the co-receptors CD4 or CD8 and their capacity to recognize processed antigenic peptides within MHC groves.

While, $CD8^+$ T cells, are a subpopulation of MHC I restricted cytotoxic T cells responsible for killing cancerous or virally infected cells in a contact-dependent manner through induction of apoptosis. $CD4^+$ T cells, are MHC II-restricted and mostly responsible for modulating both humoral and cellular immunity.

Unconventional T cells, are kind of a hybrid between adaptive and innate immunity, if on the one hand they also express TCRs, on the other hand, the repertoire is much more limited, and the nature and distribution of the recognized molecules are completely different plus that they bind their antigens directly with no need for classical MHC presentation but instead dependent on conformational shape.[43]

Intraepithelial lymphocyte (IEL) compartments, like skin, intestine and genitourinary tracts are enriched with unconventional T cells, NKTs but mostly γδ T cells. The latter, by recognizing the so-called, "stress antigens" (e.g. phospholipids and alkyl amines) helps preventing infected or transformed cells dissemination and contributes for tissue homeostasis.[43] NKTs on the other hand are mainly specialized in responding against certain types of bacteria, fungi and parasites, but recently they have also been associated with autoimmunity and immunosurveillance. NKTs, on the other hand, are mainly specialized in responding against certain types of bacteria, fungi, and parasites, but recently they have also been associated with autoimmunity and immunosurveillance. NKTs, as its name implies, share T cell traces like αβ TCR expression and NK traces like the expression of CD56 and NK1.1 marker. Similar to T cells, they must be presented with the antigen. However, instead of peptides, it will be glycolipids and instead of classical MHC presenting it will be a CD1d molecule.44 Another great advantage of this population is, depending on the environment they can be triggered to rapidly release big amounts of several cytokines like IFN-γ, IL-4, IL-10, IL-13, IL-5, GM-CSF, TNF, IL-21, and IL-17. As with conventional T cells, the authors defend this diversity to be a result of different specialized populations and the need for further studies.[45-49]

1.4.1. CD4+ T cells

As previously referred CD4 T cells, also known as T-helpers, provide help to both adaptive and innate immunity. They do it by secreting specific sets of cytokines accordingly to environmental cues, resulting in recruitment and activation of other cells. In fact, they boost, not only primary but also memory immune responses.[50] Along with the context of an immune response, mature APCs activate CD4+ T cells, resulting in proliferation and differentiation into a variety of specialized subsets.

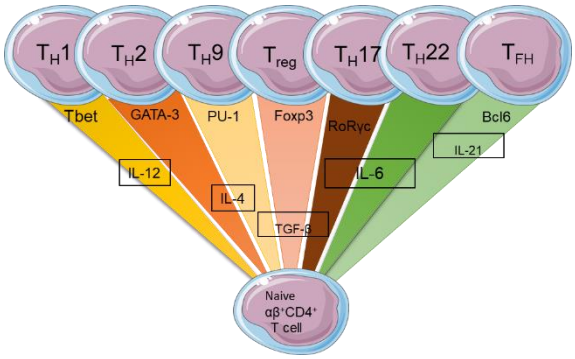


Fig.5 Illustration of the different possible $CD4^+$ T cell differentiation pathways and their associated transcription factors and inducing cytokines. Adapted from Coomes et al 2013.

The concept of differentiation was proposed almost thirty years ago by Mosmann and Coffman to explain how T-helpers could promote completely different immune responses.[51] Based on their cytokine profile they were classified into two terminally differentiated groups, Th1, and Th2 plus a controversial regulatory population, nTregs. Th1 cells are responsible for immune responses like delayed-type hypersensitivity (DTH), cellular immunity, B cell class switching to IgG2a and clearance of intracellular pathogens or transformed cells.[26] In these microenvironments, APC and NK cells are induced to produce cytokines like IL-12 and IFN-γ.[52,53] IL-12 is responsible for activating the transcription factor STAT4 which results in more IFN-γ expression, responsible for upregulating STAT1 and resulting in T-BET expression the master regulator of Th1 cells. T-BET activation,

will then lead to IL-12Rβ upregulation assuring a positive feedback for Th1 differentiation, but a negative one for Th2 and Th17 (later discussed).[54] Once differentiated they produce mostly IFN-γ and IL-2, essential for phagocytic activity and cytotoxic $CD8^+$ T cells' activation. But when its activity is not properly regulated, it results in inflammatory and autoimmune diseases, like colitis or multiple sclerosis.[55,56]

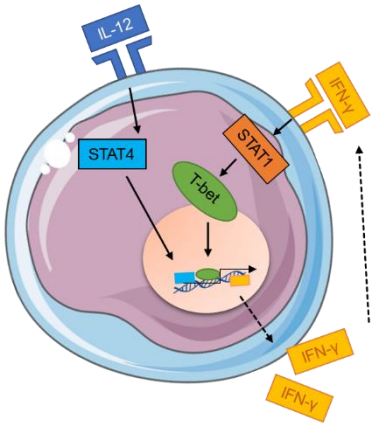


Fig.6 Illustration of $CD4^+$ T-helper 1 polarization.

Th2, on the other hand, are responsible for allergies, humoral immunity, B cell class-switching to IgG1/IgE and clearance of extracellular bacteria or parasites. Th2s are induced when after activation cytokines like IL-4 and IL-2, abundant in these microenvironments bind recently activated T cells.

IL-4 recognition then activates STAT6 resulting in GATA3 upregulation, the master regulator of Th2 cells. Together they activate a set of Th2-related gene loci, responsible for cytokine production, proliferation, and inhibition of Th1 differentiation. Other transcription factors involved are IRF4 and STAT5. While the first activates IL-4 promoter resulting in GATA3 upregulation the second is activated by IL-2 that together with GATA3 promotes IL-4 expression. Again, positive feedback is present. Upon differentiation, they will secrete IL-4, IL-5, and IL-13 which will mostly act upon innate cells, with IL-4 and IL-13 as its key cytokines.[57]

Their main targets are mostly innate cells like macrophages, basophils and mast cells, but the impact also on B cells and non-hematopoietic cells like epithelial cells present in mucosal surfaces.

Seemingly redundant on function, when in physiological conditions IL-4 and 13 have preferential roles. During a helminth infection, IL-4 is essential for IgE production and mast cell activation while IL-13 is essential for goblet cell hyperplasia, mucus production, and parasite expulsion. 58 Even when type 2 response lacks proper regulation and results in allergic diseases like asthma, IL-4, and IL-13 again despite overlapping functions have preferential roles. IL-4 favors IgE and IgG1 production, while IL-13 is essential to goblet cell hyperplasia, smooth muscle contraction, and mucus production.[59]

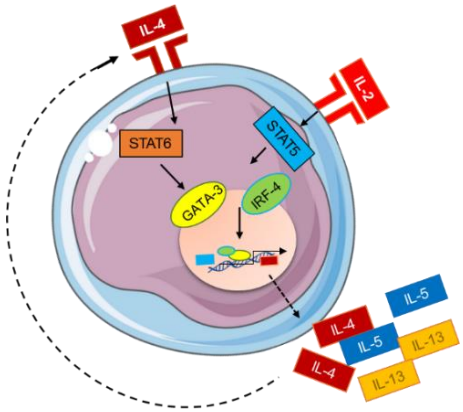


Fig.7 Illustration of $CD4^+$ T-helper 2 polarization.

Besides mutual regulation between Th1 and Th2, in the '60s, right after finding out the importance of thymus for immunity60, a population of T-cells capable of immunosuppression was proposed. However, such population was accepted only in the '90s with the induction of autoimmunity by neonatal thymectomy or transfer of T-cell populations depleted of specific cell types into lymphopenic mice.[61,62]

Named regulatory T cells (Tregs) and identified by the markers $CD5^{high}$, $CD25^+$ and $CD45RB^{low}$ this new population still missed a master regulator. [61,63]

Almost 10 years later, Sakaguchi and his laboratory finally identified Tregs' master regulator, Foxp3, a transcription factor which absence results in scurfy mice and IPEX (immune dysregulation, polyendocrinopathy, enteropathy, x-linked) syndrome, both autoimmune and inflammatory diseases. [64,65,66]

Thymic Tregs (tTregs) are the most well studied regulatory T cells, whose specific epigenetic landscape needed for lineage fate commitment, accordingly to Kitagawa et al 2017, which requires a permissive epigenetic remodeling of Tregs' specific super-enhancers. Satb1, a global genome organizer highly expressed during thymocyte development can bind these super-enhancers even within closed chromatin, activating them and assuring lineage commitment. [67] 67 Full activation of specific super-enhancers will also depend on TCR avidity and co-stimulatory signals provided by thymic epithelial and dendritic cells.[68,69] Such permissive remodelling will allow for Foxp3 expression and fixation of Tregs' specific epigenetic signature genes, that also include CD25, CTLA-4 and GITR.[70]

Immune homeostasis, peripheral tolerance, and regulation of inflammation are mostly dependent on proper T_{reg} function. This function can be either dependent on cell contact or based at cytokine secretion and metabolic disruption.[71]

Suppressive mechanisms include direct action upon T_{eff} cells through induction of apoptosis and cell cycle arrest (granzymes and galectin-1) or indirectly by manipulation of DCs function, either inducing negative signaling for T_{eff} (CTLA-4) or disruption of proper DCs-T cells interactions (neuropilin).[72-75] Also secretion of cytokines like IL-10, TGF-β, and IL-35 as well as disruption of target cell metabolism, by higher consumption of critical cytokines (IL-2) or even increased expression of proteases (CD39 and CD73) that hydrolyze ATP and compromise DCs' maturation will impact on T_{eff} function.[71,76-84]

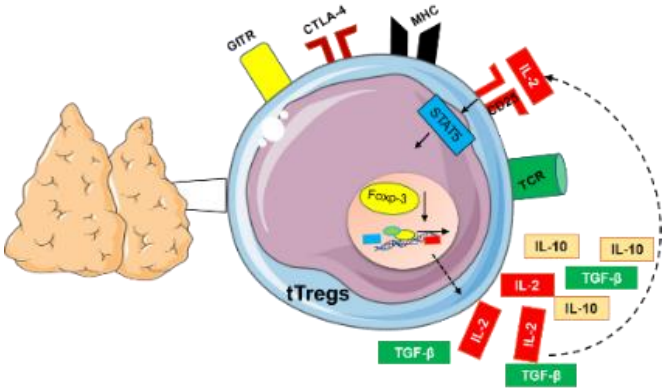


Fig8. Illustration of $CD4^+$ thymic Treg polarization.

Still, these populations were not enough to explain the growing complexity of T-helper phenotypes, which despite shared characteristics with the previous subsets presented new ones. But it took extra 5-10 years, until the characterization of these new populations which include Th17, pT_{regs}, T_{fh}, Th9, and Th22.

Th17 cells appeared in the early 2000s as a new hypothesis for the regulation of tissue damage present in both microbial infections and autoimmunity, no longer explained by the Th1/Th2 Mosmann and Coffman hypothesis.[85-90]

When they appeared, researchers considered IL-23 as the main inducer of Th17, but in 2006 three independent labs, showed that IL-23 was most important for survival and expansion while TGF-β and IL-6 were essential for polarization.[91-94]

Binding of TGF-β to its receptor activates the SMAD signaling pathway which results in both Foxp3 and ROR-γt expression.

At this point depending on microenvironmental cues, these cells can either differentiate into Th17 or a new population discovered around the same time, peripheral induced Tregs (pTregs), which we will discuss later.

So, if the microenvironment is more pro-inflammatory and has higher concentrations of IL-6, STAT3 signaling pathway becomes activated. This results in IL-21 expression initiating an IL-21/STAT3 autocrine loop responsible for a sustained

STAT3 activation and expression of Th17 master regulators, ROR-γt and ROR-α.[93,95]

ROR-γt and ROR-α will then induce IL-17A and IL-17F, which will increase inflammatory mediators, like IL-6, IL-1, IL-21, and CXCL8, as well as the recruitment of innate immune cells like neutrophils, essential for clearance and control of either extracellular bacteria or fungal infections at epithelial and mucosal barriers. ROR-γt and ROR-α also upregulates IL-23R, making Th17 responsive to environmental IL-23, produced mostly by innate cells and essential for their survival and expansion.[91-93,95,96]

However, when IL17 production becomes dysregulated it causes chronic inflammation and increased tissue damage culminating in autoimmune diseases like MS, RA, psoriasis and inflammatory bowel disease (IBD).[97]

MS considered for a long time as a Th1 dependent disease turned out to be also dependent on Th17, in a way still poorly understood. [90,98]

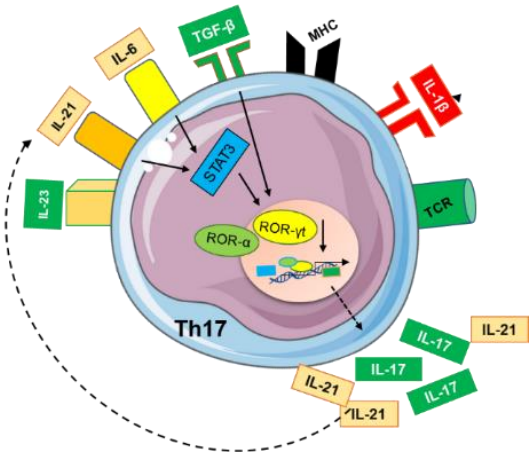


Fig 9. Illustration of $CD4^+$ T-helper 17 polarization.

Coming back to pTregs, this is a regulatory population that contrary to tTregs, can be induced in the periphery from conventional naïve CD4+ T cells if under appropriate stimulus.[99-101]

Allergens, food or even non-pathogenic microorganisms (microbiota), in both inflammatory and non-inflammatory conditions, can trigger pTregs differentiation. The main trigger is the host microbiome, with germ-free mice showing reduced numbers of pTregs.[102-104]

For pTregs differentiation, presentation of lower doses of high-affinity peptides coupled with low co-stimulation (low CD28 signaling) is crucial.[105] Also, presentation by tolerogenic APCs like gut CD103+ DCs capable of synthesizing TGF-β and retinoic acid, both inducers of Foxp3 expression together with a microenvironment rich in IL-2 and other tolerogenic soluble factors, will favor its differentiation. [99,106-108] 108Infectous tolerance mediated by tTregs will similarly contribute to pTregs induction. [109]

pTregs major advantage versus tTregs is their plasticity of Foxp3 expression, reflected in functional adaptability to evolving immune responses. They permit to balance protective immunity with tissue tolerance to help contain excessive damage without compromising pathogen clearance.[110-112]

Miyao et al. work supported a notion of time restrained inhibition sensitive to inflammation intensity or antigen availability, where pTregs can revert to Tconv cells. Less inflammation, less damage out of control, thus no need for more inhibition.[113]

De novo induced pTregs are mostly known for preventing general inflammation and contributing to both fetal and mucosal tolerance (airways and gut), however like tTregs they also contribute to autoimmunity regulation.[114-119] 119 Its main function is to ensure long term tolerance and not so much initiating it.[120,121]

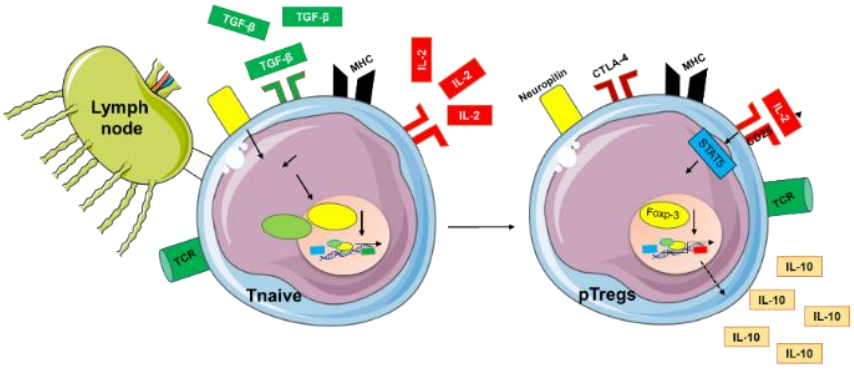


Fig 10. Illustration of $CD4^+$ peripheral induced Tregs.

Since the 60's that scientists have known $CD4^+$ T cells were indispensable for B cell memory and germinal center formation, a place for B cell somatic hypermutation, affinity maturation and class switch recombination (CSR).[122-125]

And twenty years later, during the Mosmann and Coffman Th1/Th2 period, it was believed that Th2 cells were the population assuring this help due to IL-4 and IL-10 secretion.[126,127] But later, mice data showing preferential CSR towards IgG2a isotype prompted us to consider Th1 as well. So, this would suggest an unbiased preference of T cell help, only to be contradicted, another twenty years (1999) later, with the discovery of a $CXCR5^+$ population.[128] Expression of CXCR5 in CD4+ T cells turned out to be like in B cells, the "key" for migration into follicles where its ligand CXCL13 is expressed.[128,129]

In 2000 the term T follicular helper cell (Tfh) arises, when Breitfeld et al. together with Schaerli P. et al show this population superiority in promoting B cells production of immunoglobulins.[130,131]

But it was only in 2009 with the identification of Bcl-6 as an essential factor for Tfh differentiation that these cells truly become accepted as an independent T helper subset.[132-134]

Tfh differentiation is a multi-stage, multi-factorial and highly heterogenic process, that comprises an initial priming phase, with DCs at the T-zone of secondary lymphoid organs presenting the peptides and providing co-stimulation in the form of CD80/CD86 and ICOSL in a balanced environment of IL-2 and IL-6 crucial in cell fate determination.[135,136]

IL-6 will then induce recently activated CD4+ T cells to upregulate Bcl6, T_{fh} master regulator that together with ASCL2 results in CXCR5 early expression and migration into the T-B border.[132,136-139] Here pre-T_{fh} cell will finally interact with antigen-specific B-cells and initiate the final step of commitment.[140]

This recently differentiated T_{fh} cells can now enter the follicles to establish fully operational germinal centers, allowing for somatic hypermutation and selection of high-affinity B cells culminating in memory B cells and plasma cells capable of producing antibodies with even greater affinities.[125]

At later stages, B-cells will then become the major antigen-presenting cells as opposite to DCs essential in priming phases.[141,142]

So their main function is Germinal Center (GC) development and function and when it goes well we have not only control of pathogens but also commensal microbiota.[143]

But when it goes wrong we might end up developing allergies or even autoimmunity, due to aberrant generation of autoantibodies or formation and maintenance of ectopic follicles.[144-147]

Something interesting is their involvement in cancer, which is not so unexpected if we consider all the regulatory checkpoints that may fail and result in disadvantageous mutations. But overall their ability to form and maintain germinal centers as well as promoting antibody affinity selection will be advantageous against cancer (Gu-Trantien et al.), but hazardous in autoimmunity.[148,149]

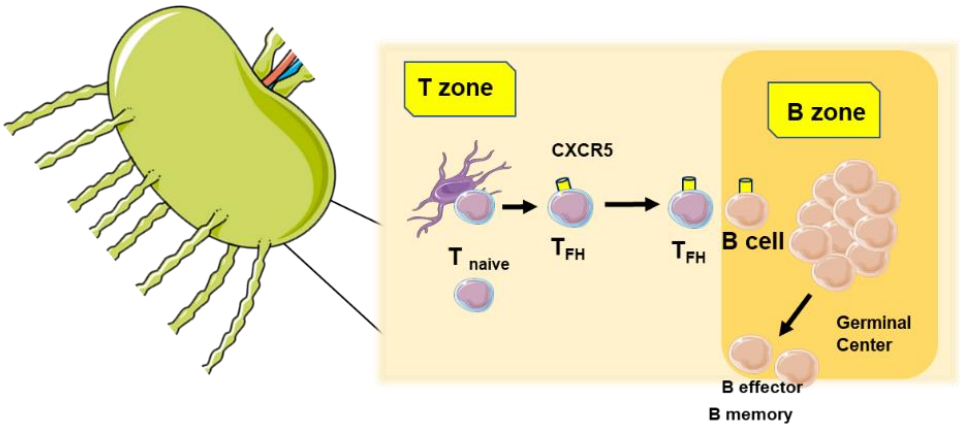


Fig 11. Illustration of T cell priming towards a T_{FH} phenotype with consequent relocation to B cell follicle and germinal center initiation.

For twenty years IL-9 was considered a Th2-derived cytokine, but only until 2008, when Veldhoen et al. and Dardalhon et al discovered a new independent IL-9 producing T cell subset.[150-153]

Named Th9, this is a population that although independent, can also be re-differentiated from Th2 if TGF-β is present and, that shares a variety of functions with the former.[151,154]

Both populations are important for resolving parasitic infections and mediating allergic inflammation.[155] However, Th9 are reported to be also involved in transplant tolerance, tumor immunity and autoimmunity.[154,156-160]

Like any other subset, it needs a proper TCR signal, co-stimulation, and a cytokine cocktail, with a preference for OX40 which selectively enhances IL-9 over IL-4 or IL-5 (NF-kB noncanonical pathway). This, in a microenvironment rich in IL-2, TGF-β, IL-4, and IL-1 culminates with the expression of PU.1 and IRF4. Both transcription factors capable of modifying chromatin at the IL9 locus and directly binding to its promotor.[161-164]

Their close relationship with the Th2 effector program favors a certain degree of plasticity to allow "fine-tuning" of responses accordingly to the need.[165] Such lack of stability or not fully understood flexibility coupled with the absence of a transcription factor are the fuel for the doubts around its identity.

The use of Licona—Limón et al IL-9 fluorescent reporter mice, would ease the doubts around this newbie T helper subset. Even because it would also exclude other sources of IL-9, like mast-cells, eosinophils, ILCs or NKT cells accounting only for T cell production and relevance on the ongoing response.[150] , In fact, EAE is one example of such situation, where other cell types might just be the source of IL-9 which is the case of Th17. Again Licona—Limón et al reporter mice would shed some light on this.

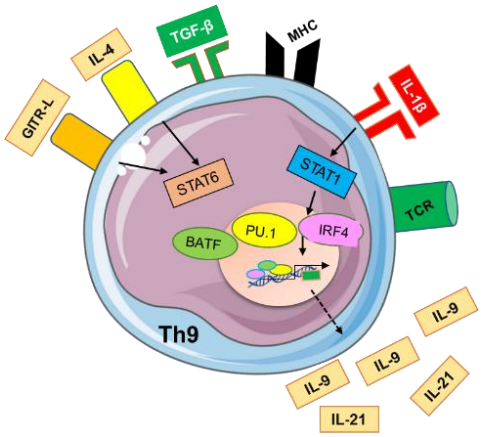


Fig 12. Illustration of CD4^{+} T-helper 9 polarization.

To shake things a little bit more, in 2009 a cytokine first discovered in 2000 which was initially attributed to Th1 cells and latter to Th17 ended up having its own lineage.[166-168] We are referring to IL-22, a cytokine with a very important role in skin homeostasis and inflammation.

Due to its ability to bind epithelial cells, which abundantly express IL-22R, it allows TH22 cell to establish a bridge between adaptive immunity and barrier organs, very important for wound healing and antimicrobial defense. [169-172] Indeed, IL-22 can trigger anti-microbial peptides (S100 proteins and defensins) and synergise with IL-17 and TNF-α to fight pathogens like *Candida albicans.*[168,169,173]

This new lineage, like any other lineage, requires antigen presentation, in this case preferentially by skin DCs', co-stimulation and a microenvironment specifically rich in TNF-β and IL-6.[174,175] With a relatively stable phenotype, these cells still lack an unquestionable master regulator, but like Th9 they also have a candidate, aryl hydrocarbon-receptor (AHR).[176]

Th22 is a population highly abundant in the skin (upper parts of the epidermis) but scarce in circulation, something explained by skin strong expression of chemokines that bind CCR4, CCR6, and CCR10 present in these cells.[168,174,177]

And what could be a good thing, left unregulated could result in autoimmunity like psoriasis (hyperkeratosis) or even potentiation of malignancies due to exacerbated anti-apoptotic effect.[178-180]

Still, many questions remain to be answered, like detailed differentiation and regulation mechanisms, dynamics regarding other IL-22 producers and better readouts of Th22 targeting treatments.

Now more than ever, with all these different T cell lineages, differentiation is perceived as a very complex and interdisciplinary process, dependent on factors such as the cytokine pool, antigen concentration, APC phenotype, and co-stimulatory stimulus.[181]

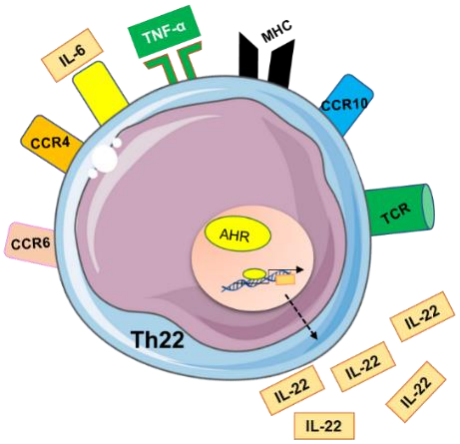


Fig 13. Illustration of CD4⁺ T-helper 22 polarization.

But if it was not enough, van Panhuys introduced an extra variable with his temporal signaling model were long term interactions producing high strength TCR signals favor Th1 and short-term interactions (weak TCR signal) with strong co-stimulation favor Th2.[182] Also induction of Th17 and Tfh are influenced by TCR strength, but while strong signals favor Tfh, for Th17 there are divergent data and for Th9 or Th22 is still unknown. [183-186]

Another aspect that has been gaining relevance is the plasticity of the CD4+ T cell population contradicting the previously accepted notion of terminal differentiation.

Until not so many years ago, each lineage characterized by the expression of a master regulator and a set of signature cytokine(s), was believed to be fully and irreversibly differentiated. Something apparently contradicted by the most recent lineages like Th9 that can be differentiated from Th2 and the well-known dichotomies of Th17/Th1 and Th17/Treg. Lineage tracing systems and phenotypic analyses coupled with TCR sequencing helped to support this concept both in mice and humans, which may suggest an evolutionary advantage to overcome the unstoppable decline of naïve T cells reservoir over time .[187-191] And such plasticity, increases adaptability upon re-challenge, propping the host fitter to face old threats in new scenarios.[189,192] The existence of such ability, of reading environmental cues and act upon that information, is the key behind T-cell plasticity.

Extracellular cues like TCR and co-stimulation strength, responsible for starting polarization, will be decoded in the form of cytosolic signalling cascades, like the PI3K-AKT-mTOR axis and later imprinted in the nucleus establishing new gene expression profiles. Depending on how strong those external cues are and how well established are those gene expression programs, T cells will either resist or remodel their functions to better fit the needs.[193,194] With a higher resistance supported for example by selective expression of cytokine receptors and SOCS proteins and higher plasticity supported by factors like a more lose methylation pattern.[195]

Metabolic programs also impact on T cell resistance or adoption of new functionalities with glicose favoring inflammatory phenotypes and fatty acids regulatory phenotypes.[196-201]

For example, PTEN deficient Tregs end up losing Foxp3 and effector cytokines expression as a result of a favored glycolytic metabolism, which normally is inhibited by PTEN.[198,199,202]

Indeed, deciphering how all these extracellular inputs drive plasticity is challenging, and the answer to clarify this complexity might just be in mathematical modeling.

Once we understand this dynamic, we will be able to implement new therapeutic strategies to manipulate immune responses accordingly to our needs. Cancer and autoimmune diseases are authentic niches of reprogramming and we might just be

able of turning the tide to our favor.[203] Upregulation of IL-10 by self-reactive Th1s under chronic stimulation (e.g. EAE model) or high antigen dosage is an example of plasticity and a way of manipulating the system.[204,205]

1.5. Autoimmunity

Over the last 30 years, western societies have shown a concerning increase of autoimmunity diseases (AD) like inflammatory bowel diseases (IBD), rheumatoid arthritis (RA), psoriasis or even multiple sclerosis (MS). Currently between 5-8% of the worldwide population suffers from at least one autoimmune disease with females being the most affected.[206]

Autoimmune diseases are a group of disorders where our immune system becomes dysregulated and begins to attack the body own tissues unable to sustain an effective tolerance. Theoretically either environmental or genetic factors could be triggers for such pathologies, however, the geo-epidemiological distribution, socioeconomic status, and impact on migrant populations (developed countries being the most affected) suggest a stronger environmental influence. Factors like better health conditions (old friend hypothesis) and increased consumption of industrial food additives (increased intestinal permeability) seem to be the major causes of this increasing inability to sustain a proper tolerance status.[206-209]

Accordingly, to the "old friend" hypothesis, the inability to induce tolerance is a consequence of insufficient exposure to probiotics and "friendly helminths", important for Treg polarization and establishment of bystander supression.[209-211] Which coupled with a disrupted intestinal barrier by food additives and a consequent leakage of immunogenic antigens will culminate in the activation of the autoimmune cascade.[208] Which involves the activity of Th1 and Th17 cells and more recently a newly identified member of the Th22 cells.

Although this sudden increase is better explained by environmental factors, people with a genetic predisposition to develop autoimmunity, like single nucleotide polymorphisms and specific mutations will be more susceptible.

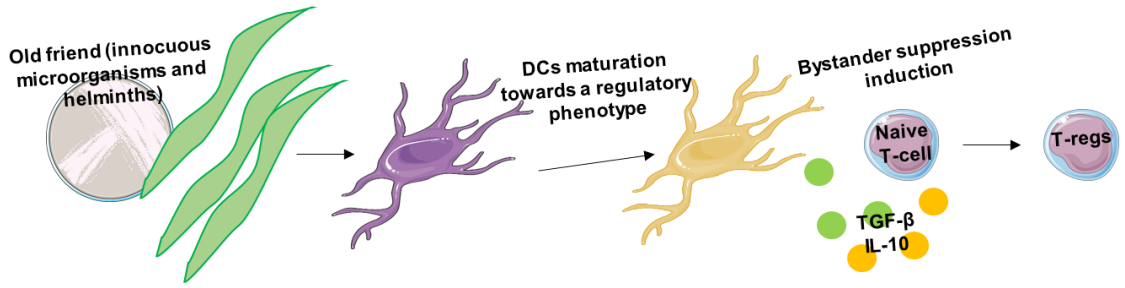


Fig 14. Bystander suppression induced by non-pathogenic microorganisms and helminths.

1.5.1. MS

Accordingly, to the Multiple Sclerosis International Federation 2013 atlas, 2.3 million people suffer from MS (mostly woman) a number expected to increase.[212-214]

MS is a chronic autoimmune and inflammatory disease that affects the central nervous system by destroying oligodendrocytes with consequent neuron demyelination and disfunction (impaired motor movement and paralysis followed by periods of remission with partial or total recovery). Its principal and most well studied immune mediators are Th1 and Th17 cells with a smaller involvement of B cells and autoantibodies production. However, recently a new population has also been reported to be involved, Th22, something yet to be clarified. [215,216]

Still lacking a definitive cause, it mostly integrates a combination of genetic susceptibility, infections, nutrition and environmental factors that in the end are enough to trigger a self-sustained autoimmune disorder associated with recurrent immune attacks on the central nervous system (CNS).[217]

So far, there is not a single diagnostic test for MS and the clinicians still require the disease to manifest in order to have a diagnose based on a combinatorial phenotype.[213]

This is becoming a big socio-economic problem, due to negative effects on health-social- and work-related issues. Disease-modifying agents (e.g. methotrexate) are being used so that acute exacerbations and episode frequencies can be diminished

and symptoms relieved.[217] However, some patients continue refractory and others eventually become non-responders, combination with mAb therapies like Natalizumab seems to be an alternative.[218-220] An alternative many times with a high price like debilitating side effects and no cure.

To better understand MS induction and pathogenesis so that new therapeutic strategies can be developed, we must use animal models and the most used/studied is the experimental autoimmune encephalomyelitis-EAE model. Without forgetting that no single model can recapitulate all aspects of MS, the fact is, all the current US Food and Drug Administration (FDA)-approved MS immunomodulatory drugs are at some extent effective in EAE and some were actually first tested on it.[221,222]

The advantages of using such model concerns mostly with the inbred genotype of laboratory mice, rapid breeding capacity, easy genetic manipulation and the existence of transgenic or knockout mice.[221] And although other models like marmoset better resemble the human immune system, factors like high costs and ethical constraints make improbable to use this non-human primate daily for compound screening.[223]

1.6. Monoclonal antibodies

Facing a new era of personalized medicine, monoclonal antibodies are at the very heart of it. Almost 40 years ago in 1986, muromonab-CD3 a mAb to prevent kidney transplant rejection had its license approved for clinical purposes.[224]
And although it was not exactly a straight forward process until our days, recently it represents a $20 billion industry with approximately 30 monoclonal antibodies approved for human therapeutics and many others under clinical evaluation.[225]

Due to a high degree of specificity and flexibility, they pose as the ideal candidates to develop new therapies against conditions like transplantation, cardiovascular diseases, infectious diseases, cancer and to remember autoimmune diseases like MS.

The major challenges mAb had to face in the early days were, inefficient models for generation and immunogenicity, but characteristics such as affinity, effector functions, and pharmacokinetics are still a concern. However, with today's technology, we can overcome many of those concerns. Using humanized antibodies or whenever possible fragments instead of all protein are two ways of minimizing immunogenicity.[226,227] Also affinity can be improved with the use of phage libraries, but high affinities are not always in our best interest, for example, to penetrate tissues lower affinities are a better alternative.[228]

As a continuously growing arm of biologicals, improved effector functions and more advantageous pharmacokinetics are essential. Thus, strategies like improved affinities for the FcγRIIIa receptor, which enhances ADCC or improved FcRn affinity which increases bioavailability in the plasma will be of great interest.[229-233]
Also, overall cost-effectiveness which was a major drawback in the past does not seem to scare big pharmaceutical companies that continue to invest in the development of mAb for both clinics and research. In fact, the main focus is on identifying new targets and maximize efficacy by adding beneficial modifications.[218]

In MS, the first mAb to get approved was Natalizumab in 2004, only to be followed by Alemtuzumab in 2013 and more recently this year by Ocrelizumab.[234,235] However, both of them are very potent drugs and severe side effects are always associated. Natalizumab, for example, was out of circulation from 2005 until 2006 due to progressive multifocal leukoencephalopathy in patients with MS and Crohn's disease. Also, Alemtuzumab had to be withdrawn for some time, but despite the associated risks for autoimmunity the benefits were bigger and EMA (European medicines agency) recommended its return to the market.[236]

1.6.1. CD6

In an era of technologies, we should be able to predict how likely a person is of developing a certain disease and if possible, act to prevent it. Over the last 15 years, genome-wide association studies (GWAS) become that tool with their ability to screen DNA sequence variations across all genome. By identifying patterns of single nucleotide polymorphisms (SNP), the units of genetic variation, these studies helped to identify genetic risk factors for common and complex diseases like MS.[237]

In 2011 after a meta-analysis of GWAS for MS, Koffler et al. identified a new susceptibility locus tagged by a SNP, rs17824933 ($p = 3.8 \times 10{-}9$), found in a block of linkage disequilibrium containing the CD6 gene. Meaning people with the genetic variant rs17824933CC were more likely to develop MS than people with the rs17824933GG allele.[238] This data came to reinforce Jager et al conclusions of 2009 were they also identified CD6 (rs17824933 SNP) out of three new MS susceptibility loci.[239] This and the fact that endothelial cells' transmigratory cups used by lymphocytes to cross the blood-brain barrier (BBB) in the CNS show co-localization of CD6 (lymphocytes) with ALCAM (endothelial cells), transforms CD6 in the perfect target for MS treatment strategies. [215,240-245]

CD6 is a transmembrane glycoprotein (105/130 kDa) mainly expressed in mature T cells but we can also find it, at a lower extent, in thymocytes, B1a cells, CD56+ NK cells, certain regions of the brain and malignant cells (ALL T cells and B-type CLL).[246-249] 249 Its structure as shown in fig.15 is composed of three extracellular

domains,1,2 and 3 and one long cytoplasmic tail (244 amino acids), that contrary to expectations does not have any catalytic activity. [246-249]

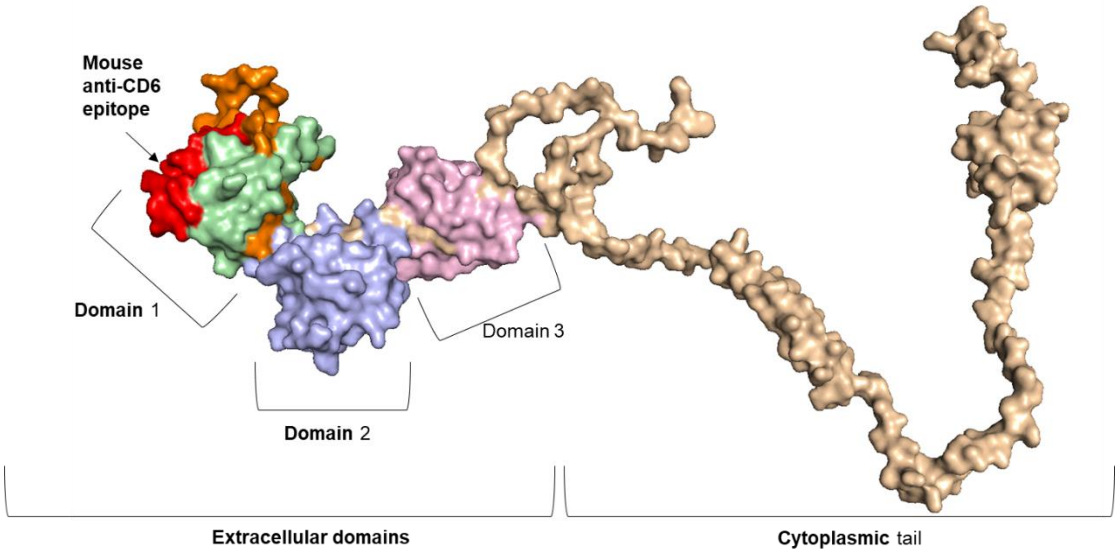


Fig.15- Mouse CD6 3D structural simulation. Mouse CD6 structural simulation based on human CD6 surface representation as published by Chappel et al 2015. In red we represent our mAb binding site, in orange and green the mutation sites necessary to "murinize" Itolizumab.

As a member of the scavenger receptor cysteine-rich superfamily (SRCR), a family known by its role in immune modulation, the same would be expected from CD6. The truth is, despite a non-catalytic cytoplasmic tail, its tail does participate in signaling transduction since it contains a Tyr-Asp-Asp-Ile motif, two proline-rich motifs, three serine/threonine rich motifs (hyperphosphorylated upon activation), three PKC and ten CK2 phosphorylation site motifs. [241,250,251] All of them potential targets for phosphorylation and/or interaction with signal-transducing effectors. In fact, upon activation serine and tyrosine residues do get phosphorylated or even hyperphosphorylated. [241,250,252-254] But, posttranslational modifications are not the only ones, post-transcriptional modifications like downmodulation of SRSF1, a transcriptional factor that binds exon 4, also occurs. This will increase exon 5

alternative splicing and consequent expression of a truncated form of CD6 that no longer bears domain 3, as seen in fig.15. [241,250,251]

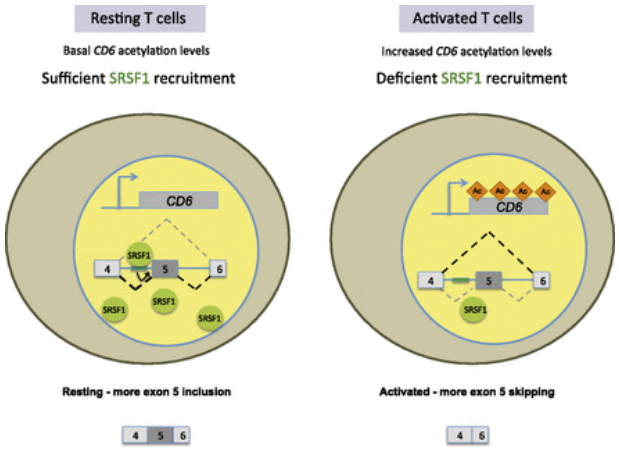


Fig.16- Model proposed by Glória V. et al 2016 illustrating *CD6* exon 5 alternative splicing regulation upon T cell activation.

So far, two ligands of CD6 (if we exclude the galectin1 and 3 to be discussed later) have been described: CD318, that binds CD6 domain 1 (d1) being expressed mostly on synovial tissues by epithelial cells but also in some tumors; and the activated leukocyte cell adhesion molecule (ALCAM) or CD166, that binds CD6 d3 and is expressed mainly by monocyte-derived cells and endothelial cells. [255-258] From the two ligands, ALCAM is the best characterized, being established that its ligation to CD6 allows for stable T cell-antigen presenting cell (APC) interactions essential for maturation of immunological synapse (IS) and consequent optimal T cell proliferation. [259-263]

ALCAM-CD6 interaction seems to have some sort of evolutive advantage since it has been so well conserved across evolution. With species like humans, mice, rat, and chicken sharing a high degree of conservation in the residues (nine by the way) used between ALCAM and CD6 to bind each other, the structural display (binding faces) and the intron/exon organization of the CD6 gene. [240,264-267]

Back in 2015, CD6 extracellular domains' crystal structure had finally been resolved by Marion Brown's team and contrary to expectations it revealed a nonlinear organization suggesting the idea of a biological relevant structure. They also showed how SNPs in the CD6 loci resulted in altered glycosylation patterns or truncated forms lacking domain 3 (risk allele rs17824933CC) both conditions compromising CD6/ALCAM interactions. [238,265,268] In fact, both human and murine T cells do express multiple CD6 isoforms, 3 extracellular and 5 intracellular. [268] Intracellular isoforms, which refer to the cytoplasmic domain, will mostly impact over signal transduction. For example, while in some cases CD6 tyrosine's are constitutively phosphorylated in others they are resistant, it even impacts over $Ca2^{+}$ influx were some isoforms do not require TCR activation for it to happen.[242]

Despite 30 years of research, CD6 was never a very straightforward study subject and it has always raised many controversies around its co-stimulatory or inhibitory role in T cell function.. [246]

Such disagreement might just be understandable by the fact that just like LAT, CD6 also acts as a signaling hub were a series of adaptor proteins like SLP76, Gads or TSAd can bind and initiate a variety of signaling cascades either negative or positive. Also its huge and highly phosphorylatable cytoplasmic tail, were many of the same kinases that bind the TCR/CD3 complex can bind, (lck fyn zap70 and itk) might either potentiate the signalling or act as decoy as suggested by Santos et al 2016.[254,269-272] Which would explain why in some cases targeting CD6 increases T cell activation and proliferation, especially under sub-optimal conditions, and in others reduce it.[259,262,269,273-276]

But controversies aside CD6 does participate in T cell activation, proliferation, and migration across the blood-brain barrier, supporting its relevance in MS treatment strategies.[259,261,262]

In fact, in the '80s and anti-CD6 mAb (anti-T12) was used for the first time against MS, but at the time, problems like immunogenicity (mouse IgM) and allergies end up putting it aside.[277-279]. Still, around the same time in Cuba another anti-CD6 mAb, ior T1 was also developed, but this one was tested in psoriasis and RA instead and although therapeutic effects were observed, it also presented the same problems.

The solution was the humanization of ior T1, and so Itolizumab was created, an improved version keeping the same CD6 recognition profile and similar affinity constant, but much less immunogenic.[280,281] In 2013 Itolizumab (IgG1) had its first clinical use approval in India for the treatment of moderate-to-severe chronic plaque psoriasis and just recently Cuba finished a phase I clinical trial in safety and clinical response for 12 weeks treatment in active RA despite previous disease-modifying therapy.[282] Itolizumab promising results in both psoriasis and RA, plus a better safety profile compared to other anti-CD6 mAbs raises the possibility of using it to treat MS.

.

1.7. Aims of the thesis

Despite controversies regarding CD6 mode of action, its involvement in T-cell activation, proliferation and migration across the blood-brain barrier is something more than accepted. [259,261,262] Thus our hypothesis was: Targeting CD6 on domain 1 prevents neurodegeneration.

As main objectives we wanted to:

- Understand the impact of CD6 targeting in the natural course of EAE (a mouse model of multiple sclerosis) but without compromising the interaction CD6-ALCAM, using for that purpose an anti-mouse mAb targeting CD6 domain 1;
- Identify the cellular and molecular mechanism underlying CD6-targeting in both murine and human cells;
- Demonstrate the equivalence between mice and human anti-CD6 mAbs

As already referred, the association of several CD6 SNPs with a higher susceptibility of MS development, plus promising results obtained with anti-human CD6d1 mAb

(Itolizumab) on other autoimmune diseases prompted CD6 targeting as the next logical strategy for the treatment of MS. Because our collaborators, who developed the anti-human CD6 also developed an equivalent murine version, we had the tools to test in-vivo how CD6 targeting, without compromising the binding CD6-ALCAM, would influence the natural course of a mouse model of MS. For that purpose, we used the experimental autoimmune encephalomyelitis (EAE) model, a model well established in our lab.

We also addressed how targeting of CD6 domain 1 would impact on T cell acquisition of effector function, survival, and proliferation. This would provide data if CD6 targeting was having a type of specific response or just affecting all T cell populations the same way. To do that we induced in-vitro Th1, Th2, Th17, and Treg cells and studied how CD6 targeting conditioned their ability to differentiate even if the environment was favorable.

To demonstrate that the anti-mouse CD6 was mimicking the anti-human CD6, we reproduced the mice in-vitro experiments with human cells to validate the relevance of the mouse reagents and in-vivo data.

Surprisingly, our results ended up uncovering an aspect of therapeutic antibodies that sometimes goes unnoticed, that different doses may present paradoxical effects. Indeed, our antibodies targeting both murine and human CD6, depending on the dose they either favor regulation, when used at lower doses, or when used at higher doses they favor Th1 induction and inhibit pTreg induction, which might result in a more inflammatory scenario. Thus, depending on the dosage, the same drug can be used in different therapeutic scenarios.

Autoimmunity

2. Itolizumab murine equivalente clone 10F12 in a model of EAE

2.1. Background

Itolizumab, is an IgG1 humanized recombinant anti-CD6 monoclonal antibody, which specifically binds the CD6 membrane distal domain 1. As shown by Alonso and collaborators this interaction will not compromise the ability of CD6 to still bind its most well studied ligand, ALCAM which binds the membrane proximal domain 3 instead. [280]

Despite limitations such as sample size, open label design, absence of a control arm or a possible impact of populational genetic background, recent clinical trials on psoriasis and rheumatoid arthritis have shown Itolizumab as a promising alternative compared to other commonly used biologicals. Characteristics like better safety profile, slightly less but still considerable efficacy coupled with smaller cost make it more attractive for “first in class” strategies than commonly used biologicals like anti-TNFα blockers. Itolizumab is commercialized in India since 2013, under the name ALZUMab,™ as a “first in class” biologic for the treatment of patients with active moderate to severe chronic plaque psoriasis.[285] Also recent reports suggesting an association between anti-TNFα blockers (infliximab, adalimumab or etanercept) and increased risk of developing central and peripheral nervous system (CNS) demyelinating disorders, supports the potential of Itolizumab as an alternative to be considered.[284,286]

Itolizumab has also been studied in the context of other autoimmune diseases, like RA, that show a higher incidence of both CD6 ligands, ALCAM and CD318, was demonstrated in the synovia of patients.[257,287] As well as in Sjogren’s syndrome and

MS, with salivary gland epithelial cells and BBB endothelium, respectively, displaying increased expression of ALCAM.

Nonetheless, and regardless of clinical trials outcomes or case report studies, the immunomodulatory effect of Itolizumab remains unclear.

Recently Yan Li, showed that DBA-1 CD6-deficient mice were resistant to EAE induction, a contradictory result if compared to other disease models like collagen-induced arthritis (CIA) where CD6 deficiency resulted in higher disease severity, but more in accordance to a model of imiquimod-induced psoriasis. [276,290,291] Such different outcomes most certainly are influenced by factors like different genetic backgrounds or even particularities intrinsic to each model.

The transition to animal models became crucial to uncover the mechanisms underlying CD6 targeting, since diseases like MS which are multifactorial need to be dissected in a whole organism context. Taking into consideration the complex myriad of functions exerted by CD6, we intended to pinpoint our approach, rather than going for complete receptor abrogation. So by directly targeting CD6 domain 1 we expected to avoid interference with other relevant biological functions. [290]

For that we used a murine mAb developed by our collaborators in Cuba trough immunization of rats with the recombinant extracellular regions of CD6. From the three selected antibodies two of them presented the highest affinity, thus we decided to use the clone 10F12 as the murine equivalent of Itolizumab.

2.2. Material and methods

Animals and in vivo experiments

C57BL/6 and OVA-specific TCR-transgenic mice (OT-II *Rag2*$^{-/-}$) were bred and maintained under specific pathogen-free conditions. Sex-matched mice, between 8 and 10 weeks of age, were used in the experiments. All experimental protocols were approved by the Local Ethics Committee and are in compliance with European Union guidelines. EAE was induced in C57Bl/6 mice by s.c. immunization with 125 µg MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK) (AnaSpec, Inc.) emulsified in complete Freund's Adjuvant (CFA) suspension (4 mg/ml mycobacteria in IFA), and

i.v. injection of 200 ng pertussis toxin (List Biological Laboratories) on days 0 and 2 following immunization. Disease severity was scored based on the following scale: 0.5-tip of tail is limp; 1-Limp tail; 1.5- hind leg inhibition; 2- weakness of hind legs; 2.5- dragging of hind legs; 3- complete paralysis of hind legs; 3.5- flattening of hind quarters with complete paralysis; 4- complete hind leg and partial front leg paralysis (minimal movement and feeding); 4.5- complete leg paralysis (absence of movement), euthanasia is recommended; 5- Death.

Histopathology

Mice were deeply anesthetized for transcardiac perfusion with PBS, followed with 4% paraformaldehyde. After perfusion, head and spinal cord were further immersed into neutral buffered formalin for 48 h. Brain and spinal cord were then removed from the bone, trimmed and routinely processed for paraffin embedding. Sections with 4 µm were stained with hematoxylin-eosin and Luxol fast blue, and screened by a pathologist blinded to experimental groups, in a Leica DM2500 microscope coupled to a Leica MC170 HD microscope camera. Semi-quantification of inflammation and demyelination were performed using a 5-tier system with 0–4 grading scale: 0, absent; 1, minimal; 2, mild; 3, moderate; 4, marked.

mAbs and flow cytometry

Anti-mouse CD6 d1 (10F12) as well as isotype control (rat IgG) were produced at the Centro de Inmunología Molecular (Havana, Cuba). Anti-IL4 (11B11), and anti-IFNγ mAbs were produced at IMM (Lisbon, Portugal) using Integra CL1000 flasks (IBS Integra Biosciences, Chur, Switzerland), purified by 50% (w/v) ammonium sulfate precipitation, dialyzed against PBS, and purity was checked by native and SDS gel electrophoresis. Murine single cell suspensions were stained with CD4-PE (GK1.5, eBioscience); CD4 APC-eFluor® 780 (RM4-5 eBioscience); TCRβ APC-eFluor® 780 (H57-597 eBioscience); CD25 PE-Cy7 (PC61.5 eBioscience); IFN-γ FITC (XMG1.2 eBioscience); IL-13 PE (eBio13A eBioscience); IL-17 PE (ebio17B7 eBioscience); Foxp3 APC (FJK-16s eBioscience), anti-rat IgG Biotin (eBioscience) and Streptavidin PE (eBioscience), CD6 PE (BX222 Biolegend). Cell viability was

detected with Live/Dead Fixable Aqua Dead Cell Stain Kit (Life Technologies) and Annexin V Apoptosis Detection Kit (eBioscience). CellTrace™ Violet Cell Proliferation Kit was used for cell proliferation assessment accordingly to manufacturer protocol (Thermofisher Scientific #C34557). In some studies, cytokine production was assessed following 4 h stimulation with 50 ng/ml PMA, 500 ng/ml ionomycin, 10 µg/ml brefeldin (all three from Sigma Aldritch) and 0.66µl/ml golgistop™ (BD Biosciences). Cells were permeabilized with eBioscience kit (# A25866A).

Recombinant mouse extracellular CD6 protein

Murine soluble CD6 (Gly17-Thr398) was provided by INVIGATE GmbH, Jena, Germany (www.invigate.com). The recombinant protein is derived from HEK 293 cells and comprises C-terminally fused HA-Tag (YPYDVPDYA), BirA-Tag (GLNDIFEAQKIEWH) and His-Tag (HHHHHH).

T-cell activation and polarization (murine cells)

OVA-specific $CD4^+$ T cells were magnetically sorted with CD4 (L3T4) microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) from OTII-$Rag2^{-/-}$ mice, with a purity greater than 90%. T cells were cultured for 4 days and activated with bone marrow-derived dendritic cells (BMDCs) (at a 2:1 ratio) [292] and 10µM $OVA_{323-339}$ peptide (Eurogentec) or with 3 µg/ml plate bound anti-CD3 and 2 µg/ml soluble anti-CD28 (OKT3 and 37.51, eBioscience). For Th1 polarization the medium was supplemented with 5 ng/ml IL-2 and 10 ng/ml IL-12 (both from Peprotech), and 0.5 mg/ml anti-IL-4 (11B11). For Treg polarization, we added 5 ng/ml IL-2 and 5 ng/m TGF-β (R&D). For Th17 polarization the medium included 10 ng/ml IL-1β and 20 ng/ml IL-6 (both from Peprotech), 1 ng/m TGF-β (R&D), and 0.5 mg/ml anti-IFNγ (R46A2). Finally, for Th2 polarization we added 5 ng/ml IL-2, 10 ng/ml IL-4 (Peprotech), and 0.5 mg/ml anti-IFNγ.

Statistical analysis

Statistical significance was calculated using nonparametric Mann-Whitney *U* test, and Kruskal-Wallis one-way analysis of variance, *p* values of <0.05 were considered statistically significant (**p*<0.05, ***p*<0.01, ****p*<0.001). Results are presented as mean ± SEM.

2.3. Results:

2.3.1.1. Targeting CD6 d1 leads to anti-inflammatory effects exclusively at low doses.

In 2011, a GWAS meta-analysis identified the CD6 locus, as a new susceptibility locus for MS (*rs17824933*^{CC} -risk allele).[238,239] This finding, together with new data showing CD6-deficient mice to resist EAE induction [290], prompted us to investigate whether antibodies targeting CD6 d1 could prevent neuroinflammation. To address this, we used an established model of EAE, which consisted in a subcutaneous immunization with MOG-CFA into C57Bl/6 mice followed by pertussis toxin administration **(Figure 1A)**. Our lab has previously shown that it is possible to prevent EAE by using non-depleting antibodies against CD4, through favouring peripheral Tregs induction. [293] Because of that we used the YTS177 (a non-depleting pro-tolerogenic anti-CD4) as our positive control (**Figure 1B**). In our experimental setting we decided to titrate our antibody and evaluate the impact of different dosages in the natural course of EAE. We observed that mice were only partially protected from a severe form of EAE if treated with the lowest dose of anti-CD6 d1 mAb (**Figure 1C**). While on the other hand, the high doses of anti-CD6 d1 mAb did not show any beneficial effect, as seen in **fig.1C**

Although low-dose of anti-CD6 reduced EAE severity it was not sufficient to completely annul inflammatory changes observed by histopathology, or to completely abolish infiltration of the CNS with lymphocytes (Fig. 1D).

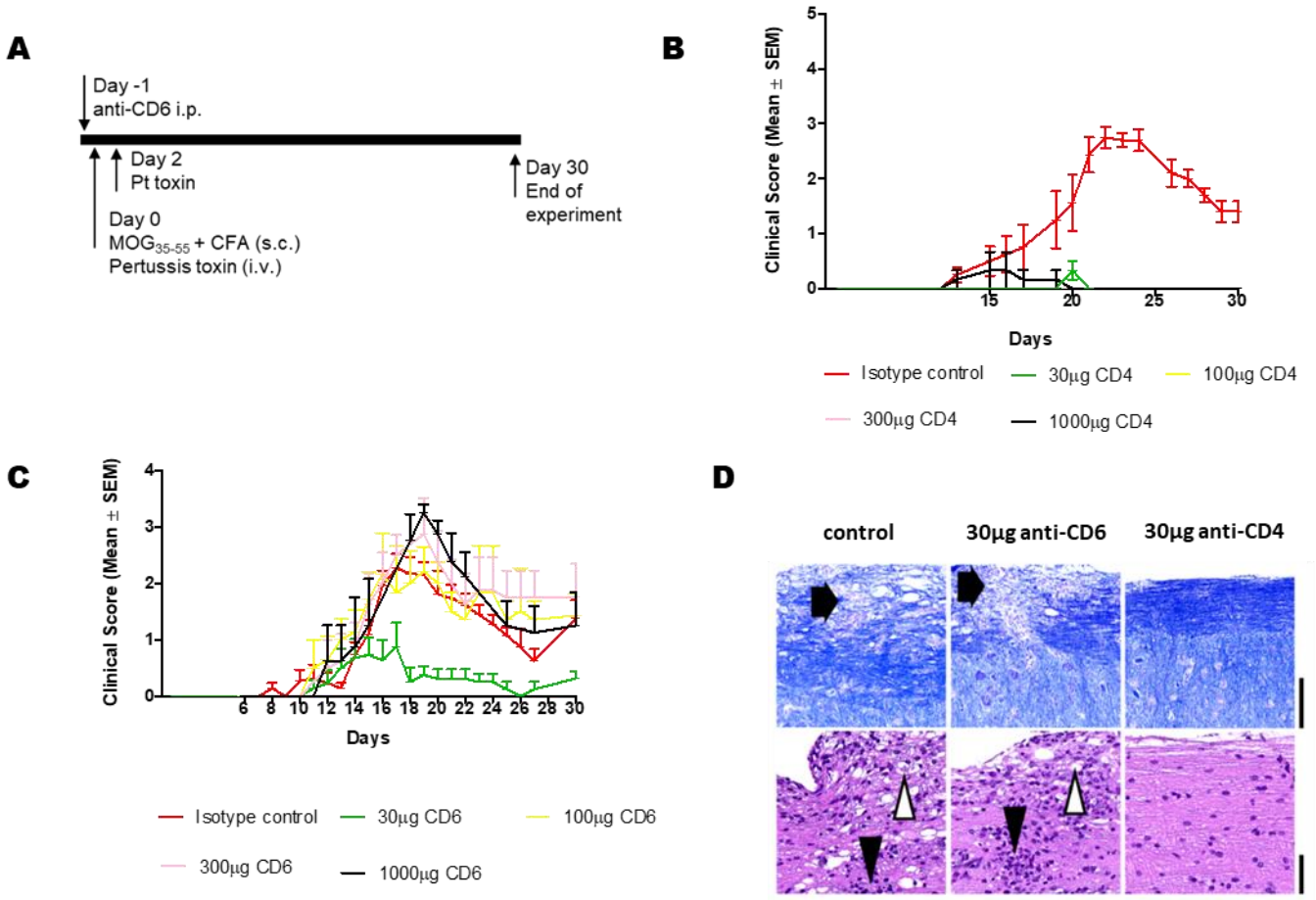


Figure 1- A low dose of anti-CD6 Mab (10F12) prevents the onset of EAE. (A) C57BL/6 mice were immunized with MOG and treated with different doses of anti-CD6, or an isotype control at day 0. **(B)** Clinical score of mice treated with different doses of non-depleting anti-CD4 (YTS177), on the day before MOG35–55 immunization. All mice treated with anti-CD4 were protected from EAE (n=5 per group). (c) Clinical score for each concentration of anti-CD6 and control group of mice immunized with MOG35–55 peptide are shown as mean values ± SEM, pooled data from three independent experiments. Mice treated with 30µg anti-CD6 (n=11) were protected from EAE. However, mice treated with 100µg anti-CD6 (n=8), or greater doses (n=4 per group), developed EAE with disease severity and incidence similar to the control group (n=15). (d) Longitudinal sections of spinal cord from mice 22days after MOG35–55 immunization. In the Luxol fast blue stained section (upper panel), mice treated with anti-CD6 but not anti-CD4 show demyelination of the peripheral spinal cord white matter (black arrow), similar to control mice (original magnification 10×, bar 250µm). In the hematoxylin-eosin-stained section (lower panel), mice treated with anti-CD6 but not anti-CD4

also show an intense mononuclear inflammatory infiltration of the peripheral white matter, with macrophage-rich areas that include numerous myelin-containing phagocytes (white arrowhead), and with fewer lymphocytes (black arrowhead), similar to control (original magnification 40×, bar 50μ).

2.3.1. Anti-mouse CD6 d1 mAb (clone 10F12) modulates T cell differentiation by favouring Th1, in detriment of T-regulatory cells.

Previous data from our lab showed protection from EAE in presence of CD4-blockade, due to expansion of peripheral Tregs, along with inhibition of effector T cells (Th1 and Th17). As a consequence, we decided to investigate the impact of CD6 d1 targeting on T cell polarization.[293]

As a source of antigen specific naïve T-cells we sorted $CD4^+$ OVA TCR-transgenic cells from OT-II.$Rag^{-/-}$ mice and demonstrated that more than 95% of $CD4^+$ T cells constitutively bear CD6 on its surface, similarly to $CD8^+$ T cells (Figure 2).

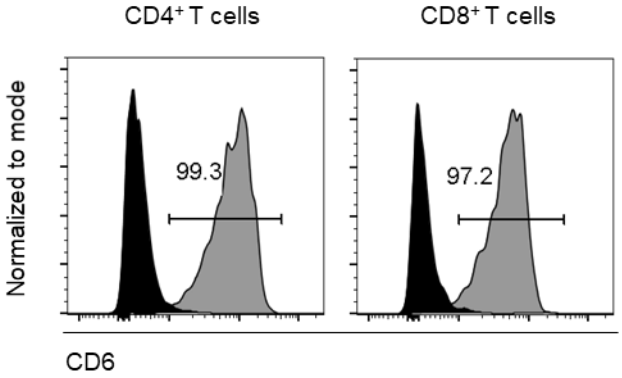


Figure 2-*Virtually all murine $CD4^+$ and* $CD8^+$ *T cells express CD6.* C57Bl/6 splenocytes were stained for CD6. Histograms represent the percentage of $CD4^+$ (left) and $CD8^+$ (right) cells that express CD6. Gated on $CD3^+$ lymphocytes.

To activate the sorted naïve OVA-specific $CD4^+$ T-cells, we used BMDCs loaded with OVA peptide, in presence of the appropriate cytokine milieu to drive the intended functional polarization, namely Tregs, Th1, Th2 or Th17.

What we observed was a dose-dependent suppression of Tregs polarization, as shown in **fig. 3A and B,** as evaluated by a decrease in Foxp3 expression. Both cell viability and proliferative ability remained unaffected as presented in **fig.3C and D,**

as no change in cell numbers of the culture was observed (**Figure 3D**). Interestingly, a trend showing less cells undergoing increased rounds of cell division was observed, thus resulting in an accumulation of cells at intermediate divisions whenever high doses of anti-CD6 d1 were being used (Figure 3E).

Also, we observed that under Th1 polarizing conditions, naïve T-cells in presence of high doses of anti-CD6 d1 displayed an increased polarization of a Th1 phenotype as shown in **fig. 3F and G,** as evaluated by IFNγ expression. Again, no significant impact on survival or proliferation was observed (**Figure 3H-J**).

Regarding polarization towards a Th17 or Th2 phenotype, no differences were observed, as judged by the expression of IL-17 and Il-13, respectively (**Figure 3K**).

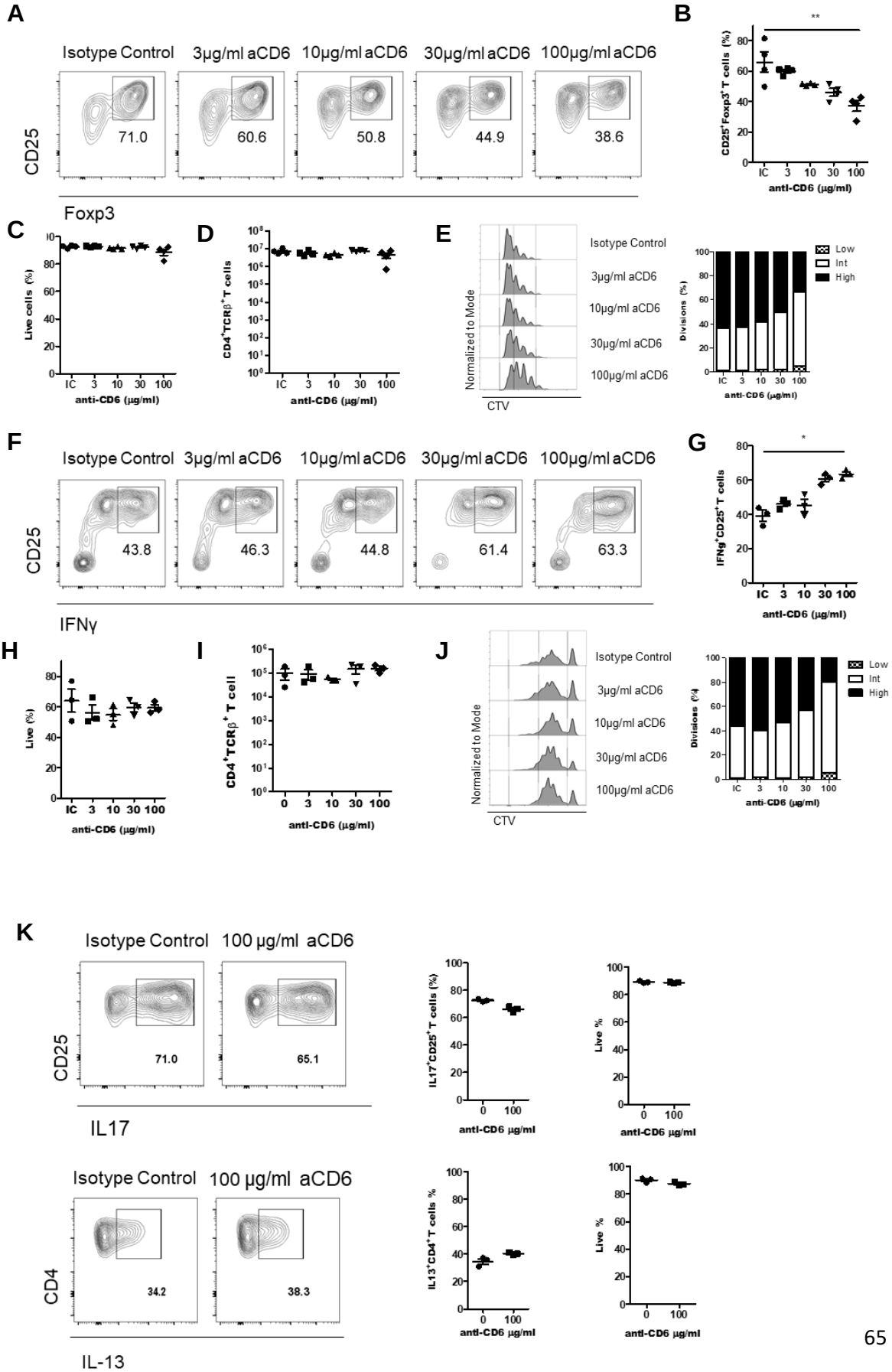

A
Isotype Control
3μg/ml aCD6
10μg/ml aCD6
30μg/ml aCD6
100μg/ml aCD6
CD25
71.0
60.6
50.8
44.9
38.6
B
CD25+Foxp3+ T cells (%)
anti-CD6 (μg/ml)
C
Foxp3
Live cells (%)
anti-CD6 (μg/ml)
D
CD4+TCRβ+ T cells
anti-CD6 (μg/ml)
E
Normalized to Mode
CTV
Isotype Control
3μg/ml aCD6
10μg/ml aCD6
30μg/ml aCD6
100μg/ml aCD6
Divisions (%)
Low
Int
High
anti-CD6 (μg/ml)
F
Isotype Control
3μg/ml aCD6
10μg/ml aCD6
30μg/ml aCD6
100μg/ml aCD6
CD25
43.8
46.3
44.8
61.4
63.3
IFNγ
G
IFNg+CD25+ T cells
anti-CD6 (μg/ml)
H
Live (%)
anti-CD6 (μg/ml)
I
CD4+TCRβ+ T cell
anti-CD6 (μg/ml)
J
Normalized to Mode
CTV
Isotype Control
3μg/ml aCD6
10μg/ml aCD6
30μg/ml aCD6
100μg/ml aCD6
Divisions (%)
Low
Int
High
anti-CD6 (μg/ml)
K
Isotype Control
100 μg/ml aCD6
CD25
71.0
65.1
IL17
IL17+CD25+ T cells (%)
anti-CD6 μg/ml
Live %
anti-CD6 μg/ml
Isotype Control
100 μg/ml aCD6
CD4
34.2
38.3
IL-13
IL13+CD4+ T cells %
anti-CD6 μg/ml
Live %
anti-CD6 μg/ml

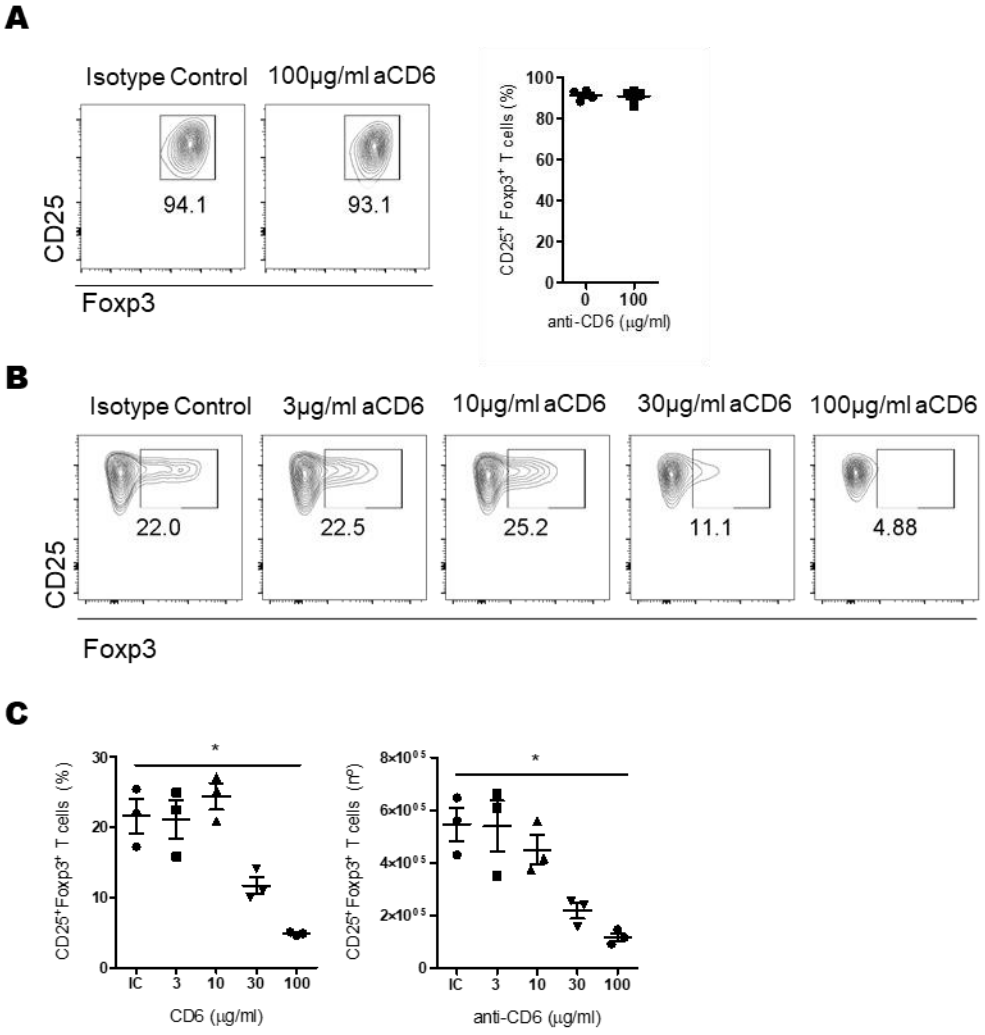
Figure 3. *CD6-targeting increases Th1 polarization, while inhibiting Treg differentiation.* OVA-specific TCR-transgenic OT-II.Rag$^{-/-}$ sorted CD4^{+} T cells were cultured for 4 days in a 2:1 ratio with bone marrow derived dendritic cells (BMDCs) in Th1 and Treg polarising conditions. **(A, B)** Representative flow cytometry dotplots and scatter plots showing the percentage of CD25^{+}Foxp3^{+} T cells within CD4^{+}TCRβ^{+} T cells at the end of Treg polarizing cultures with different doses of anti-CD6 (10F12) or 100 µg/ml isotype control (IC). **(C)** Survival of CD4 T cells at the end of culture. **(D)** Number of CD4 T cells recovered at the end of the culture. **(E)** Representative histograms showing CTV dilution of T cells following culture and bar graph displaying the frequency of cells within gates representing low, intermediate and high proliferation, as displayed in the histograms. **(F, G)** Representative flow cytometry dotplots and scatter plots showing the percentage of CD4^{+}IFNγ^{+} T cells in Th1-polarizing conditions. **(H)** Viability of CD4 T cells under Th1 polarizing conditions. **(I)** Number of CD4 cells recovered at the end of culture. **(J)** T cell proliferation under Th1 polarizing conditions. **(K)** Representative dotplots and scatter plots showing the percentage of T cells producing IL-17 (top) or IL-13 (bottom) following culture under Th17 and Th2 polarizing conditions, respectively, as well as cell viability (right). Statistical tests: Kruskal-Wallis and Mann-Whitney. Representative of three independent experiments, each with n=3. *p<0.05 **p<0.01

2.3.1.2. Alteration of T cell functional specialization by anti-CD6 d1 is influenced by the abrogation of CD6-CD166 interactions

Because the observed impact of anti-CD6 d1 on Treg and Th1 polarization could have resulted from ordinary steric hindrance, we decided to investigate this.

To do that, we induced peripheral Tregs under direct stimulation with anti-CD3 and anti-CD28, so that CD166, the ligand for CD6 d3 would be absent. We observed that adding anti-CD6 d1 in these conditions did not change the ability of naïve CD4 T cells to polarize towards a Treg phenotype, as shown by expression of Foxp3 in **fig. 4A**. However, considering that different activation strategies may impact differently on polarization, we decided to use a more physiological strategy, having used BMDC as APCs for that effect. Knowing that CD166 is now present, we used either soluble CD6 or soluble CD166 as strategies to prevent CD166 on APCs from interacting with CD6 on T cells. If the effect of anti-CD6 d1 on T cells to polarize towards a Treg phenotype could be attributed to mere steric hindrance, we would expect similar outcomes, specifically a dose dependent inhibition in expression of Foxp3. But while using soluble CD6, resulted in a dose-dependent reduction of Treg polarization

similar to the one observed with anti-CD6 (**Figure 4B, C**), using soluble CD166 showed no impact on Tregs' polarization. Thus we conclude, that anti-CD6 modulation of T cell functional specialization upon activation is influenced by the displacement of CD6-CD166 interactions but other mechanisms are involved and requires further studies.



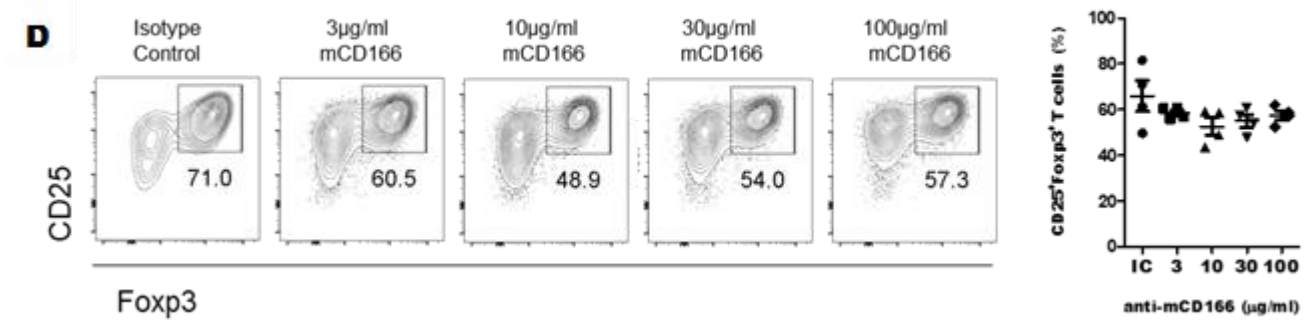


Figure 4. *$CD4^+$ T cell polarization is influenced by CD166 binding.* ***(A)*** *$CD25^+Foxp3^+$ T cells within $CD4^+TCR\beta^+$ T cells at the end of 4-days culture of OVA-specific TCR-transgenic OT-II.$Rag^{-/-}$ CD4 T cells with plate-bound anti-CD3 and anti-CD28 under Treg polarizing conditions. Anti-CD6 did not alter the frequency of induced $Foxp3^+$ T cells.* ***(B)*** *OT-II.$Rag^{-/-}$ CD4 T cells were cultured in a 2:1 ratio with BMDC for 4 days under Treg polarizing conditions with increasing concentrations of soluble CD6. Representative dot plots and* ***(C)*** *graphs showing the frequency and number of $CD25^+Foxp3^+$ T cells within $CD4^+TCR\beta^+$ T cells. Data are representative of two independent experiments (n=4).* ***(D)*** *OT-II.Rag-/- CD4 T cells were cultured in a 2:1 ratio with BMDCs for 4 days under Tregs polarizing conditions with increasing concentrations of mCD166 soluble protein. Representative dotplots and graph showing the frequency of CD25+Foxp3+ T cells within $CD4^+TCR\beta^+$ T cells. Data representative of two independent experiments (n=4).*

2.4. Discussion

Overall, accordingly to our data targeting CD6 d1 with a mAb, differently impacts T cell functional specialization, and while Treg differentiation is inhibited by increasing concentrations of anti-CD6 d1, Th1 differentiation is favoured.

This might explain our *in vivo* data, where animals treated with high doses of anti-CD6 d1 at the time of EAE induction, present a level of disease severity equivalent or even higher than the control group. Such dosage effect resembles what was reported on ongoing clinical trials with a human version of anti-CD6 d1, Itolizumab, for the treatment of RA [283]. These studies suggested that patients treated with the highest dose of Itolizumab had lower term responses compared to low dosage treatment.

Our initial hypothesis for the mechanism of action of anti-CD6 d1 was a possible steric hindrance effect due to antibody size, thereby resulting in disruption of CD6-CD166 interactions. To test this, we used both soluble CD6 and soluble CD166 so that interactions between T cells' CD6 and APCs' CD166 would be disrupted. Our hypothesis was only partially supported, with the treatment of soluble CD6 showing an outcome similar to the one observed with anti-CD6 d1, while the treatment with soluble CD166 did not impact on T cells ability to polarize. The observed modulatory property was only detectable under physiologic conditions, meaning that supra-physiologic conditions, like activation with anti-CD3 and anti-CD28, result in such a commitment that anti-CD6 d1 no longer affects T cells' functional polarization. Our observations are supported by the work of Consuegra-Fernandez et al 2017, where they also showed that under contact-independent and supraphysiological conditions there is no difference for *in vitro* Treg induction, either for $CD6^{-/-}$ or for $CD6^{+/+}$ T cells. [294]

Because no major alterations regarding proliferation or viability were observed, we expect the impact of anti-CD6 d1 to be a subtle and specific one. This dose dependent impact, where somewhat paradoxically low doses are protective, while

high doses are detrimental, prompted us to explore its potential for disease models other than EAE, with both different kinetics and underlying mechanisms.

Cancer

Cancer

3. Clone 10F12, the murine equivalent of itolizumab, as a therapeutic agent in a model of Breast Cancer.

3.1. Background

When CD6 was first discovered in the 80's, as a differentiation antigen recognized by a monoclonal antibody named 12.1, it was known to be expressed in T-cells, although it was also detected in B cells under malignant conditions, namely chronic lymphocytic leukemias (CLL) and lymphosarcoma cell leukemias (LSCL).[1]

The main strategies against leukemia relate to bone marrow or stem cell transplantation, and back in the 90's the previously referred mab T12 was used as a mean to reduce the incidence of graft versus host disease after alllogeneic transplantation, mostly due to its depleting properties. Indeed, this treatment approach was so effective that it even reduced the necessity for immune suppressive drugs for most of the patients.[2-4] Meanwhile, another anti-CD6 mab targeting a different epitope, but lacking depleting properties, was discovered in Cuba, ior-t1.[5]

However ior-t1 was somewhat paradoxical in terms of its effect, as while in B-CLL it promoted cell survival through regulation of the Bcl-2/Bax ratio, in cutaneous T-cell lymphoma it resulted in clinical and histopathological regression of lesions.[6,7] Because ior-t1, originally developed as a murine monoclonal antibody, was humanized in the early 2000's to minimize issues like imunogenecity and allergies, while preserving antigen affinity.[10] This newly humanized ior-t1, named Itolizumab, exhibited enhanced selectivity as evaluated by its effect on B-CLL with selective apoptosis induction of transformed lymphocytes and without a detectable impact on healthy cells.[11] Indeed CLL patients who received a weekly dose of 0.8mg/kg for 12 weeks showed at least preliminary clinical or haematological improvement. [12]

In addition, both clinical and in-vitro studies, suggest that lack of CD6 in leukemic cells increased the apoptotic effect of other therapeutic drugs.[13,14]

Another aspect is also the involvement of both CD6 ligands, ALCAM and CD318 in tumour progression and development. While ALCAM assists cancer cells in migration and angiogenesis, absence of CD318 (CD318KO) results in enhanced tumour growth.[15,16]

As referred in chapter 2, the effect of anti-CD6 d1 was dependent on the dose used; therefore, and because higher doses showed an increase in Th1 polarization, while reducing induction of Tregs, we sought to investigate what would the effect of a high dose be in a model of cancer.

For that we used a breast cancer model, that according to the "The HUMAN PROTEIN ATLAS" (in 2018) would display a more favourable prognostic with higher CD6 expression. So, we tested high systemic doses as well as a high *in-situ* cumulative dose and investigated how this would impact tumour growth and immune infiltrating populations.

3.2. Material and methods

Cell lines and culture

Breast tumour cell line E0771 (C57BL/6 genetic background) was obtained from the laboratory of Bruno Silva Santos Lab, IMM Portugal and maintained in DMEM medium containing 10% (v/v) fetal calf serum (FCS) and penicillin-streptomycin 10000U/ml 1% (v/v) (GIBCO), until they reached log growth phase (<50% confluence). Then they were collected and kept at $0{,}5x10^6$ cells/50µl for in vivo experiments.

Animals and in vivo experiments

C57BL/6 were bred and maintained under specific pathogen-free conditions. Sex-matched mice, between 6 and 12 weeks of age, were used in the experiments. All experimental protocols were approved by the Local Ethics Committee and follow European Union guidelines. The E0771 syngeneic breast carcinoma model was introduced through s.c injection of $0{,}5x10^6$ cells into the second bottom left nipple.

To better follow tumour growth, the tumour site was shaved 5-7 days post inoculation. The animals received either treatment or IC when tumour reached 100 mm^3. Mice were divided as follows into 4 groups, with 4 animals each: control group: injected with IC (rat IgG); second group: injected every two days with 6 intra-tumour cumulative doses of anti-CD6 d1 (40µg/mice); third group: injected with one single dose i.p. of anti-CD6 d1 at 100µg/mice; fourth group: injected with one single dose i.p. of anti-CD6 d1 at 1mg/mice. To measure tumour volume a caliper was used, and the following formula applied Volume=(Vmin^(2*Vmax))/2. When tumours reached a volume of 1000mm^3, usually around 20 to 21 days post-inoculation, animals were sacrificed. Draiing lymph nodes (DLN-mesenchymal) and tumour tissues were harvested, and mononuclear cells were purified for phenotypic and cytokine profile analyses *in vitro.*

mAbs and flow cytometry

Anti-mouse CD6 d1 (10F12), as well as isotype control (rat IgG), were produced at the CIM (Havana, Cuba). Murine single cell suspensions were stained with CD4-PB (RM4-4, biolegend); CD3-PercPCy5.5 (145-2C11 ebioscience); CD25 PE-Cy7 (PC61.5 ebioscience); CD45.2-APC-Cy7 (104 ebioscience); CD6-PE (BX222 biolegend); IFN-γ BV711 (XMG1.2 BD biosciences); IL-17A-Alexa Fluor® 488 (BL168 biolegend); Foxp3 APC (FJK-16s ebioscience). Cell viability was detected with Live/Dead Fixable Aqua Dead Cell Stain Kit (Life Technologies) and Annexin V Apoptosis Detection Kit (ebioscience). Cytokine production was assessed following 4 h stimulation with 50 ng/ml PMA, 500 ng/ml ionomycin, 10 µg/ml brefeldin (all three from Sigma Aldritch) and 0.66µl/ml golgistop™ (BD Biosciences). Cells were permeabilized with eBioscience kit (#) A25866A.

Statistical analysis

Statistical significance was calculated using nonparametric Mann-Whitney *U* test, and Kruskal-Wallis one-way analysis of variance, *p* values of <0.05 were considered

statistically significant (*p<0.05, **p<0.01, ***p<0.001). Results are presented as mean ± SEM.

3.3. Results:

3.3.1. *Intra-tumour treatment with anti-CD6 d1 Mab (10F12) shows a tendency to reduce tumour growth rate.*

Because previous studies using Itolizumab as a therapeutic strategy in malignancies did show clinical improvement, and because our data have also shown that high doses favour Th1 differentiation while inhibiting Treg, we decided to test 10F12 mAb in a well-established model of breast cancer. In collaboration with the lab of Bruno Silva Santos, we induced the E0771 breast cancer model by s.c. injection of exponentially growing E0771 breast cells into C57Bl/6 female mice (**Figure 1A**). In mice treated intra-tumourly with anti-CD6 d1 a tendency for slower tumour growth rate was observed, although not statistically significant when compared to the control group (**Figure 1B**). Regarding the i.p. administration of anti-CD6 d1 and the impact of the higher doses on tumour growth we observed a transient decrease (around day 15), which became unobservable towards the end of the experiment, contrary to the intra-tumour treatment.

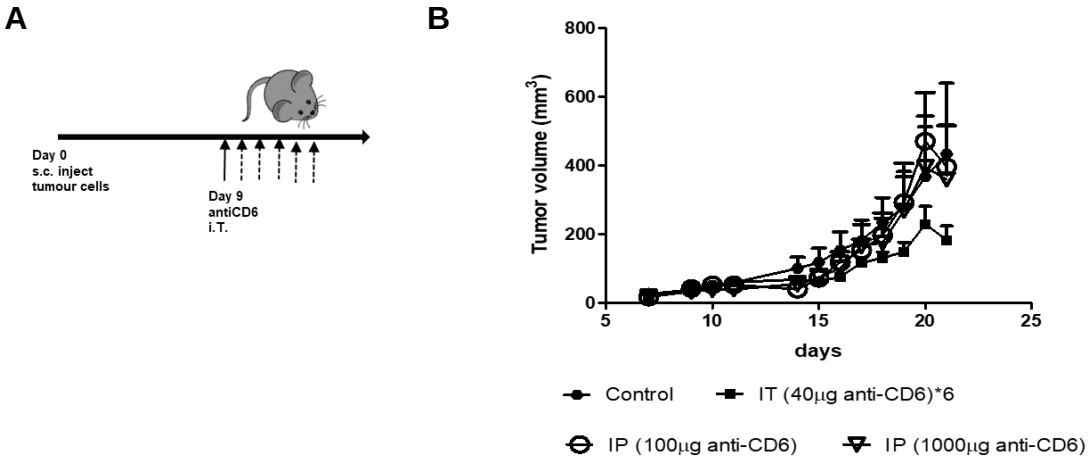


Figure 1. *Intra-tumour Treatment with anti-CD6 mAb (10F12) suggests a delay in tumour growth.* C57Bl/6 mice (5 mice per group) were injected s.c. with $0,5*10^6$ E0771 breast carcinoma cells at day 0 and treated with the indicated doses of anti-CD6, or an isotype control, for 6 consecutive days starting day 9. Mice treated with six doses of 40µg anti-CD6 intra-tumor show a decrease in tumor volume compared to the control. Results are represented as the mean tumor size (mm^3). Data are representative of two independent experiments.

3.1.1. *Dose and route of administration of anti-CD6 d1 suggest an impact over tumour infiltrating populations.*

Following our *in-vivo* results (**Figure 1**) we investigated how anti-CD6 d1 treatments were specifically impacting on $CD4^+$ T cell populations. For that we collected both tumour and draining lymph nodes (mesenchymal) and purified the infiltrating lymphocytes, which were stained for CD45, CD4, CD3, Foxp3, IL-17 and IFNγ expression. Following treatment with anti-CD6 d1 we detected no signal for IFNγ, but observed a trend in the increased expression of IL-17, in $CD25^+Foxp3^+$ and $CD25^-Foxp3^+$ T cell populations as can be seen in (**Fig2 A**), with a more pronounced effect visible on the $CD25^+$ group (**Fig2 A, C and D).** Treatment with anti-CD6 d1 also suggested a decrease in the $CD25^+Foxp3^+$ T cell population, more specifically with the *in-situ* cumulative dosage and 1mg i.p. per mouse (**Figure 2 A, B**), and even though it was not statistically significant, it is merits further investigation.

Regarding tumour infiltration, as shown in **fig. 2E** we could not detect any impact due to anti-CD6 d1 therapy. Nonetheless, we could observe a generalized reduction in the median fluorescence intensity (MFI) of CD25 for all experimental conditions, suggestive of an impact on cellular activation, more evident in the higher dose of 1 mg/mouse, as shown in **fig. 2F**.

When analyzing DLN infiltrating populations no impact was observed on $CD25^+Foxp3^+$ regulatory T cells (Tregs) regardless of treatment strategy, as shown in f**ig.3.** Worthy of note was the increased trend in the expression of IL-17 producing Tregs under all anti-CD6 d1 treatment strategies (**Fig. 3C**), even though in a non-statistically significant manner.

Accordingly, treatment with anti-CD6 d1 also did not impair the ability of $CD4^+$ T cells to infiltrate DLN (**fig. 3D)** as observed with tumour tissues (**Fig. 2E**). However, regarding activation of $CD4^+$ T cell populations in DLNs, it appears that only *in-situ* cumulative doses and the highest dose of 1mg/mouse of anti-CD6 d1 could reduce activation as shown in f**ig. 3E** through a decrease of CD25 MFI levels. Although this data is somewhat preliminary, it suggests a negative impact of anti-CD6 d1 mab over tumour infiltrating regulatory T cells, coupled with an increasing ability to secrete IL-17. Also, it seems to suggest that *in-situ* cumulative doses or a very high systemic dose, e.g. 1mg/animal, were the best strategies.

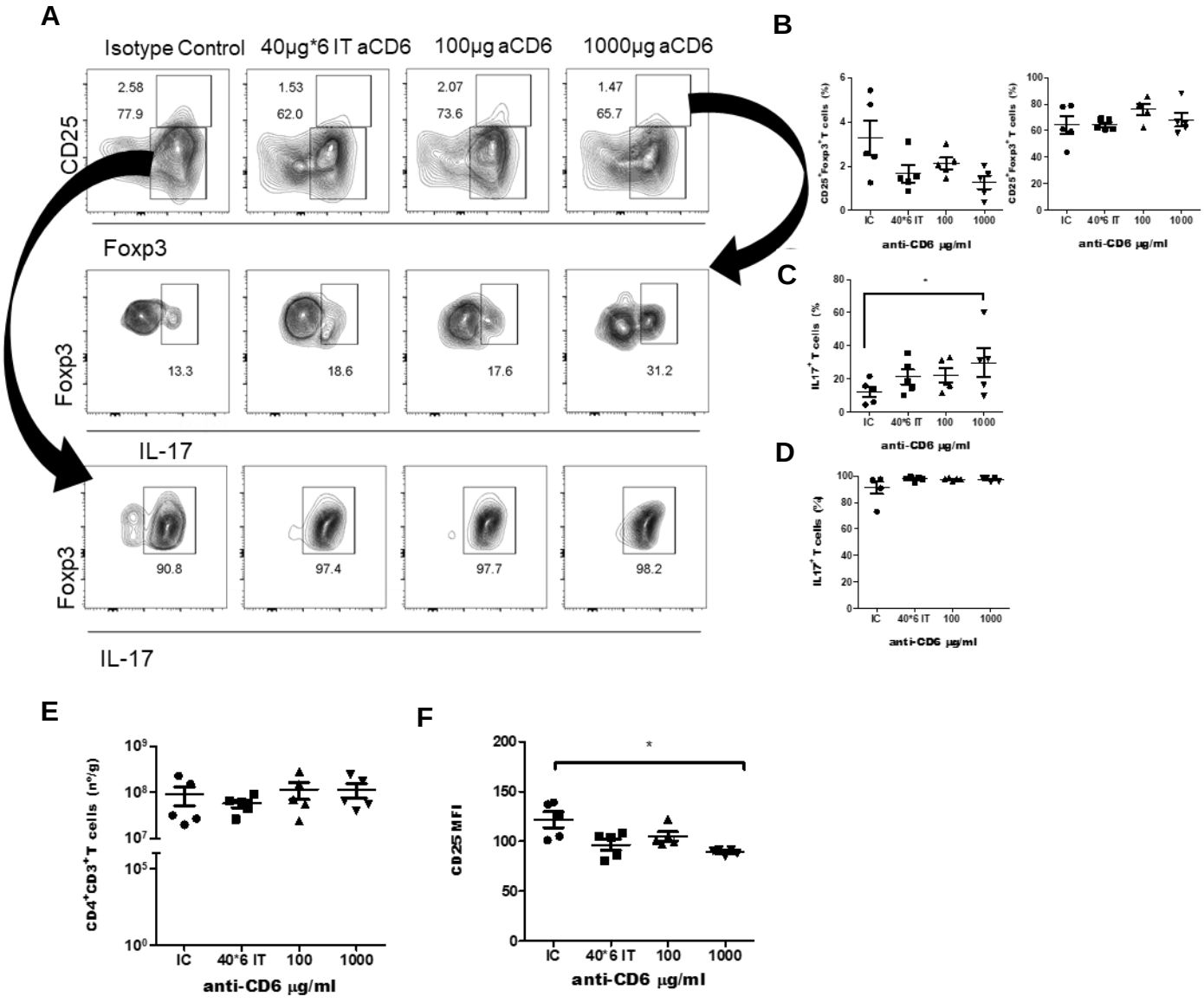

A
Isotype Control
40μg*6 IT aCD6
100μg aCD6
1000μg aCD6
CD25
2.58
77.9
1.53
62.0
2.07
73.6
1.47
65.7
Foxp3
Foxp3
13.3
18.6
17.6
31.2
IL-17
Foxp3
90.8
97.4
97.7
98.2
IL-17
B
C
D
IC
40*6 IT
100
1000
anti-CD6 μg/ml
E
CD4+CD3+T cells (n°/g)
F
CD25 MFI

Figure 2. *Local treatment and high systemic doses suggest an impact over activation and the phenotype of T cells infiltrating the tumour.* C57Bl/6 mice (5 mice per group) were injected with 0,5*10^6 E0771 breast carcinoma cells s.c. and treated with different doses of anti-CD6, or an isotype control at day 0. **A)** Representative counterplots of tumour infiltrating regulatory T cells either activated $CD25^+Foxp3^+$ or not $CD25^-Foxp3^+$ and the expression of IL-17 on each subset. **B)** Scatter plot representing the percentages for both $CD25^+Foxp3^+$ and $CD25^-Foxp3^+$ regulatory T cell populations. **C)** Scatter plot showing the percentage of IL-17 producers inside $CD25^+Foxp3^+$ T cells. **D)** Scatter plot showing the percentage of IL-17 producers inside $CD25^-Foxp3^+$ T cells. **E)** Scatter plot representing the number of infiltrating $CD4^+CD3^+$ T cells per gram of tumour tissue under each experimental condition. **F)** Scatter plot showing how treatment strategy impacted over T-cell activation, as shown by the median fluorescence intensity of CD25 expression. Statistical tests: Kruskal-Wallis * p<0,05. Preliminary data.

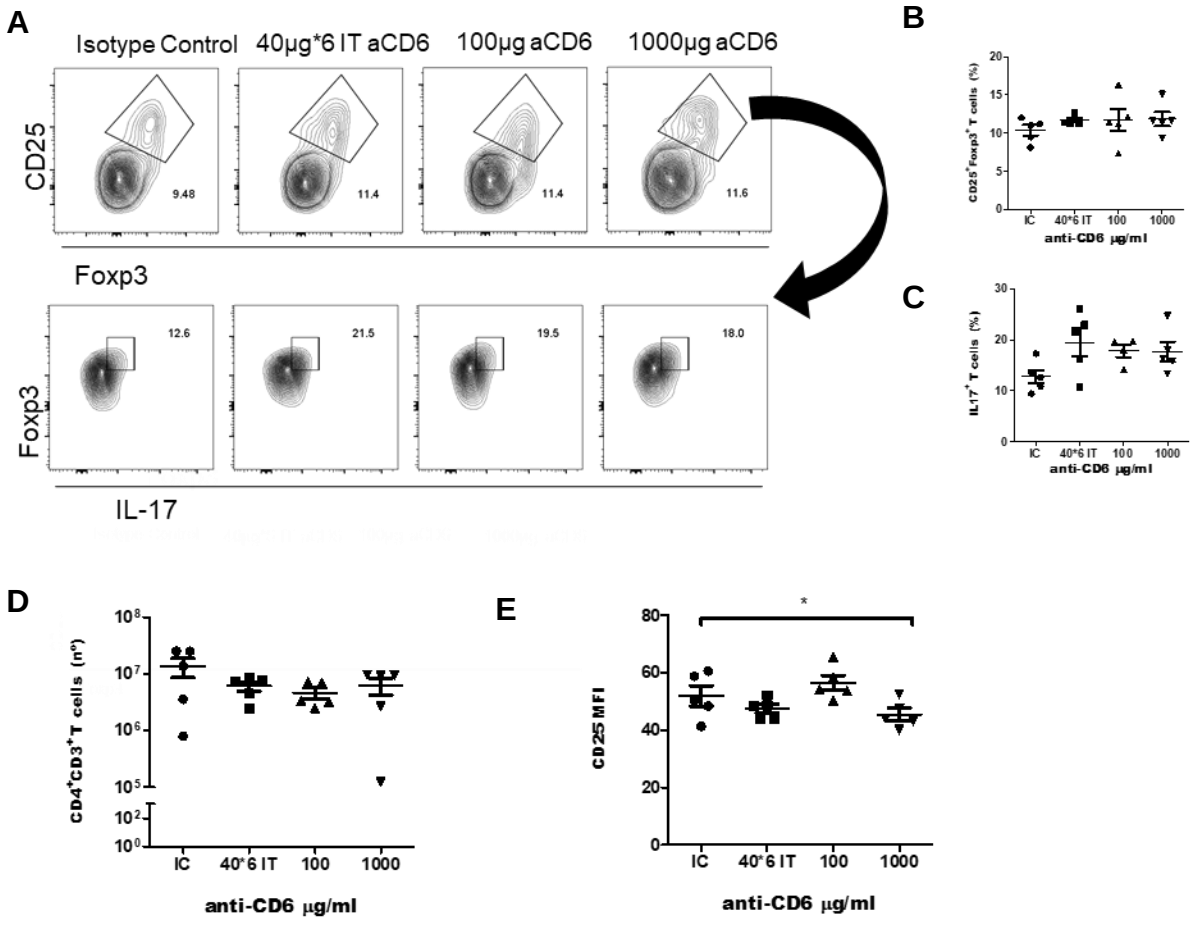


Figure 3. *Treatment with anti-CD6 d1 has minimal impact over draining lymph nodes (DLN) CD4⁺T cell populations.* C57Bl/6 mice (5 mice per group) were injected with 0,5*10^6 E0771 breast carcinoma cells s.c. and treated with different doses of anti-CD6, or an isotype control at day 0. **A)** Representative counterplots of DLN infiltrating $CD25^+Foxp3^+$ regulatory T cells and their expression of IL-17. **B)** Scatter plot representing the percentages for both $CD25^+Foxp3^+$ regulatory T cels. **C)** Scatter plot showing the percentage of IL-17 producers inside $CD25^+Foxp3^+$ T cells. **D)** Scatter plot representing the number of infiltrating $CD4^+CD3^+$ T cells per gram of tumour tissue under each experimental condition. **F)** Scatter plot showing how treatment strategy impacted over T-cell activation, as shown by the median fluorescence intensity of CD25 expression. Statistical tests: Kruskal-Wallis * p<0,05. Preliminary data.

3.4. Discussion

Overall, anti-CD6 d1 mAb seems to reduce tumour growth as observed in **fig.1,** if given in *in-situ* cumulative doses.

Although not conclusive, due to the lack of statistical significance, our preliminary data from **fig.2** might provide a possible explanation for such observation, which consists in the reduction of $CD25^+Foxp3^+$ regulatory T cells (**Figure 2A, B**) and an increased ability to secrete IL-17 compared to control group. (**Figure 2A, C**).

These observations were restricted to tumour tissue, not being observed in phenotypically equivalent cell populations present in the DLNs (**Figure 3**), with the exception of IL-17 expression that was also increased (**Figure 3C**).

According to the literature the presence of Tregs at the tumour microenvironment correlates with a poor prognosis for several types of cancer, including breast cancer.[306] In addition, even though for some types of cancer their ability to control inflammation through IL-10 expression is an advantage, eventually, and after continuous inflammatory stimulus, they end up switching to expression of IL-17.[307] Indeed in our observations the treatment strategy inducing a higher increment in IL-17 secretion (1mg/animal i.p.) (**Figure 2C**), was the one that impacted tumour growth i*n-vivo* (day 21) equivalent to the control.

The presence of an IL17Foxp3 double positive $CD4^+$ T cell population has already been reported for several groups.[308-310] This is a population known for being induced through MAPK activation pathways which induce IL1β, then resulting in this double positive phenotype.[308,311] According to the work of Ibáñez et al 2006 activation of CD6 with its ligand will result in the activation of the three MAPK cascades, which, despite the unquestionable need for further studies, seems to go in line with our preliminary observations.[312]

During our studies we also observed a lack of impact on the infiltrating $CD4^+CD3^+$ T cells, both in tumour tissue as well as in DLNs (**Figures 2E and 3D**), but we did notice a negative impact on the activation, as measured by CD25 MFI levels (**Figures 2F and 3E**) when *in-situ* cumulative dosage or very high doses like 1mg/mouse were used. Accordingly, the work of Carrasco et al 2017 demonstrated how CD6 down-modulation inversely correlates with CD25 upregulation; thus, and despite this data was generated from human cells, we might consider to further investigate if the opposite is also true, meaning if higher CD6 expression could result in CD25 downregulation.[302]

Although our data suggests a protective effect by *in-situ* delivery of cumulative doses of anti-CD6 d1, and that this effect might be correlated with a decrease in tumour infiltrating Tregs, this is just preliminary data and requires further validation. However, it is interesting to observe the persistence of a negative effect caused by CD6 d1 targeting over the regulatory T cell population as referred on chapter I.

Itolizumab

Itolizumab

4. Itolizumab, a humanised monoclonal antibody against CD6 d1.

4.1. Background

As previously referred in chapter I, Itolizumab is an IgG1 humanized recombinant monoclonal antibody targeting CD6 domain 1, but contrary to ior T1, its murine counterpart, it does not cause immunogenicity and retains its ability to bind its ligand, ALCAM.[280] In fact, not only does it preserve the therapeutic profile of ior T1, as well as the process of humanization improved the side effect profile, most probably related to immunogenicity issues.[313,314]

With a selective impact over T cells, this immunomodulatory mAb, with a molecular weight of 148kDa and 449 amino acids (a.a.) in length, is composed of two heavy and two light chains plus a disulphide bond.[284,314,315] Commercialized in India since 2013 by Biocon Ltd, it appeared in the markets as Alzumab™ a preservative-free, single-use 25 mg/5 mL colourless buffered solution (pH 7.0±0.5) for intravenous (iv) injection.[284]

This new biological has proven to be highly advantageous as a therapeutic strategy against psoriasis and rheumatoid arthritis, even when compared to other commonly used biologicals, like anti-TNFα blockers.[283,284] To support this, factors like better safety profile, comparable efficacy and lower costs, made Itolizumab a good candidate for “first line” therapeutic strategies.[285] But other aspects like sample size, open label design, absence of a control arm or a possible impact of populational genetic background over trials’ readout, must be sorted out.

Thus, given the good results of Itolizumab in both psoriasis and RA therapeutics and genome wide association studies (GWAS) linking some *CD6* specific polymorphisms to MS development[238,239,316,317] , it became relevant to study if CD6 targeting indeed improved disease outcome and which T cell populations were being affected.[318 319]

In chapter I we show that by using a murine version of Itolizumab, when low doses were being administered, we could observe a significant decrease in disease severity (**Figure 1 of chapter I**). Also, we verified *in-vitro* that while CD6 targeting was reducing the ability of T-cells to polarize towards Tregs, for Th1s it was actually improving its polarization (**Figure 3, chapter I**). And although it lacked statistical significance, also on **chapter II on fig. 2A and B** we could observe a tendency for less infiltrating Tregs.

Our animal data combined with data from RA patients, where the better clinical benefit and long lasting improvements came from the lower doses, prompted us to study if Itolizumab had the same dose effect over regulatory and helper 1 T cell populations.[283,320]

This parallelism between murine and human data might be important as a proof of concept for further studies using Itolizumab on other autoimmune diseases as MS.

4.2. Material and Methods

mAbs and flow cytometry

Single cells suspensions were stained with CD4-PE (RPA-T4); CD4-FITC (OKT4); CD3-PE (OKT3); CD25-PE-Cy7 (BC96); CD45RA-APC-eFluor® 780 (HI100); IFN-γ-PerCP-Cy5.5 (4S.B3); IL-13 PerCP-Cy5.5 (JES10-5A2); IL-17-APC (eBio64DEC17); Foxp3-APC (PCH101), CD6-FITC (BL-CD6) and anti-human IgG-APC-Cy7. Cell viability was detected with Live/Dead Fixable Aqua Dead Cell Stain Kit (Life Technologies). CellTrace™ Violet Cell Proliferation Kit was used for cell proliferation assessment according to the manufacturer's protocol (Thermofisher). In some studies, cytokine production was assessed following 4 h stimulation with 50 ng/ml PMA, 500 ng/ml ionomycin, 10 µg/ml brefeldin A (all from Sigma Aldritch) and 0.66/ml Golgistop™ (BD Biosciences). Cells were permeabilized with eBioscience kit (# A25866A).

T-cell activation and polarization

Peripheral blood mononuclear cells (PBMCs) were isolated from blood or buffy-coats from healthy volunteers provided by Instituto Português do Sangue e Transplantação (IPST) by Ficoll gradient (Sigma Aldritch) using SepMate™ (STEMCELL Technologies). Naïve T cells ($CD4^+CD3^+CD25^-CD45RA^+$) were then sorted with FACS Aria III (BD Biosciences). Irradiated (25 Gy) PBMCs (iPBMCs) or Raji cells (donated by Alexandre do Carmo lab, i3S Porto) were used as APCs. $CD4^+$ cells were cultured with iPBMCs (at 1:2 ratio) and 1 µg/ml of soluble anti-CD3 (OKT3); with 3 µg/ml plate bound anti-CD3 (OKT3) and 2 µg/ml soluble anti-CD28 (CD28.2) or mix of superantigens 1ug/ml, SEB (Sigma Aldrich), SEE, SEA and TSST-1 (Toxin Technology). For Th1 polarization the medium was supplemented with 10 ng/ml IL-2, 2.5 ng/ml IL-12, and 5 µg/ml anti-IL-4 (11B11) mAb (all from Peprotech). For Treg polarization we added 10 ng/ml IL-2 and 10 ng/m TGF-β (R&D).

Statistical analysis

Statistical significance was calculated using nonparametric Mann-Whitney *U* test, and Kruskal-Wallis one-way analysis of variance, *p* values of <0.05 were considered significant (*p<0.05, **p<0.01, ***p<0.001). Results are presented as mean ± SEM.

4.3. Results

4.3.1.1. In humans, Itolizumab modulates T cell differentiation by favouring Th1 in detriment of T-regulatory cells, just like in mice.

To investigated whether Itolizumab could also influence the acquisition of effector functions by activated human CD4 T cells, we used sort-purified naïve CD4 T cells, and as observed in mice, it was not necessary to discriminate populations based on CD6 expression, as around 98% of $CD4^+$ T cells constitutively bear CD6 on its surface (**Figure 1**).

Because we could not use TCR specific populations (like in mice), instead we stimulated them with soluble anti-CD3 in the presence of antigen presenting cells (APCs). We decided to use anti-CD3, because the presence of Itolizumab under allogeneic stimulatory conditions inhibited T cell activation and, consequently, their ability to enter division almost completely as shown in **fig.2**.

Thus, by adding soluble anti-CD3 to irradiated peripheral blood mononuclear cells (PBMCs), we could overcome the negative impact of Itolizumab over T cell activation/proliferation, as shown on f**igure 3A** through the profile of CTV dilution. Also, we could see (**Figure 3B)** that under these stimulatory conditions, survival was not significantly affected.

Then, we could proceed to investigate the impact of Itolizumab over T cell functional specialization, just like we did with murine cells. So we cultured sorted naïve $CD4^+$ human T cells under Tregs or Th1 favoring conditions also with titrated anti-CD6 d1 (Itolizumab).

Once again, as shown in **fig. 4A and B**, a dose-dependent reduction in the frequency of induced Tregs was observed, with no significant impact on cell viability (**Figure 4C**), with a small, although significant, impairment on cell proliferation at higher doses of Itolizumab (**Figure 4D, E**).

As for the Th1 favoring conditions, a dose dependent increase of Th1 polarized cells was observed instead (**Figure 4F, G**). Regarding survival and proliferation, an impact to the one described for Tregs was again observed (**Figure 4H-J**).

Overall murine and human data seem to be concordant regarding the impact of CD6 d1 targeting.

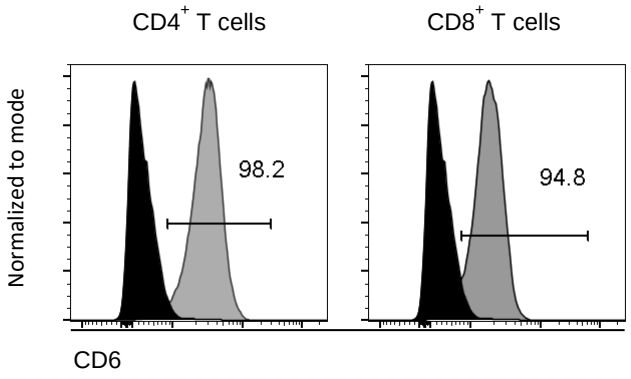


Figure 1- *Virtually all human CD4⁺ T cells express CD6.* Peripheral blood mononuclear cells (PBMCs) from healthy donors gated on CD3⁺ lymphocytes showing the percentage of positive CD6⁺ T cells within CD4⁺ (left) and CD8⁺ (right) populations.

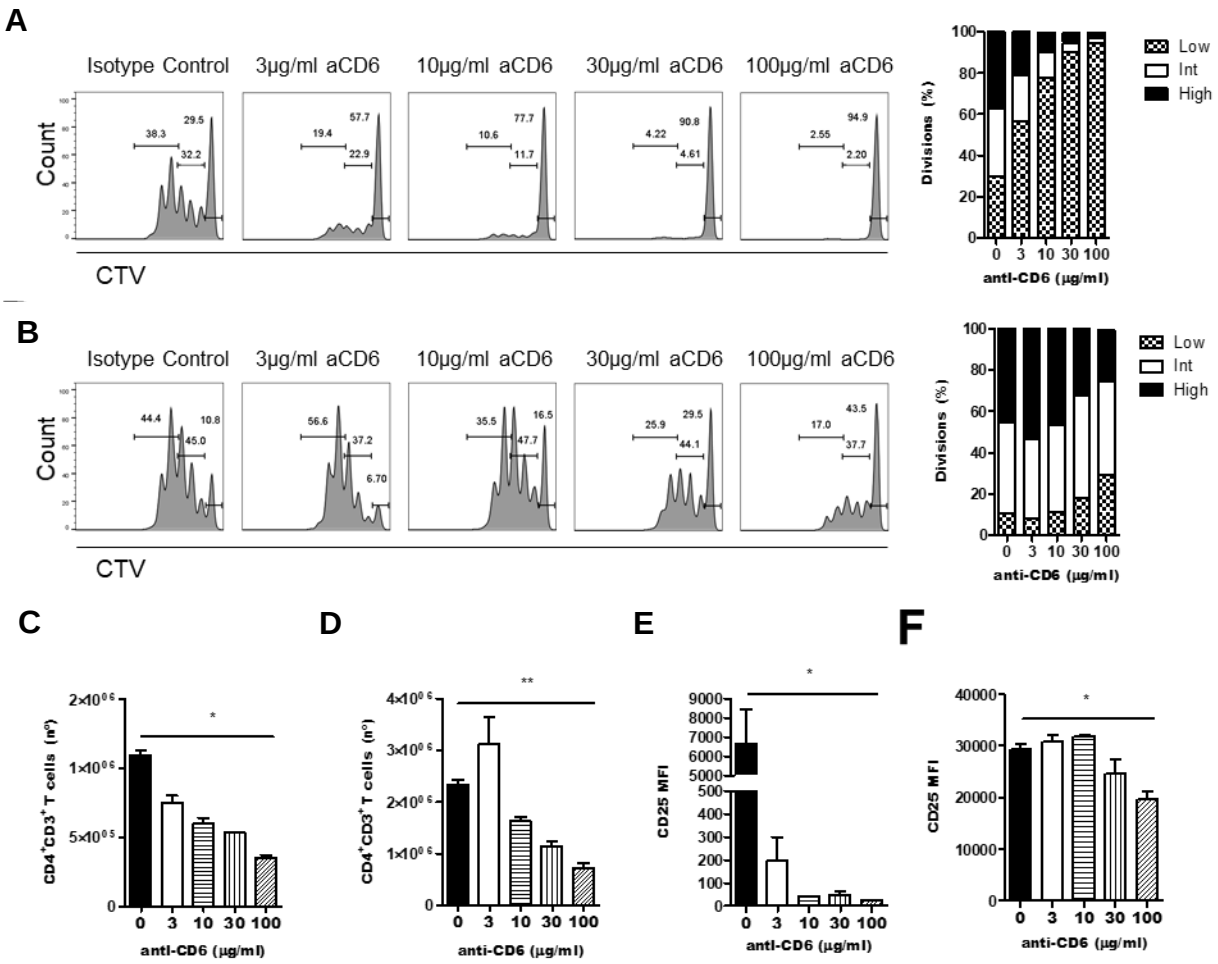


Figure 2- *Itolizumab reduces both proliferation and survival compromising T cell entry into division.* $CD4^+CD3^+CD25^-CD45RA^+$ sorted peripheral blood mononuclear cells (PBMC) from healthy donors were used as source of naïve T cells (responders) while irradiated PBMCs as stimulators. The CD4 T cells were labelled with CTV and stimulated with iPBMCs only (**A, C**) or with iPBMCs and anti-CD3 (**B,D**), in the presence of IL-2 for five days. (**A**) Itolizumab inhibits T cell entry into division in MLR conditions, (**B**) but activation with anti-CD3 partially recovers that inhibition. (**E,F**) Itolizumab induces CD25 downregulation as measured by the MFI, with anti-CD3 (**F**) only having a positive impact in CD25 expression. Data are representative of three independent experiments. Statistical test: Kruskal-Wallis p**>0.01 p*>0.05

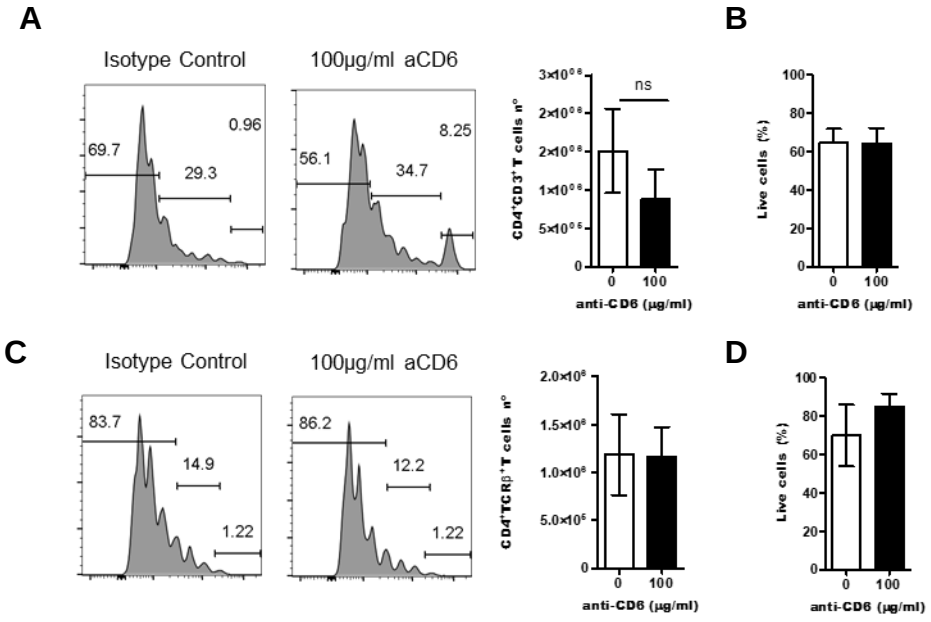


Figure 3. *Itolizumab has no significant impact in proliferation or viability under direct CD3 stimulation.* Peripheral blood mononuclear cells (PBMCs) from healthy donors were used as source of naïve T cells (responders). The naive T cells were labelled with CTV and stimulated with irradiated PBMC plus anti-CD3 **(A)** or with anti-CD3 and anti-CD28 in the absence of stimulating cells **(C)**. Responder naïve CD4 T cells were cultured for 5 days in a ratio of 1:2 with the irradiated PBMCs (C) in non-polarizing conditions with IL-2. **(A, C)**

Representative histograms of cultures with and without itolizumab and bar graphs showing the number of recovered CD4 T cells (left) and their viability (B,D). Statistical test: Mann-Whitney. Data are representative of three independent experiments, each of them run in triplicate.

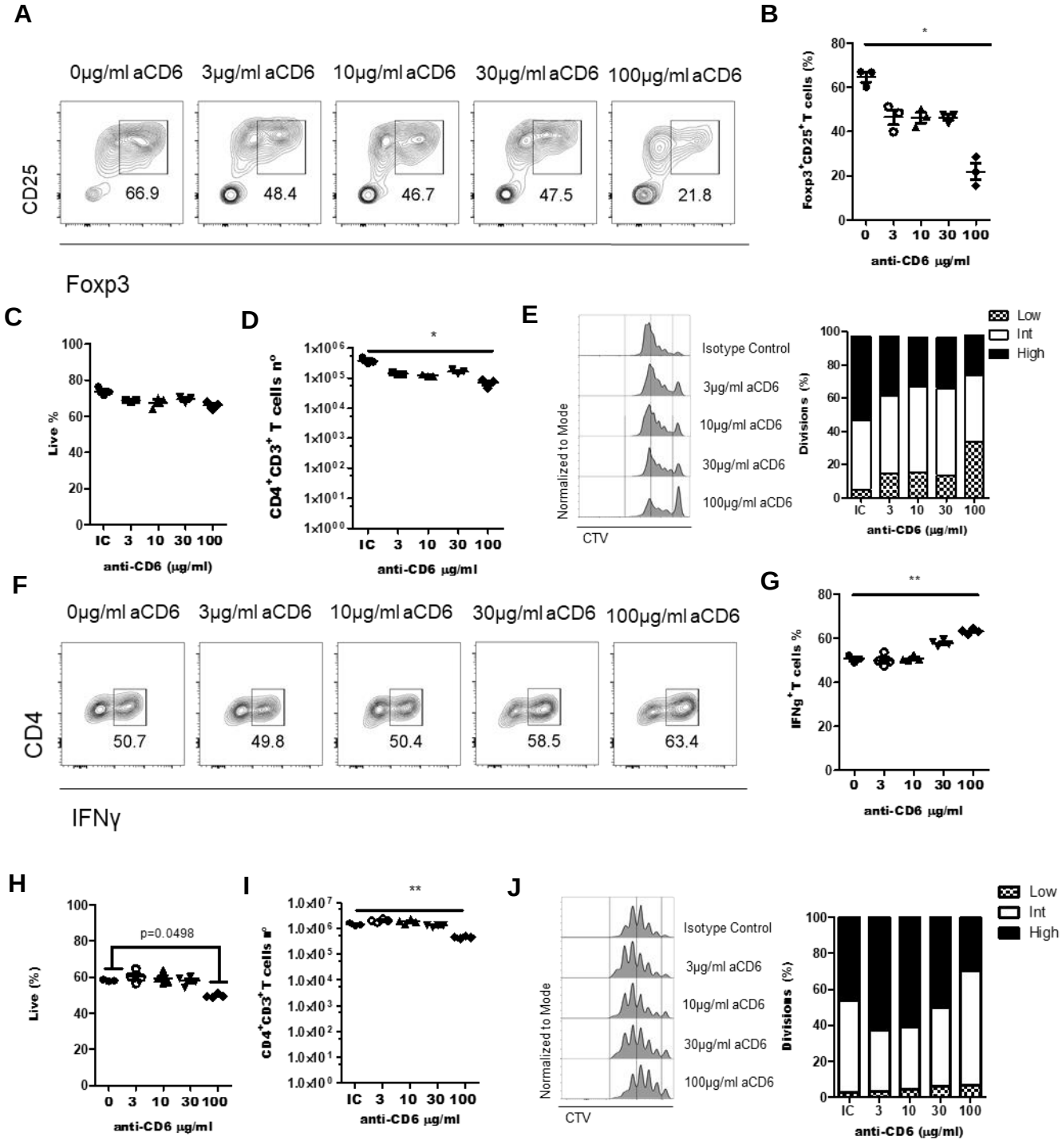


Figure 4. *Itolizumab inhibits Treg polarization while enhancing acquisition of Th1 phenotype.* **(A-E)** PBMCs from healthy donors were used as source of naïve CD4 T cells (responders), labelled with CTV and stimulated 5 days with syngeneic irradiated PBMC (at a 1:2 ratio) with added anti-CD3, TGF-β, and IL-2 for Treg polarization; or **(F-J)** with IL-12, IL-2 and anti-IL4 for Th1 polarization. **(A)** Representative contourplots of $CD25^{+}Foxp3^{+}$ Treg cells and **(B)** graph with pooled data. **(C)** Viability of CD4 T cells at the end of the culture in Treg polarizing conditions and **(D)** number of recovered cells. **(E)** Representative histograms showing T cell proliferation (CTV dilution) under Treg polarizing conditions and pooled data with the frequency of T cells within the represented low, intermediate and high cell division gate. **(F)** Representative contour plots of IFNγ producing CD4 T cells and **(G)** graph with pooled data. **(H-J)** Viability, number of recovered CD4 T cells, and T cell proliferation at the end of cultures under Th1-polarizing conditions. Data are representative of two independent experiments, each of them run in triplicate. Statistical test: Kruskal-Wallis. *p<0.05 **p<0.01

4.3.1.1. Impact of Itolizumab over T cell functional specialization suggests a dependence on the nature of the activation stimulus.

To investigate if the impact of CD6 targeting over human T cells' functional specialization was due to a putative disruption of CD6-CD166 interactions, we repeated the *in-vitro* assays, to exclude the CD166 stimulus we didn't use APCs (**Figure 5**). Instead we used anti-CD3 and anti-CD28 as a direct activation strategy, and the absence of CD6-CD166 interactions did not result in any altered functional specialization when in the presence of Itolizumab (**Figure 5**). This data went in accordance with the literature (Rodriguez et al 2012), that the impact of CD6 d1 targeting is CD166 independent. [314] Also the absence of ALCAM did not seem to affect either proliferation or survival as seen in **fig. 3C, D**.

Similar to the murine data, **(Chapter I, Fig.4**) we observed that the strategy used for activation is important for a putative impact of Itolizumab over T cell functional specialization and activating capacity. With superantigen activation (SAg) the impact of Itolizumab over functional differentiation was lost, just like what happened with anti-CD3/anti-CD28. Also, both strategies impacted differently on T cell activation status as shown in **fig.6** by CD25 MFI, with superantigen Itolizumab increasing the activation (**Figure 6A**) and with CD3/anti-CD28 stimulation decreasing it (**Figure 6D**).

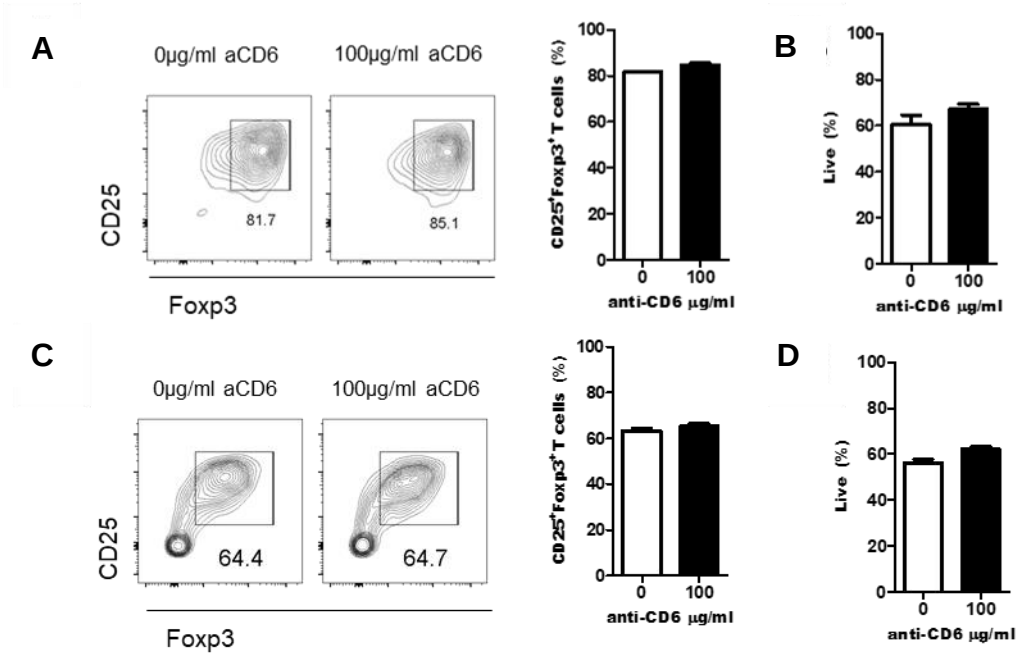


Figure 5- *Itolizumab does not impact Treg polarization when stimulated either with anti-CD28 and anti-CD3 or with SAg***.** Peripheral blood mononuclear cells (PBMCs) from healthy donors were used as source of naïve T cells (responders). **(A, B) N**aive T cells were stimulated with anti-CD3 and anti-CD28, in presence of Treg polarizing conditions for five days. **(B)** Bar graph showing the survival of CD4 T cells. **(C. D) N**aive T cells were stimulated with either Raji plus SAg mix 1µg/ml **(C)** Naïve CD4 T cells were cultured in a ratio of 1:1 with Raji cells in Treg polarizing conditions for 4 days **(D)** Bar graph showing the survival of CD4 T cells. Data representative of three independent experiments.

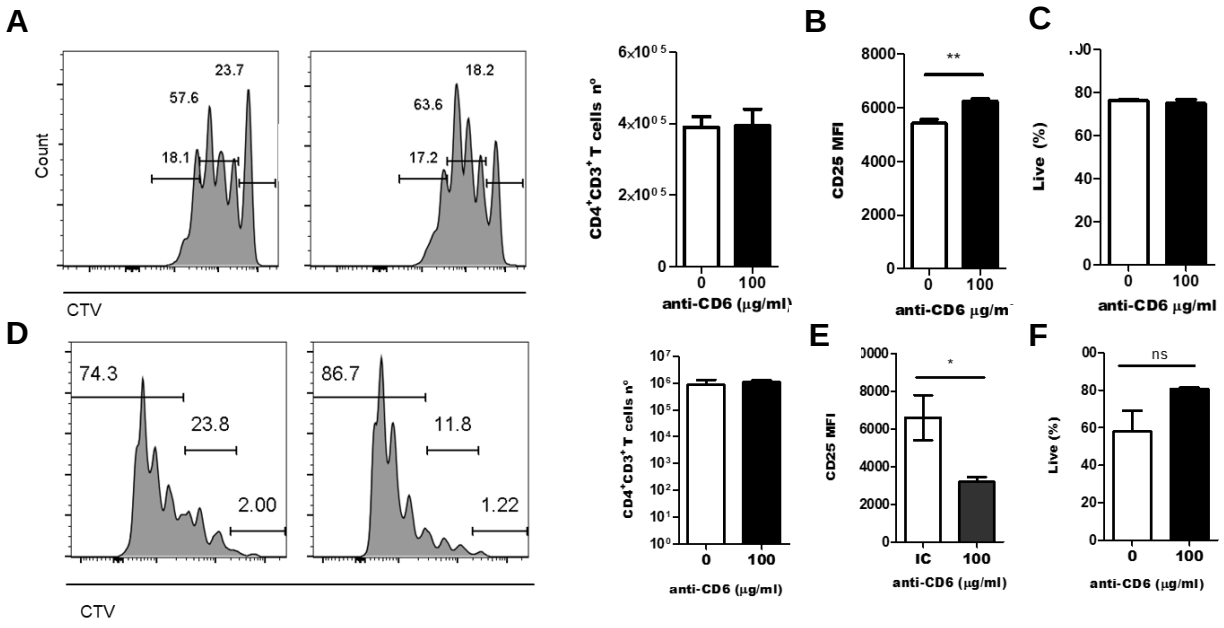


Figure 6- *Activation strategy is relevant for the impact of Itolizumab on CD4⁺ T cell activation***.** $CD4^+CD3^+CD25^-CD45RA^+$ sorted peripheral blood mononuclear cells (PBMC) from healthy donors were used as source of naïve T cells (responders) while Raji cells were used as APCs. Naïve sorted CD4 T cells were labelled with CTV and activated either with (**A-C**) SAg mix in the presence of IL-2 for 4/5 days or with (**D-F**) anti-CD3 & anti-CD28. (**A, D**) CD6-targeting with Itolizumab showed no impact on T cell proliferation (assessed by CTV dilution), and no reduction in the number of CD4 T cells recovered at the end of the culture. **(B)** When activated with a Sag mix and treated with Itolizumab, CD4 T cells showed an increase in the CD25 median fluorescence intensity (MFI), however **(E)**, when activated with anti-CD3/anti-CD28 there is a reduction in CD25 MFI. (**C, F**) No impact was observed on cell survival. Data are representative of two independent experiments. Statistical test: Kruskal-Wallis.

4.4. Discussion

Again, our results, support the idea that CD6 targeting with a mAb against domain 1, which is the case of Itolizumab, results in a negative impact over Tregs polarization, while favouring a Th1 phenotype polarization.

Similarly, very important is that, just as in mice, this is a dose dependent effect, again highlighting the importance of dosage. However contrary to mice we noticed a small, yet significant, impact over proliferation. Regarding survival, both the murine clone 10F12 and Itolizumb did not negatively impact T cell survival when compared to control group as seen in **fig.4**.

Because in humans we could not activate our purified naïve $CD4^+$ T cells in an antigen specific manner, as we did in mice, we decided to use a mixed lymphocyte reaction (MLR). The MLR strategy mimics an activation resembling the TCR recognition of nominal peptide epitopes presented by self-MHC molecules, hypothetically maintaining the assays as physiological as possible.[321] However, as shown in **fig.2,** when we targeted CD6 with Itolizumab, we observed that the T cells, although alive, were not activating, in opposition to what happened when anti-CD3 was added.[321] So, in light of this observation, we decided to include anti-CD3, and with this new activating strategy not only did the cells activate (**Figure 2**), but also the CD6 targeting impact observed in murine cells was reproduceable with human cells (**Figure 4**), e.g. high doses of Itolizumab increased Th1 differentiation, while decreasing peripheral Treg induction.

Indeed, Nair et al 2010 had previously shown CD6 co-stimulation involvement in Th1 commitment, thus supporting our observations.[273]

Despite no impact on overall proliferation, we were able to detect a dose dependent cell division arrest at intermediate divisions (**Figure 4E, J**) which goes in line with Bughani et al 2017, where a microarray analysis demonstrated that Itolizumab impacts over cell cycle.[322]

As in mice we also wanted to investigate in human if targeting CD6 d1 with Itolizumab was causing some kind of steric hindrance over CD6 d3 interaction with its ligand ALCAM. For that we activated our T cells without APCs, using anti-CD3 and anti-CD28 so that ALCAM would not be present and just like in mice, we registered no difference between the experimental and control groups (**Figure 5C, D**), suggesting the impact of Itolizumab over differentiation was independent of CD6 d3-ALCAM interactions. However, just like with murine cells a different more physiological activation strategy might have had the opposite effect, as supported by the the work

of Bughani et al 2017, where they showed that while Itolizumab F(ab') fragments cannot disrupt CD6-ALCAM interactions, full Itolizumab can.[322]

During our experiments we noticed another, very important, aspect regarding the impact of Itolizumab over functional specialization and T cell activation. Indeed, depending on the type of activation strategy, Itolizumab affected polarization or not. If we used iPBMCs and anti-CD3 (**Figure 4**) we had lower Treg differentiation, however if we used anti-CD3/anti-CD28 or a SAg mix and APCs we would lose that negative impact (**Figure 5).** That cannot be attributed solely to an interference over CD6 d3- ALCAM interactions, since both had the same outcome and the first was lacking ALCAM. Moreover, the impact over T cell activation by both strategies was different, with our anti-CD3/anti-CD28 results being similar to the ones of Bughani et al 2017, showing less CD25 expression when treated with Itolizumab. Again, if different activation strategies were used, as we show in **fig.6** Itolizumab, instead of decreasing CD25 expression, it increases it.

This data suggest that the signalling and activation pathways affected by Itolizumab can be exposed using different activation strategies. Future studies will then be required to identify how Itolizumab specifically affects signalling elements and how that will affect activation and consequently functional specialization.

In fact, it is already documented in the literature that TCR activation and consequent ZAP70 phosphorylation facilitates the interaction of CD6 with the TCR, with the physical binding of CD6 and the CD3 complex mediated through Lck.[271] Also, just like LAT, CD6 behaves as a signalling hub important for SLP-76 and Vav1 recruitment, both of them adaptor proteins.[254,323] And it will be the ability of CD6 to hyper-phosphorylate tyrosine, serine and threonine residues on its cytoplasmic tail that will allow SLP76 binding, thus resulting in a time and dose dependent activation of MAPKinases.[312,322]

Overall, our results show that therapeutic antibodies, specifically anti-CD6 d1, are dosage sensitive, meaning that only when used at high doses they do prevent Treg induction, while favouring Th1 differentiation at lower doses, just like in mice.

This impact over functional specificity might be related to different levels of activation favouring alternative polarization phenotypes [182].

This observation highlights the relevance of dosage, drawing our attention to the fact that the same drug might be useful for different therapeutic targets depending on the dose used.

General Discussion

General discussion

Summarizing, we show that targeting CD6 d1 with a monoclonal antibody allows, in a dose dependent manner, specific modulation of T cell functional specialization. Indeed, in both mice and humans we verified that when we use high doses of anti-CD6 d1 mAb, induction of peripheral Tregs is impaired while induction of Th1 cells is favoured.

We also noticed that the strategy used to activate T cells influenced how anti-CD6 d1 affected the T cells. T cell activation with strategies like anti-CD3/anti-CD28 or SAg mix, does not allow anti-CD6 d1 impact over T cell functional specialization to be detectable. Probably, and this obviously requires further investigation, due to the signalling pathways differently used by these strategies or to a supra-physiologic stimulation that masks the impact of anti-CD6 d1.

Overall, we show that anti-CD6 d1, a therapeutic antibody with an already commercially available version, may present paradoxical effects depending on the dose at which it is used and all because of a distinct impact over CD4 T cells functional specialization. Such dose selection becomes crucial as it is the case of EAE, where only low doses were protective while high doses may even exacerbate disease severity. The relevance of dosage in this scenario seems to suggest that the same compound depending on its dose may be therapeutically useful for several diseases. Because of that, we decided to explore its impact on a mouse model of breast cancer, which despite the lack of statistical significance, suggested a protective effect with *in-situ* delivery of anti-CD6 d1 cumulative doses, possibly due to higher availability at the tumour site.

Clinical trials using the human version of anti-CD6 d1, Itolizumab, to treat RA suggested a preferential use of lower dosages compared to high doses since they allowed for longer term responses. [6] This goes in accordance with our mice results where we also observed a dose dependent effect. In fact, it is interesting to notice that when high doses are used not only did we observe an increase in disease severity *in-vivo,* but also an increase in Th1 polarization coupled with a decrease in Treg polarization *in-vitro.*

Because we suspected that anti-CD6 d1 impact over T cell functional specialization would be no more than a consequence of steric hindrance compromising CD6-CD166 ligation, we decided to investigate this. Our murine results partially supported this hypothesis. Also we observed that the anti-CD6 d1 immunomodulatory effect is lost when physiologic conditions are not met, meaning supra-physiologic stimulation like anti-CD3 and anti-CD28 activation masks the immunomodulatory impact of anti-CD6. Observations supported by the literature, where Consuegra-Fernandez et al 2017 also shows that under contact-independent and supra-physiological activating conditions the differences observed for *in vitro* Treg induction, between $CD6^{-/-}$ and $CD6^{+/+}$ T cells, disappear. [24]

Such an impact, sensitive to supra-physiological conditions and that seems to be directly associated with activation signalling, highlights its specific nature instead of a pan impact usually correlated with major alterations over proliferation and viability which is not the case.

The somewhat paradoxical nature of anti-CD6 d1 in a model of EAE, where low doses were protective, and high doses detrimental, shed the hypothesis for a beneficial impact over other disease models should different dosages be applied.

We decided to explore that possibility and to achieve this we used a mouse model of breast cancer, the E0771 breast cancer cell line model. Indeed, according, to the literature, breast cancer belongs to a list of cancer types where infiltration of Tregs in the tumours correlates with a poor prognosis, and since CD6 targeting with high doses reduced the induction of Tregs, we investigated the impact of CD6 targeting in this scenario. [25]

We observed that only when administering *in-situ* cumulative doses of anti-CD6 d1, we were able to slow down tumour growth. Such observation, although not statistically significant, might be explained by a tumour tissue restricted reduction of infiltrating $CD25^{+}Foxp3^{+}$ regulatory T cells and an increased ability to secrete IL-17 compared to control group. Several groups have already reported the existence of an IL17Foxp3 double positive $CD4^{+}$ T cell population, known for being induced through MAPK activation pathways, which induces IL1β thus resulting in this phenotype. [26-29] Indeed, Ibáñez et al 2006 have shown that CD6 activation with its

ligand results in the activation of three MAPK cascades, which, despite the need for further studies, appears to go in line with our preliminary observations.[21]

When *in-situ* cumulative doses or even doses like 1mg/mouse were tested, we noticed a negative impact over activation as measured by CD25 MFI levels similarly to Carrasco et al 2017. There they also showed a correlation between CD6 immune-modulation and CD25 expression, and although this is humans, we might contemplate the possibility of investigating it and verify if higher human CD6 expression also results in CD25 downregulation.[30]

So, the use of high doses in the form of *in-situ* cumulative dosage, although not statistically significant and preliminary, points to a protective effect against breast cancer development in this mouse model. Despite the obvious necessity for validation, it was interesting to observe that the negative impact of CD6 d1 targeting over the regulatory T cell population still persisted as previously shown by *in-vitro* data from chapter I.

Indeed, even when transposed to human cells, anti-CD6 d1 (Itolizumab) continued to diminish Tregs polarization *in-vitro.* But not only did it diminish Treg polarization, it also, like in mice, favoured Th1 polarization, again, in a dose dependent manner.

Therefore, the dose factor was crucial for the impact of anti-CD6 d1 mAb over T cell functional specialization. However, and contrary to mice, Itolizumab did significantly impact proliferation, despite its small amplitude. Moreover, now in line with published mice data, increasing treatment doses caused cell cycle arrest at intermediate divisions as shown by CTV plots.[17] Survival, on its turn, was unaffected by Itolizumab treatment both in humans and mice.

To support even more this parallelism between mice and human anti-CD6 d1, we also investigated a possible impact attributable to steric hindrance. Thus, we activated T cells without APCs, using instead anti-CD3 and anti-CD28 so that ALCAM would not be present. Again, just as in mice, no difference between the experimental and the control group was detected. This, although not enough, suggested that Itolizumab impacts T cell differentiation not only through disruption of CD6 d3-ALCAM interactions, as supported by Bughani et al 2017, but also in

accordance with Oliveira et al. 2012 the binding of anti-CD6 might be *per se* enough to impact activation signalling and proliferation[23,17].

For more than 50 years, the mixed lymphocyte reactions (MLR) was considered as one of the most physiological ways of activating T cells. When considering human cells, where antigen specific activation is very difficult, MLR becomes even more relevant. [15] However, when we targeted CD6 with Itolizumab, T cells did not activate what was only overcame after adding anti-CD3 to the cultures.[15] Addition of anti-CD3 allowed not only to finally activate T cells, but also to reproduce mice data, where Itolizumab also increased Th1 induction and decreased Treg induction in a dose dependent manner. In fact, in 2010 Nair et al also showed CD6 co-stimulation involvement in Th1 commitment, thereby supporting the observations.[16]

As just pointed out, the activation strategy turned out to be a crucial element of anti-CD6 d1 impact over T cell functional specialization. This happens because depending on the type of activation, Itolizumab may or may not interfere with T cell polarization. So, if we used iPBMCs with anti-CD3, Itolizumab would decrease Treg polarization, but if instead we used anti-CD3/anti-CD28, or even a SAg mix and APCs, Itolizumab would no longer impact on polarization. This differential impact over polarization could not be only a consequence of steric hindrance, causing CD6 d3- ALCAM interactions to disrupt, since both conditions masked Itolizumab impact on polarization and the first had no ALCAM at all but the second had. Also, the impact over T cell activation itself was different depending on the strategy used. If we used anti-CD3/anti-CD28, like Bughani et al 2017, Itolizumab would induce less CD25 expression, whereas if we used a SAg mix instead, we would increase CD25 expression.

By using different activation strategies, we were then able to expose several signalling and activation pathways affected by Itolizumab. To answer the question of which are the signal elements and pathways affected by CD6 targeting and how these will specifically impact on T cell activation and polarization, further investigation is pivotal. If we go through the literature, we know already that TCR activation and ZAP70 phosphorylation facilitates CD6 interaction with the TCR, which is expected since CD6 physically interacts with the CD3 complex through

direct contact with Lck.[18]Also, the role of CD6 as a signalling hub appears to be important for SLP-76 and Vav1 recruitment, both of them adaptor proteins.[19,20] Indeed, it will be the ability of CD6 to hyper-phosphorylate tyrosine, serine and threonine residues on its very long cytoplasmic tail that will be responsible for SLP76 recruitment, and, consequently, for the time and dose dependent activation of MAPKinases.[17,21]

In summary, our data shows that therapeutic antibodies, specifically anti-CD6 d1, display dosage dependent immunomodulatory effects, and only when used at high doses do they prevent Treg induction, while favouring Th1 differentiation at lower doses. This impact over functional specificity might be related to different levels of activation favouring alternative polarization phenotypes [22].

This stands out the importance of dosage, drawing our attention to the fact that the same drug might be useful for different therapeutic targets depending on the dose. Considering our results are consensual between human and mice, data obtained from mice anti-CD6 d1 can be used as a pre-clinical proof of concept.

References

References

5. References

1. Eberl G. Immunity by equilibrium. *Nat Rev Immunol.* 2016;16(8):524-532.
2. Turvey SE, Broide DH. Innate immunity. *J Allergy Clin Immunol.* 2010;125(2 Suppl 2):S24-32.
3. Janeway CA, Medzhitov R. Innate immune recognition. *Annu Rev Immunol.* 2002;20:197-216.
4. Gay NJ, Symmons MF, Gangloff M, Bryant CE. Assembly and localization of Toll-like receptor signalling complexes. *Nat Rev Immunol.* 2014;14(8):546-558.
5. Gay NJ, Gangloff M. Structure and function of Toll receptors and their ligands. *Annu Rev Biochem.* 2007;76:141-165.
6. Nesargikar PN, Spiller B, Chavez R. The complement system: history, pathways, cascade and inhibitors. *European Journal of Microbiology & Immunology.* 2012;2(2):103-111.
7. Kaplan A, Lee MW, Wolf AJ, et al. Direct Antimicrobial Activity of IFN-β. *The Journal of Immunology.* 2017;198(10):4036-4045.
8. Marichal T, Mesnil C, Bureau F. Homeostatic Eosinophils: Characteristics and Functions. *Frontiers in Medicine.* 2017;4:101.
9. Jacobsen EA, Helmers RA, Lee JJ, Lee NA. The expanding role(s) of eosinophils in health and disease. *Blood.* 2012;120(19):3882-3890.
10. Zook EC, Kee BL. Development of innate lymphoid cells. *Nat Immunol.* 2016;17(7):775-782.
11. Klose CS, Artis D. Innate lymphoid cells as regulators of immunity, inflammation and tissue homeostasis. *Nat Immunol.* 2016;17(7):765-774.
12. Rivera A, Siracusa MC, Yap GS, Gause WC. Innate cell communication kick-starts pathogen-specific immunity. *Nat Immunol.* 2016;17(4):356-363.
13. Breedveld A, Groot Kormelink T, van Egmond M, de Jong EC. Granulocytes as modulators of dendritic cell function. *J Leukoc Biol.* 2017;102(4):1003-1016.
14. Behring, E. v. K, S. *Ueber das Zustandekommen der Diphtherie-Immunität und der Tetanus-Immunität bei Thieren*. In: Philipps-Universität Marburg; 1890.
15. Ehrlich P. Nobel Lecture , December 11, 1908. In. Nobel lectures, Physiology

or Medicine, 1901–1921 Elsevier; 1967.
16. Tiselius A, Kabat EA. AN ELECTROPHORETIC STUDY OF IMMUNE SERA AND PURIFIED ANTIBODY PREPARATIONS. *J Exp Med.* 1939;69(1):119-131.
17. FAGRAEUS A. The plasma cellular reaction and its relation to the formation of antibodies in vitro. *J Immunol.* 1948;58(1):1-13.
18. COONS AH, LEDUC EH, CONNOLLY JM. Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. *J Exp Med.* 1955;102(1):49-60.
19. COOPER MD, PETERSON RD, GOOD RA. DELINEATION OF THE THYMIC AND BURSAL LYMPHOID SYSTEMS IN THE CHICKEN. *Nature.* 1965;205:143-146.
20. Tonegawa S. Somatic generation of antibody diversity. *Nature.* 1983;302(5909):575-581.
21. Jackson KJL, Kidd MJ, Wang Y, Collins AM. The Shape of the Lymphocyte Receptor Repertoire: Lessons from the B Cell Receptor. *Frontiers in Immunology.* 2013;4:263.

22. Bassing CH, Swat W, Alt FW. The mechanism and regulation of chromosomal V(D)J recombination. *Cell.* 2002;109 Suppl:S45-55.
23. Stavnezer J, Schrader CE. Ig heavy chain class switch recombination: mechanism and regulation. *Journal of immunology (Baltimore, Md : 1950).* 2014;193(11):5370-5378.
24. Williams JW, Tjota MY, Sperling AI. The contribution of allergen-specific IgG to the development of th2-mediated airway inflammation. *J Allergy (Cairo).* 2012;2012:236075.
25. Sideras P, Bergstedt-Lindqvist S, Severinson E. Partial biochemical characterization of IgG1-inducing factor. *Eur J Immunol.* 1985;15(6):593-598.
26. Toellner K-M, Luther SA, Sze DMY, et al. T Helper 1 (Th1) and Th2 Characteristics Start to Develop During T Cell Priming and Are Associated with an Immediate Ability to Induce Immunoglobulin Class Switching. *The Journal of Experimental Medicine.* 1998;187(8):1193-1204.
27. Mantis NJ, Rol N, Corthesy B. Secretory IgA's complex roles in immunity and mucosal homeostasis in the gut. *Mucosal Immunol.* 2011;4(6):603-611.
28. Tangye SG, Ma CS, Brink R, Deenick EK. The good, the bad and the ugly - TFH cells in human health and disease. *Nat Rev Immunol.* 2013;13(6):412-426.
29. Kurosaki T, Kometani K, Ise W. Memory B cells. *Nat Rev Immunol.* 2015;15(3):149-159.
30. Inlay MA, Bhattacharya D, Sahoo D, et al. Ly6d marks the earliest stage of B-cell specification and identifies the branchpoint between B-cell and T-cell development. *Genes Dev.* 2009;23(20):2376-2381.
31. Richie Ehrlich LI, Serwold T, Weissman IL. In vitro assays misrepresent in vivo lineage potentials of murine lymphoid progenitors. *Blood.* 2011;117(9):2618-2624.
32. Petrie HT, Zúñiga-Pflücker JC. Zoned out: functional mapping of stromal signaling microenvironments in the thymus. *Annu Rev Immunol.* 2007;25:649-679.
33. Takahama Y. Journey through the thymus: stromal guides for T-cell development and selection. *Nat Rev Immunol.* 2006;6(2):127-135.
34. Balciunaite G, Ceredig R, Rolink AG. The earliest subpopulation of mouse thymocytes contains potent T, significant macrophage, and natural killer cell but no B-lymphocyte potential. *Blood.* 2005;105(5):1930-1936.
35. Ciofani M, Knowles GC, Wiest DL, von Boehmer H, Zúñiga-Pflücker JC. Stage-specific and differential notch dependency at the alphabeta and gammadelta T lineage bifurcation. *Immunity.* 2006;25(1):105-116.
36. von Boehmer H. Unique features of the pre-T-cell receptor alpha-chain: not just a surrogate. *Nat Rev Immunol.* 2005;5(7):571-577.
37. Ciofani M, Zúñiga-Pflücker JC. Notch promotes survival of pre-T cells at the beta-selection checkpoint by regulating cellular metabolism. *Nat Immunol.* 2005;6(9):881-888.
38. Porritt HE, Gordon K, Petrie HT. Kinetics of steady-state differentiation and mapping of intrathymic-signaling environments by stem cell transplantation in nonirradiated mice. *J Exp Med.* 2003;198(6):957-962.
39. Klein L, Hinterberger M, Wirnsberger G, Kyewski B. Antigen presentation in the thymus for positive selection and central tolerance induction. *Nat Rev Immunol.* 2009;9(12):833-844.
40. Anderson MS, Su MA. AIRE expands: new roles in immune tolerance and beyond. *Nature Reviews Immunology.* 2016;16:247.
41. Sakaguchi S, Wing K, Yamaguchi T. Dynamics of peripheral tolerance and immune regulation mediated by Treg. *Eur J Immunol.* 2009;39(9):2331-2336.
42. Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. *Nature reviews Immunology.* 2013;13(4):227-242.

43. Roberts S, Girardi M. Conventional and Unconventional T Cells. In: Gaspari AA, Tyring SK, eds. *Clinical and Basic Immunodermatology.* London: Springer London; 2008:85-104.
44. MacDonald HR. NKT cells: In the beginning…. *European Journal of Immunology.* 2007;37(S1):S111-S115.
45. Godfrey DI, Kronenberg M. Going both ways: immune regulation via CD1d-dependent NKT cells. *J Clin Invest.* 2004;114(10):1379-1388.
46. Gumperz JE, Miyake S, Yamamura T, Brenner MB. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J Exp Med.* 2002;195(5):625-636.
47. Lee PT, Benlagha K, Teyton L, Bendelac A. Distinct functional lineages of human V(alpha)24 natural killer T cells. *J Exp Med.* 2002;195(5):637-641.
48. Coquet JM, Kyparissoudis K, Pellicci DG, et al. IL-21 is produced by NKT cells and modulates NKT cell activation and cytokine production. *J Immunol.* 2007;178(5):2827-2834.
49. Michel ML, Keller AC, Paget C, et al. Identification of an IL-17-producing NK1.1(neg) iNKT cell population involved in airway neutrophilia. *J Exp Med.* 2007;204(5):995-1001.
50. Novy P, Quigley M, Huang X, Yang Y. CD4 T Cells Are Required for CD8 T Cell Survival during Both Primary and Memory Recall Responses. *The Journal of Immunology.* 2007;179(12):8243-8251.
51. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol.* 1986;136(7):2348-2357.
52. Kaliński P, Hilkens CM, Wierenga EA, Kapsenberg ML. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. *Immunol Today.* 1999;20(12):561-567.
53. Agaugue S, Marcenaro E, Ferranti B, Moretta L, Moretta A. Human natural killer cells exposed to IL-2, IL-12, IL-18, or IL-4 differently modulate priming of naive T cells by monocyte-derived dendritic cells. *Blood.* 2008;112(5):1776-1783.
54. Thieu VT, Yu Q, Chang HC, et al. Signal transducer and activator of transcription 4 is required for the transcription factor T-bet to promote T helper 1 cell-fate determination. *Immunity.* 2008;29(5):679-690.
55. Acharya S, Timilshina M, Jiang L, et al. Amelioration of Experimental autoimmune encephalomyelitis and DSS induced colitis by NTG-A-009 through the inhibition of Th1 and Th17 cells differentiation. *Sci Rep.* 2018;8(1):7799.
56. O'Connor RA, Prendergast CT, Sabatos CA, et al. Cutting edge: Th1 cells facilitate the entry of Th17 cells to the central nervous system during experimental autoimmune encephalomyelitis. *J Immunol.* 2008;181(6):3750-3754.
57. Walker JA, McKenzie ANJ. TH2 cell development and function. *Nat Rev Immunol.* 2018;18(2):121-133.
58. Bao K, Reinhardt RL. The differential expression of IL-4 and IL-13 and its impact on type-2 immunity. *Cytokine.* 2015;75(1):25-37.
59. Grünig G, Warnock M, Wakil AE, et al. Requirement for IL-13 Independently of IL-4 in Experimental Asthma. *Science (New York, NY).* 1998;282(5397):2261-2263.
60. Gershon RK, Kondo K. Cell interactions in the induction of tolerance: the role of thymic lymphocytes. *Immunology.* 1970;18(5):723-737.
61. Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol.* 2004;22:531-562.
62. Shevach EM. The resurrection of T cell-mediated suppression. *J Immunol.* 2011;186(7):3805-3807.

63. Shevach EM. Regulatory T cells in autoimmmunity*. *Annu Rev Immunol.* 2000;18:423-449.
64. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science.* 2003;299(5609):1057-1061.
65. Brunkow ME, Jeffery EW, Hjerrild KA, et al. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet.* 2001;27(1):68-73.
66. Bennett CL, Christie J, Ramsdell F, et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nat Genet.* 2001;27(1):20-21.
67. Cai S, Han HJ, Kohwi-Shigematsu T. Tissue-specific nuclear architecture and gene expression regulated by SATB1. *Nat Genet.* 2003;34(1):42-51.
68. Lio CW, Hsieh CS. A two-step process for thymic regulatory T cell development. *Immunity.* 2008;28(1):100-111.
69. Tai X, Erman B, Alag A, et al. Foxp3 transcription factor is proapoptotic and lethal to developing regulatory T cells unless counterbalanced by cytokine survival signals. *Immunity.* 2013;38(6):1116-1128.
70. Kitagawa Y, Ohkura N, Kidani Y, et al. Guidance of regulatory T cell development by Satb1-dependent super-enhancer establishment. *Nat Immunol.* 2017;18(2):173-183.
71. Li Z, Li D, Tsun A, Li B. FOXP3+ regulatory T cells and their functional regulation. *Cellular And Molecular Immunology.* 2015;12:558.
72. Velaga S, Ukena SN, Dringenberg U, et al. Granzyme A Is Required for Regulatory T-Cell Mediated Prevention of Gastrointestinal Graft-versus-Host Disease. *PLOS ONE.* 2015;10(4):e0124927.
73. Garín MI, Chu CC, Golshayan D, Cernuda-Morollón E, Wait R, Lechler RI. Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells. *Blood.* 2007;109(5):2058-2065.
74. Oderup C, Cederbom L, Makowska A, Cilio CM, Ivars F. Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression. *Immunology.* 2006;118(2):240-249.
75. Sarris M, Andersen KG, Randow F, Mayr L, Betz AG. Neuropilin-1 expression on regulatory T cells enhances their interactions with dendritic cells during antigen recognition. *Immunity.* 2008;28(3):402-413.
76. Kearley J, Barker JE, Robinson DS, Lloyd CM. Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent. *J Exp Med.* 2005;202(11):1539-1547.
77. Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *J Exp Med.* 1999;190(7):995-1004.
78. Nakamura K, Kitani A, Strober W. Cell contact-dependent immunosuppression by CD4(+)CD25(+) regulatory T cells is mediated by cell surface-bound transforming growth factor beta. *J Exp Med.* 2001;194(5):629-644.
79. Wahl SM, Swisher J, McCartney-Francis N, Chen W. TGF-beta: the perpetrator of immune suppression by regulatory T cells and suicidal T cells. *J Leukoc Biol.* 2004;76(1):15-24.
80. Collison LW, Workman CJ, Kuo TT, et al. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature.* 2007;450(7169):566-569.
81. Thornton AM, Shevach EM. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med.* 1998;188(2):287-296.
82. Bopp T, Becker C, Klein M, et al. Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression. *J Exp Med.* 2007;204(6):1303-1310.

83. Deaglio S, Dwyer KM, Gao W, et al. Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression. *J Exp Med.* 2007;204(6):1257-1265.
84. Kobie JJ, Shah PR, Yang L, Rebhahn JA, Fowell DJ, Mosmann TR. T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5'-adenosine monophosphate to adenosine. *J Immunol.* 2006;177(10):6780-6786.
85. Harrington LE, Hatton RD, Mangan PR, et al. Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. *Nat Immunol.* 2005;6(11):1123-1132.
86. Gran B, Chu N, Zhang GX, et al. Early administration of IL-12 suppresses EAE through induction of interferon-gamma. *J Neuroimmunol.* 2004;156(1-2):123-131.
87. Voorthuis JA, Uitdehaag BM, De Groot CJ, Goede PH, van der Meide PH, Dijkstra CD. Suppression of experimental allergic encephalomyelitis by intraventricular administration of interferon-gamma in Lewis rats. *Clin Exp Immunol.* 1990;81(2):183-188.
88. Billiau A, Heremans H, Vandekerckhove F, et al. Enhancement of experimental allergic encephalomyelitis in mice by antibodies against IFN-gamma. *J Immunol.* 1988;140(5):1506-1510.
89. Willenborg DO, Fordham SA, Staykova MA, Ramshaw IA, Cowden WB. IFN-gamma is critical to the control of murine autoimmune encephalomyelitis and regulates both in the periphery and in the target tissue: a possible role for nitric oxide. *J Immunol.* 1999;163(10):5278-5286.
90. Langrish CL, Chen Y, Blumenschein WM, et al. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med.* 2005;201(2):233-240.
91. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity.* 2006;24(2):179-189.
92. Mangan PR, Harrington LE, O'Quinn DB, et al. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature.* 2006;441(7090):231-234.
93. Bettelli E, Carrier Y, Gao W, et al. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature.* 2006;441(7090):235-238.
94. Elson CO, Cong Y, Weaver CT, et al. Monoclonal anti-interleukin 23 reverses active colitis in a T cell-mediated model in mice. *Gastroenterology.* 2007;132(7):2359-2370.
95. Ivanov II, McKenzie BS, Zhou L, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell.* 2006;126(6):1121-1133.
96. McGeachy MJ, Cua DJ. Th17 Cell Differentiation: The Long and Winding Road. *Immunity.* 2008;28(4):445-453.
97. Jin W, Dong C. IL-17 cytokines in immunity and inflammation. *Emerging Microbes &Amp; Infections.* 2013;2:e60.
98. Park H, Li Z, Yang XO, et al. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol.* 2005;6(11):1133-1141.
99. Sun CM, Hall JA, Blank RB, et al. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med.* 2007;204(8):1775-1785.
100. Mucida D, Kutchukhidze N, Erazo A, Russo M, Lafaille JJ, Curotto de Lafaille MA. Oral tolerance in the absence of naturally occurring Tregs. *J Clin Invest.* 2005;115(7):1923-1933.
101. Coombes JL, Siddiqui KR, Arancibia-Cárcamo CV, et al. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med.* 2007;204(8):1757-1764.

102. Strauch UG, Obermeier F, Grunwald N, et al. Influence of intestinal bacteria on induction of regulatory T cells: lessons from a transfer model of colitis. *Gut.* 2005;54(11):1546-1552.
103. Ostman S, Rask C, Wold AE, Hultkrantz S, Telemo E. Impaired regulatory T cell function in germ-free mice. *Eur J Immunol.* 2006;36(9):2336-2346.
104. Ishikawa H, Tanaka K, Maeda Y, et al. Effect of intestinal microbiota on the induction of regulatory CD25+ CD4+ T cells. *Clin Exp Immunol.* 2008;153(1):127-135.
105. Taylor PA, Friedman TM, Korngold R, Noelle RJ, Blazar BR. Tolerance induction of alloreactive T cells via ex vivo blockade of the CD40:CD40L costimulatory pathway results in the generation of a potent immune regulatory cell. *Blood.* 2002;99(12):4601-4609.
106. Li MO, Sanjabi S, Flavell RA. Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. *Immunity.* 2006;25(3):455-471.
107. Gottschalk RA, Corse E, Allison JP. TCR ligand density and affinity determine peripheral induction of Foxp3 in vivo. *J Exp Med.* 2010;207(8):1701-1711.
108. Idoyaga J, Fiorese C, Zbytnuik L, et al. Specialized role of migratory dendritic cells in peripheral tolerance induction. *J Clin Invest.* 2013;123(2):844-854.
109. Qin S, Cobbold SP, Pope H, et al. "Infectious" transplantation tolerance. *Science.* 1993;259(5097):974.
110. Zhou L, Chong MM, Littman DR. Plasticity of CD4+ T cell lineage differentiation. *Immunity.* 2009;30(5):646-655.
111. Zhou X, Bailey-Bucktrout S, Jeker LT, Bluestone JA. Plasticity of CD4(+) FoxP3(+) T cells. *Curr Opin Immunol.* 2009;21(3):281-285.
112. Bailey-Bucktrout SL, Bluestone JA. Regulatory T cells: stability revisited. *Trends Immunol.* 2011;32(7):301-306.
113. Miyao T, Floess S, Setoguchi R, et al. Plasticity of Foxp3(+) T cells reflects promiscuous Foxp3 expression in conventional T cells but not reprogramming of regulatory T cells. *Immunity.* 2012;36(2):262-275.
114. Hsu P, Santner-Nanan B, Dahlstrom JE, et al. Altered decidual DC-SIGN+ antigen-presenting cells and impaired regulatory T-cell induction in preeclampsia. *Am J Pathol.* 2012;181(6):2149-2160.
115. Rowe JH, Ertelt JM, Xin L, Way SS. Pregnancy imprints regulatory memory that sustains anergy to fetal antigen. *Nature.* 2012;490(7418):102-106.
116. Curotto de Lafaille MA, Kutchukhidze N, Shen S, Ding Y, Yee H, Lafaille JJ. Adaptive Foxp3+ regulatory T cell-dependent and -independent control of allergic inflammation. *Immunity.* 2008;29(1):114-126.
117. Josefowicz SZ, Niec RE, Kim HY, et al. Extrathymically generated regulatory T cells control mucosal TH2 inflammation. *Nature.* 2012;482(7385):395-399.
118. Samstein RM, Josefowicz SZ, Arvey A, Treuting PM, Rudensky AY. Extrathymic generation of regulatory T cells in placental mammals mitigates maternal-fetal conflict. *Cell.* 2012;150(1):29-38.
119. Lathrop SK, Bloom SM, Rao SM, et al. Peripheral education of the immune system by colonic commensal microbiota. *Nature.* 2011;478(7368):250-254.
120. Getts DR, Turley DM, Smith CE, et al. Tolerance induced by apoptotic antigen-coupled leukocytes is induced by PD-L1+ and IL-10-producing splenic macrophages and maintained by T regulatory cells. *J Immunol.* 2011;187(5):2405-2417.
121. Jones A, Opejin A, Henderson JG, et al. Peripherally Induced Tolerance Depends on Peripheral Regulatory T Cells That Require Hopx To Inhibit Intrinsic IL-2 Expression. *J Immunol.* 2015;195(4):1489-1497.

122. Miller JF, Mitchell GF. Cell to cell interaction in the immune response. I. Hemolysin-forming cells in neonatally thymectomized mice reconstituted with thymus or thoracic duct lymphocytes. *J Exp Med.* 1968;128(4):801-820.
123. MacLennan IC. Germinal centers. *Annu Rev Immunol.* 1994;12:117-139.
124. Allen CD, Okada T, Cyster JG. Germinal-center organization and cellular dynamics. *Immunity.* 2007;27(2):190-202.
125. Victora GD, Nussenzweig MC. Germinal centers. *Annu Rev Immunol.* 2012;30:429-457.
126. Mosmann TR, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol.* 1989;7:145-173.
127. Banchereau J, Bazan F, Blanchard D, et al. The CD40 antigen and its ligand. *Annu Rev Immunol.* 1994;12:881-922.
128. Ansel KM, McHeyzer-Williams LJ, Ngo VN, McHeyzer-Williams MG, Cyster JG. In vivo-activated CD4 T cells upregulate CXC chemokine receptor 5 and reprogram their response to lymphoid chemokines. *J Exp Med.* 1999;190(8):1123-1134.
129. Förster R, Mattis AE, Kremmer E, Wolf E, Brem G, Lipp M. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell.* 1996;87(6):1037-1047.
130. Breitfeld D, Ohl L, Kremmer E, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med.* 2000;192(11):1545-1552.
131. Schaerli P, Willimann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med.* 2000;192(11):1553-1562.
132. Nurieva RI, Chung Y, Martinez GJ, et al. Bcl6 mediates the development of T follicular helper cells. *Science.* 2009;325(5943):1001-1005.
133. Johnston RJ, Poholek AC, DiToro D, et al. Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science.* 2009;325(5943):1006-1010.
134. Yu D, Rao S, Tsai LM, et al. The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity.* 2009;31(3):457-468.
135. Tubo NJ, Pagán AJ, Taylor JJ, et al. Single naive CD4+ T cells from a diverse repertoire produce different effector cell types during infection. *Cell.* 2013;153(4):785-796.
136. Choi YS, Eto D, Yang JA, Lao C, Crotty S. Cutting edge: STAT1 is required for IL-6-mediated Bcl6 induction for early follicular helper cell differentiation. *J Immunol.* 2013;190(7):3049-3053.
137. Choi YS, Kageyama R, Eto D, et al. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity.* 2011;34(6):932-946.
138. Liu X, Chen X, Zhong B, et al. Transcription factor achaete-scute homologue 2 initiates follicular T-helper-cell development. *Nature.* 2014;507(7493):513-518.
139. Liu X, Lu H, Chen T, et al. Genome-wide Analysis Identifies Bcl6-Controlled Regulatory Networks during T Follicular Helper Cell Differentiation. *Cell Rep.* 2016;14(7):1735-1747.
140. Vinuesa CG, Cyster JG. How T cells earn the follicular rite of passage. *Immunity.* 2011;35(5):671-680.
141. Baumjohann D, Preite S, Reboldi A, et al. Persistent antigen and germinal center B cells sustain T follicular helper cell responses and phenotype. *Immunity.* 2013;38(3):596-605.
142. Deenick EK, Chan A, Ma CS, et al. Follicular helper T cell differentiation requires continuous antigen presentation that is independent of unique B cell signaling. *Immunity.* 2010;33(2):241-253.

143. Wei M, Shinkura R, Doi Y, Maruya M, Fagarasan S, Honjo T. Mice carrying a knock-in mutation of Aicda resulting in a defect in somatic hypermutation have impaired gut homeostasis and compromised mucosal defense. *Nat Immunol.* 2011;12(3):264-270.
144. Liang HE, Reinhardt RL, Bando JK, Sullivan BM, Ho IC, Locksley RM. Divergent expression patterns of IL-4 and IL-13 define unique functions in allergic immunity. *Nat Immunol.* 2011;13(1):58-66.
145. Simpson N, Gatenby PA, Wilson A, et al. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. *Arthritis Rheum.* 2010;62(1):234-244.
146. Szabo K, Papp G, Barath S, Gyimesi E, Szanto A, Zeher M. Follicular helper T cells may play an important role in the severity of primary Sjögren's syndrome. *Clin Immunol.* 2013;147(2):95-104.
147. Morita R, Schmitt N, Bentebibel SE, et al. Human blood CXCR5(+)CD4(+) T cells are counterparts of T follicular cells and contain specific subsets that differentially support antibody secretion. *Immunity.* 2011;34(1):108-121.
148. Gu-Trantien C, Loi S, Garaud S, et al. $CD4^+$ follicular helper T cell infiltration predicts breast cancer survival. *J Clin Invest.* 2013;123(7):2873-2892.
149. Bindea G, Mlecnik B, Tosolini M, et al. Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity.* 2013;39(4):782-795.
150. Schmitt E, Klein M, Bopp T. Th9 cells, new players in adaptive immunity. *Trends Immunol.* 2014;35(2):61-68.
151. Veldhoen M, Uyttenhove C, van Snick J, et al. Transforming growth factor-beta 'reprograms' the differentiation of T helper 2 cells and promotes an interleukin 9-producing subset. *Nat Immunol.* 2008;9(12):1341-1346.
152. Dardalhon V, Awasthi A, Kwon H, et al. IL-4 inhibits TGF-beta-induced Foxp3+ T cells and, together with TGF-beta, generates IL-9+ IL-10+ Foxp3(-) effector T cells. *Nat Immunol.* 2008;9(12):1347-1355.
153. Gessner A, Blum H, Röllinghoff M. Differential regulation of IL-9-expression after infection with Leishmania major in susceptible and resistant mice. *Immunobiology.* 1993;189(5):419-435.
154. Li J, Chen S, Xiao X, Zhao Y, Ding W, Li XC. IL-9 and Th9 cells in health and diseases-From tolerance to immunopathology. *Cytokine Growth Factor Rev.* 2017;37:47-55.
155. Licona-Limón P, Henao-Mejia J, Temann AU, et al. Th9 Cells Drive Host Immunity against Gastrointestinal Worm Infection. *Immunity.* 2013;39(4):744-757.
156. Purwar R, Schlapbach C, Xiao S, et al. Robust tumor immunity to melanoma mediated by interleukin-9-producing T cells. *Nat Med.* 2012;18(8):1248-1253.
157. Lu Y, Hong S, Li H, et al. Th9 cells promote antitumor immune responses in vivo. *J Clin Invest.* 2012;122(11):4160-4171.
158. Kara EE, Comerford I, Bastow CR, et al. Distinct chemokine receptor axes regulate Th9 cell trafficking to allergic and autoimmune inflammatory sites. *J Immunol.* 2013;191(3):1110-1117.
159. Jäger A, Dardalhon V, Sobel RA, Bettelli E, Kuchroo VK. Th1, Th17, and Th9 effector cells induce experimental autoimmune encephalomyelitis with different pathological phenotypes. *J Immunol.* 2009;183(11):7169-7177.
160. Pang N, Xu J, Qu J, et al. Peripheral blood Th9 cells reconstitution and its relationship with acute graft-versus-host disease after matched-sibling peripheral blood hematopoietic stem cell transplantation. *Am J Transl Res.* 2017;9(8):3623-3632.

161. Staudt V, Bothur E, Klein M, et al. Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity.* 2010;33(2):192-202.
162. Chang HC, Sehra S, Goswami R, et al. The transcription factor PU.1 is required for the development of IL-9-producing T cells and allergic inflammation. *Nat Immunol.* 2010;11(6):527-534.
163. Goswami R, Jabeen R, Yagi R, et al. STAT6-dependent regulation of Th9 development. *J Immunol.* 2012;188(3):968-975.
164. Perumal NB, Kaplan MH. Regulating Il9 transcription in T helper cells. *Trends Immunol.* 2011;32(4):146-150.
165. Hegazy AN, Peine M, Helmstetter C, et al. Interferons direct Th2 cell reprogramming to generate a stable GATA-3(+)T-bet(+) cell subset with combined Th2 and Th1 cell functions. *Immunity.* 2010;32(1):116-128.
166. Dumoutier L, Van Roost E, Ameye G, Michaux L, Renauld JC. IL-TIF/IL-22: genomic organization and mapping of the human and mouse genes. *Genes Immun.* 2000;1(8):488-494.
167. Eyerich S, Eyerich K, Cavani A, Schmidt-Weber C. IL-17 and IL-22: siblings, not twins. *Trends Immunol.* 2010;31(9):354-361.
168. Eyerich S, Eyerich K, Pennino D, et al. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *J Clin Invest.* 2009;119(12):3573-3585.
169. Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R. IL-22 increases the innate immunity of tissues. *Immunity.* 2004;21(2):241-254.
170. Nograles KE, Zaba LC, Guttman-Yassky E, et al. Th17 cytokines interleukin (IL)-17 and IL-22 modulate distinct inflammatory and keratinocyte-response pathways. *Br J Dermatol.* 2008;159(5):1092-1102.
171. Wolk K, Witte E, Wallace E, et al. IL-22 regulates the expression of genes responsible for antimicrobial defense, cellular differentiation, and mobility in keratinocytes: a potential role in psoriasis. *Eur J Immunol.* 2006;36(5):1309-1323.
172. Boniface K, Bernard FX, Garcia M, Gurney AL, Lecron JC, Morel F. IL-22 inhibits epidermal differentiation and induces proinflammatory gene expression and migration of human keratinocytes. *J Immunol.* 2005;174(6):3695-3702.
173. Liang SC, Tan XY, Luxenberg DP, et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *J Exp Med.* 2006;203(10):2271-2279.
174. Duhen T, Geiger R, Jarrossay D, Lanzavecchia A, Sallusto F. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nat Immunol.* 2009;10(8):857-863.
175. Fujita H, Nograles KE, Kikuchi T, Gonzalez J, Carucci JA, Krueger JG. Human Langerhans cells induce distinct IL-22-producing CD4+ T cells lacking IL-17 production. *Proc Natl Acad Sci U S A.* 2009;106(51):21795-21800.
176. Trifari S, Kaplan CD, Tran EH, Crellin NK, Spits H. Identification of a human helper T cell population that has abundant production of interleukin 22 and is distinct from T(H)-17, T(H)1 and T(H)2 cells. *Nat Immunol.* 2009;10(8):864-871.
177. Riis JL, Johansen C, Vestergaard C, Bech R, Kragballe K, Iversen L. Kinetics and differential expression of the skin-related chemokines CCL27 and CCL17 in psoriasis, atopic dermatitis and allergic contact dermatitis. *Exp Dermatol.* 2011;20(10):789-794.
178. Jiang R, Tan Z, Deng L, et al. Interleukin-22 promotes human hepatocellular carcinoma by activation of STAT3. *Hepatology.* 2011;54(3):900-909.

179. Nograles KE, Zaba LC, Shemer A, et al. IL-22-producing "T22" T cells account for upregulated IL-22 in atopic dermatitis despite reduced IL-17-producing TH17 T cells. *J Allergy Clin Immunol.* 2009;123(6):1244-1252.e1242.
180. Xu X, Tang Y, Guo S, et al. Increased intratumoral interleukin 22 levels and frequencies of interleukin 22-producing CD4+ T cells correlate with pancreatic cancer progression. *Pancreas.* 2014;43(3):470-477.
181. Tao X, Constant S, Jorritsma P, Bottomly K. Strength of TCR signal determines the costimulatory requirements for Th1 and Th2 CD4+ T cell differentiation. *J Immunol.* 1997;159(12):5956-5963.
182. van Panhuys N. TCR Signal Strength Alters T-DC Activation and Interaction Times and Directs the Outcome of Differentiation. *Front Immunol.* 2016;7:6.
183. Kang J-O, Lee J-B, Chang J. Cholera Toxin Promotes Th17 Cell Differentiation by Modulating Expression of Polarizing Cytokines and the Antigen-Presenting Potential of Dendritic Cells. *PLOS ONE.* 2016;11(6):e0157015.
184. Purvis HA, Stoop JN, Mann J, et al. Low-strength T-cell activation promotes Th17 responses. *Blood.* 2010;116(23):4829-4837.
185. Hwang S, Palin AC, Li L, et al. TCR ITAM multiplicity is required for the generation of follicular helper T-cells. *Nature Communications.* 2015;6:6982.
186. Iezzi G, Sonderegger I, Ampenberger F, Schmitz N, Marsland BJ, Kopf M. CD40-CD40L cross-talk integrates strong antigenic signals and microbial stimuli to induce development of IL-17-producing CD4+ T cells. *Proc Natl Acad Sci U S A.* 2009;106(3):876-881.
187. Zhou X, Bailey-Bucktrout SL, Jeker LT, et al. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat Immunol.* 2009;10(9):1000-1007.
188. Hirota K, Duarte JH, Veldhoen M, et al. Fate mapping of IL-17-producing T cells in inflammatory responses. *Nat Immunol.* 2011;12(3):255-263.
189. Becattini S, Latorre D, Mele F, et al. T cell immunity. Functional heterogeneity of human memory CD4$^+$ T cell clones primed by pathogens or vaccines. *Science.* 2015;347(6220):400-406.
190. Han A, Glanville J, Hansmann L, Davis MM. Linking T-cell receptor sequence to functional phenotype at the single-cell level. *Nat Biotechnol.* 2014;32(7):684-692.
191. O'Shea JJ, Paul WE. Mechanisms underlying lineage commitment and plasticity of helper CD4+ T cells. *Science.* 2010;327(5969):1098-1102.
192. Lee YK, Turner H, Maynard CL, et al. Late developmental plasticity in the T helper 17 lineage. *Immunity.* 2009;30(1):92-107.
193. Williams CL, Schilling MM, Cho SH, et al. STAT4 and T-bet are required for the plasticity of IFN-γ expression across Th2 ontogeny and influence changes in Ifng promoter DNA methylation. *J Immunol.* 2013;191(2):678-687.
194. van den Ham HJ, de Boer RJ. Cell division curtails helper phenotype plasticity and expedites helper T-cell differentiation. *Immunol Cell Biol.* 2012;90(9):860-868.
195. Huynh A, DuPage M, Priyadharshini B, et al. Control of PI(3) kinase in Treg cells maintains homeostasis and lineage stability. *Nat Immunol.* 2015;16(2):188-196.
196. Pearce EL, Poffenberger MC, Chang CH, Jones RG. Fueling immunity: insights into metabolism and lymphocyte function. *Science.* 2013;342(6155):1242454.
197. Frauwirth KA, Riley JL, Harris MH, et al. The CD28 signaling pathway regulates glucose metabolism. *Immunity.* 2002;16(6):769-777.
198. Macintyre AN, Gerriets VA, Nichols AG, et al. The glucose transporter Glut1 is selectively essential for CD4 T cell activation and effector function. *Cell Metab.* 2014;20(1):61-72.

199. Basu S, Hubbard B, Shevach EM. Foxp3-mediated inhibition of Akt inhibits Glut1 (glucose transporter 1) expression in human T regulatory cells. *J Leukoc Biol.* 2015;97(2):279-283.
200. Michalek RD, Gerriets VA, Jacobs SR, et al. Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory CD4+ T cell subsets. *J Immunol.* 2011;186(6):3299-3303.
201. Gerriets VA, Kishton RJ, Nichols AG, et al. Metabolic programming and PDHK1 control CD4+ T cell subsets and inflammation. *J Clin Invest.* 2015;125(1):194-207.
202. Shrestha S, Yang K, Guy C, Vogel P, Neale G, Chi H. Treg cells require the phosphatase PTEN to restrain TH1 and TFH cell responses. *Nat Immunol.* 2015;16(2):178-187.
203. DuPage M, Bluestone JA. Harnessing the plasticity of CD4(+) T cells to treat immune-mediated disease. *Nat Rev Immunol.* 2016;16(3):149-163.
204. Saraiva M, Christensen JR, Veldhoen M, Murphy TL, Murphy KM, O'Garra A. Interleukin-10 production by Th1 cells requires interleukin-12-induced STAT4 transcription factor and ERK MAP kinase activation by high antigen dose. *Immunity.* 2009;31(2):209-219.
205. Neumann C, Heinrich F, Neumann K, et al. Role of Blimp-1 in programing Th effector cells into IL-10 producers. *J Exp Med.* 2014;211(9):1807-1819.
206. Selmi C. The worldwide gradient of autoimmune conditions. *Autoimmun Rev.* 2010;9(5):A247-250.
207. Parks CG, Miller FW, Pollard KM, et al. Expert panel workshop consensus statement on the role of the environment in the development of autoimmune disease. *Int J Mol Sci.* 2014;15(8):14269-14297.
208. Lerner A, Matthias T. Changes in intestinal tight junction permeability associated with industrial food additives explain the rising incidence of autoimmune disease. *Autoimmun Rev.* 2015;14(6):479-489.
209. Rook GA, Adams V, Hunt J, Palmer R, Martinelli R, Brunet LR. Mycobacteria and other environmental organisms as immunomodulators for immunoregulatory disorders. *Springer Semin Immunopathol.* 2004;25(3-4):237-255.
210. Rachmilewitz D, Katakura K, Karmeli F, et al. Toll-like receptor 9 signaling mediates the anti-inflammatory effects of probiotics in murine experimental colitis. *Gastroenterology.* 2004;126(2):520-528.
211. Rook GA, Brunet LR. Microbes, immunoregulation, and the gut. *Gut.* 2005;54(3):317-320.
212. Goldenberg MM. Multiple Sclerosis Review. *Pharmacy and Therapeutics.* 2012;37(3):175-184.
213. Hauser SL GD. *Multiple sclerosis and other demyelinating diseases.* 17th ed. New York: McGraw-Hill Medical; 2008.
214. Federation MSI. Multiple Sclerosis International Federation. Atlas of MS 2013. Published 2013 Accessed2018.
215. Lee BP, Imhof BA. Lymphocyte transmigration in the brain: a new way of thinking. *Nat Immunol.* 2008;9(2):117-118.
216. Hoglund RA, Maghazachi AA. Multiple sclerosis and the role of immune cells. *World J Exp Med.* 2014;4(3):27-37.
217. BAC C. *Multiple sclerosis.* New York: Lange Medical Books/McGraw-Hill Medical; 2007.
218. Liu JKH. The history of monoclonal antibody development – Progress, remaining challenges and future innovations. *Annals of Medicine and Surgery.* 2014;3(4):113-116.
219. Mouzaki A, Koutsokera M, Dervilli Z, et al. Remitting–relapsing multiple sclerosis patient refractory to conventional treatments and bone marrow transplantation who responded to natalizumab. *International Journal of General Medicine.* 2010;3:313-320.

220. Correia I, Batista S, Galego O, et al. Long-term effectiveness and safety of natalizumab in a Portuguese population. *Int Immunopharmacol.* 2017;46:105-111.
221. Robinson AP, Harp CT, Noronha A, Miller SD. The experimental autoimmune encephalomyelitis (EAE) model of MS: utility for understanding disease pathophysiology and treatment. *Handbook of clinical neurology.* 2014;122:173-189.
222. Baker D, Amor S. Experimental autoimmune encephalomyelitis is a good model of multiple sclerosis if used wisely. *Mult Scler Relat Disord.* 2014;3(5):555-564.
223. 't Hart BA, Gran B, Weissert R. EAE: imperfect but useful models of multiple sclerosis. *Trends Mol Med.* 2011;17(3):119-125.
224. Leavy O. Therapeutic antibodies: past, present and future. *Nat Rev Immunol.* 2010;10(5):297.
225. Maggon K. Monoclonal antibody "gold rush". *Curr Med Chem.* 2007;14(18):1978-1987.
226. Harding FA, Stickler MM, Razo J, DuBridge RB. The immunogenicity of humanized and fully human antibodies: residual immunogenicity resides in the CDR regions. *MAbs.* 2010;2(3):256-265.
227. Goulet A, Dorvillius M, Pèlegrin A, Barbet J, Baty D. Pharmacokinetic and tumor-seeking properties of recombinant and nonrecombinant anti-carcinoembryonic antigen antibody fragments. *Int J Cancer.* 2002;100(3):367-374.
228. Thurber GM, Schmidt MM, Wittrup KD. Antibody tumor penetration: transport opposed by systemic and antigen-mediated clearance. *Adv Drug Deliv Rev.* 2008;60(12):1421-1434.
229. Yamane-Ohnuki N, Satoh M. Production of therapeutic antibodies with controlled fucosylation. *MAbs.* 2009;1(3):230-236.
230. Allen JG, Mujacic M, Frohn MJ, et al. Facile Modulation of Antibody Fucosylation with Small Molecule Fucostatin Inhibitors and Cocrystal Structure with GDP-Mannose 4,6-Dehydratase. *ACS Chem Biol.* 2016;11(10):2734-2743.
231. Wang W, Wang EQ, Balthasar JP. Monoclonal antibody pharmacokinetics and pharmacodynamics. *Clin Pharmacol Ther.* 2008;84(5):548-558.
232. Kuo TT, Aveson VG. Neonatal Fc receptor and IgG-based therapeutics. *MAbs.* 2011;3(5):422-430.
233. Hinton PR, Xiong JM, Johlfs MG, Tang MT, Keller S, Tsurushita N. An engineered human IgG1 antibody with longer serum half-life. *J Immunol.* 2006;176(1):346-356.
234. Monoclonal Antibodies Approved by the EMA and FDA for Therapeutic Use (status 2017). http://www.actip.org/products/monoclonal-antibodies-approved-by-the-ema-and-fda-for-therapeutic-use/. Accessed.
235. Roche's OCREVUS (ocrelizumab) approved in the European Union for relapsing forms of multiple sclerosis and primary progressive multiple sclerosis. https://www.roche.com/media/store/releases/med-cor-2018-01-12.htm. Accessed.
236. Rommer PS, Dudesek A, Stüve O, Zettl UK. Monoclonal antibodies in treatment of multiple sclerosis. *Clinical and Experimental Immunology.* 2014;175(3):373-384.
237. Bush WS, Moore JH. Chapter 11: Genome-Wide Association Studies. *PLOS Computational Biology.* 2012;8(12):e1002822.
238. Kofler DM, Severson CA, Mousissian N, De Jager PL, Hafler DA. The CD6 multiple sclerosis susceptibility allele is associated with alterations in CD4+ T cell proliferation. *J Immunol.* 2011;187(6):3286-3291.
239. De Jager PL, Jia X, Wang J, et al. Meta-analysis of genome scans and replication identify CD6, IRF8 and TNFRSF1A as new multiple sclerosis susceptibility loci. *Nat Genet.* 2009;41(7):776-782.

240. Bowen MA, Whitney GS, Neubauer M, et al. Structure and chromosomal location of the human CD6 gene: detection of five human CD6 isoforms. *J Immunol.* 1997;158(3):1149-1156.
241. Robinson WH, Neuman de Vegvar HE, Prohaska SS, Rhee JW, Parnes JR. Human CD6 possesses a large, alternatively spliced cytoplasmic domain. *Eur J Immunol.* 1995;25(10):2765-2769.
242. Kobarg J, Whitney GS, Palmer D, Aruffo A, Bowen MA. Analysis of the tyrosine phosphorylation and calcium fluxing of human CD6 isoforms with different cytoplasmatic domains. *Eur J Immunol.* 1997;27(11):2971-2980.
243. Cayrol R, Wosik K, Berard JL, et al. Activated leukocyte cell adhesion molecule promotes leukocyte trafficking into the central nervous system. *Nat Immunol.* 2008;9(2):137-145.
244. Robinson WH, Prohaska SS, Santoro JC, Robinson HL, Parnes JR. Identification of a mouse protein homologous to the human CD6 T cell surface protein and sequence of the corresponding cDNA. *J Immunol.* 1995;155(10):4739-4748.
245. Pal A, Romain PL, Singer NG, Fox D, Stavnezer J. Mouse CD6: sequence of cDNA and expression of mRNA. *Immunol Lett.* 1996;49(1-2):133-137.
246. <1981 Hansen et al CD6 discover.pdf>.
247. Braun M, Muller B, ter Meer D, et al. The CD6 scavenger receptor is differentially expressed on a CD56 natural killer cell subpopulation and contributes to natural killer-derived cytokine and chemokine secretion. *J Innate Immun.* 2011;3(4):420-434.
248. Mayer B, Funke I, Seed B, Riethmuller G, Weiss E. Expression of the CD6 T lymphocyte differentiation antigen in normal human brain. *J Neuroimmunol.* 1990;29(1-3):193-202.
249. Konno A, Ahn JS, Kitamura H, et al. Tissue distribution of CD6 and CD6 ligand in cattle: expression of the CD6 ligand (CD166) in the autonomic nervous system of cattle and the human. *J Leukoc Biol.* 2001;69(6):944-950.
250. Aruffo A, Melnick MB, Linsley PS, Seed B. The lymphocyte glycoprotein CD6 contains a repeated domain structure characteristic of a new family of cell surface and secreted proteins. *J Exp Med.* 1991;174(4):949-952.
251. da Gloria VG, Martins de Araujo M, Mafalda Santos A, et al. T cell activation regulates CD6 alternative splicing by transcription dynamics and SRSF1. *J Immunol.* 2014;193(1):391-399.
252. Swack JA, Mier JW, Romain PL, Hull SR, Rudd CE. Biosynthesis and post-translational modification of CD6, a T cell signal-transducing molecule. *J Biol Chem.* 1991;266(11):7137-7143.
253. Wee S, Schieven GL, Kirihara JM, Tsu TT, Ledbetter JA, Aruffo A. Tyrosine phosphorylation of CD6 by stimulation of CD3: augmentation by the CD4 and CD2 coreceptors. *J Exp Med.* 1993;177(1):219-223.
254. Hassan NJ, Simmonds SJ, Clarkson NG, et al. CD6 regulates T-cell responses through activation-dependent recruitment of the positive regulator SLP-76. *Mol Cell Biol.* 2006;26(17):6727-6738.
255. Joo YS, Singer NG, Endres JL, et al. Evidence for the expression of a second CD6 ligand by synovial fibroblasts. *Arthritis Rheum.* 2000;43(2):329-335.
256. Bowen MA, Patel DD, Li X, et al. Cloning, mapping, and characterization of activated leukocyte-cell adhesion molecule (ALCAM), a CD6 ligand. *J Exp Med.* 1995;181(6):2213-2220.
257. Enyindah-Asonye G, Li Y, Ruth JH, et al. CD318 is a ligand for CD6. *Proc Natl Acad Sci U S A.* 2017;114(33):E6912-E6921.
258. Bowen MA, Bajorath J, Siadak AW, et al. The amino-terminal immunoglobulin-like domain of activated leukocyte cell adhesion molecule binds specifically to the membrane-proximal

scavenger receptor cysteine-rich domain of CD6 with a 1:1 stoichiometry. *J Biol Chem.* 1996;271(29):17390-17396.
259. Zimmerman AW, Joosten B, Torensma R, Parnes JR, van Leeuwen FN, Figdor CG. Long-term engagement of CD6 and ALCAM is essential for T-cell proliferation induced by dendritic cells. *Blood.* 2006;107(8):3212-3220.
260. Te Riet J, Helenius J, Strohmeyer N, Cambi A, Figdor CG, Muller DJ. Dynamic coupling of ALCAM to the actin cortex strengthens cell adhesion to CD6. *J Cell Sci.* 2014;127(Pt 7):1595-1606.
261. Gimferrer I, Calvo M, Mittelbrunn M, et al. Relevance of CD6-mediated interactions in T cell activation and proliferation. *J Immunol.* 2004;173(4):2262-2270.
262. Hassan NJ, Barclay AN, Brown MH. Frontline: Optimal T cell activation requires the engagement of CD6 and CD166. *Eur J Immunol.* 2004;34(4):930-940.
263. Whitney GS, Starling GC, Bowen MA, Modrell B, Siadak AW, Aruffo A. The membrane-proximal scavenger receptor cysteine-rich domain of CD6 contains the activated leukocyte cell adhesion molecule binding site. *J Biol Chem.* 1995;270(31):18187-18190.
264. Bowen MA, Bajorath J, D'Egidio M, et al. Characterization of mouse ALCAM (CD166): the CD6-binding domain is conserved in different homologs and mediates cross-species binding. *Eur J Immunol.* 1997;27(6):1469-1478.
265. Chappell PE, Garner LI, Yan J, et al. Structures of CD6 and Its Ligand CD166 Give Insight into Their Interaction. *Structure.* 2015;23(8):1426-1436.
266. Skonier JE, Bodian DL, Emswiler J, Bowen MA, Aruffo A, Bajorath J. Mutational analysis of the CD6 ligand binding domain. *Protein Eng.* 1997;10(8):943-947.
267. Bodian DL, Skonier JE, Bowen MA, et al. Identification of residues in CD6 which are critical for ligand binding. *Biochemistry.* 1997;36(9):2637-2641.
268. Castro MA, Oliveira MI, Nunes RJ, et al. Extracellular isoforms of CD6 generated by alternative splicing regulate targeting of CD6 to the immunological synapse. *J Immunol.* 2007;178(7):4351-4361.
269. Breuning J, Brown MH. T Cell Costimulation by CD6 Is Dependent on Bivalent Binding of a GADS/SLP-76 Complex. *Mol Cell Biol.* 2017;37(11).
270. Hem CD, Ekornhol M, Granum S, Sundvold-Gjerstad V, Spurkland A. CD6 and Linker of Activated T Cells are Potential Interaction Partners for T Cell-Specific Adaptor Protein. *Scand J Immunol.* 2017;85(2):104-112.
271. Roncagalli R, Hauri S, Fiore F, et al. Quantitative proteomics analysis of signalosome dynamics in primary T cells identifies the surface receptor CD6 as a Lat adaptor-independent TCR signaling hub. *Nat Immunol.* 2014;15(4):384-392.
272. Castro MA, Nunes RJ, Oliveira MI, et al. OX52 is the rat homologue of CD6: evidence for an effector function in the regulation of CD5 phosphorylation. *J Leukoc Biol.* 2003;73(1):183-190.
273. Nair P, Melarkode R, Rajkumar D, Montero E. CD6 synergistic co-stimulation promoting proinflammatory response is modulated without interfering with the activated leucocyte cell adhesion molecule interaction. *Clin Exp Immunol.* 2010;162(1):116-130.
274. Osorio LM, Garcia CA, Jondal M, Chow SC. The anti-CD6 mAb, IOR-T1, defined a new epitope on the human CD6 molecule that induces greater responsiveness in T cell receptor/CD3-mediated T cell proliferation. *Cell Immunol.* 1994;154(1):123-133.
275. Oliveira MI, Gonçalves CM, Pinto M, et al. CD6 attenuates early and late signaling events, setting thresholds for T-cell activation. *Eur J Immunol.* 2012;42(1):195-205.
276. Orta-Mascaro M, Consuegra-Fernandez M, Carreras E, et al. CD6 modulates thymocyte selection and peripheral T cell homeostasis. *J Exp Med.* 2016;213(8):1387-1397.

277. Hafler DA, Fallis RJ, Dawson DM, Schlossman SF, Reinherz EL, Weiner HL. Immunologic responses of progressive multiple sclerosis patients treated with an anti-T-cell monoclonal antibody, anti-T12. *Neurology.* 1986;36(6):777-784.
278. Kirkman RL, Araujo JL, Busch GJ, et al. Treatment of acute renal allograft rejection with monoclonal anti-T12 antibody. *Transplantation.* 1983;36(6):620-626.
279. Reinherz EL, Geha R, Rappeport JM, et al. Reconstitution after transplantation with T-lymphocyte-depleted HLA haplotype-mismatched bone marrow for severe combined immunodeficiency. *Proc Natl Acad Sci U S A.* 1982;79(19):6047-6051.
280. Alonso R, Huerta V, de Leon J, et al. Towards the definition of a chimpanzee and human conserved CD6 domain 1 epitope recognized by T1 monoclonal antibody. *Hybridoma (Larchmt).* 2008;27(4):291-301.
281. Roque-Navarro L, Mateo C, Lombardero J, et al. Humanization of predicted T-cell epitopes reduces the immunogenicity of chimeric antibodies: new evidence supporting a simple method. *Hybrid Hybridomics.* 2003;22(4):245-257.
282. Rodríguez PC, Prada DM, Moreno E, et al. The anti-CD6 antibody itolizumab provides clinical benefit without lymphopenia in rheumatoid arthritis patients: results from a 6-month, open-label Phase I clinical trial. *Clin Exp Immunol.* 2018;191(2):229-239.
283. Hernandez P, Moreno E, Aira LE, Rodriguez PC. Therapeutic Targeting of CD6 in Autoimmune Diseases: A Review of Cuban Clinical Studies with the Antibodies IOR-T1 and Itolizumab. *Curr Drug Targets.* 2016;17(6):666-677.
284. Menon R, David BG. Itolizumab - a humanized anti-CD6 monoclonal antibody with a better side effects profile for the treatment of psoriasis. *Clin Cosmet Investig Dermatol.* 2015;8:215-222.
285. Pai G SN. Biologic armamentarium in psoriasis. . In: Asian J Pharm Clin Res 2016 65–72.
286. Kemanetzoglou E, Andreadou E. CNS Demyelination with TNF-α Blockers. *Curr Neurol Neurosci Rep.* 2017;17(4):36.
287. Levesque MC, Heinly CS, Whichard LP, Patel DD. Cytokine-regulated expression of activated leukocyte cell adhesion molecule (CD166) on monocyte-lineage cells and in rheumatoid arthritis synovium. *Arthritis Rheum.* 1998;41(12):2221-2229.
288. Consuegra-Fernández M, Lin F, Fox DA, Lozano F. Clinical and experimental evidence for targeting CD6 in immune-based disorders. *Autoimmun Rev.* 2018.
289. Le Dantec C, Alonso R, Fali T, et al. Rationale for treating primary Sjogren's syndrome patients with an anti-CD6 monoclonal antibody (Itolizumab). *Immunol Res.* 2013;56(2-3):341-347.
290. Li Y, Singer NG, Whitbred J, Bowen MA, Fox DA, Lin F. CD6 as a potential target for treating multiple sclerosis. *Proc Natl Acad Sci U S A.* 2017;114(10):2687-2692.
291. Consuegra-Fernández M, Julià M, Martínez-Florensa M, et al. Genetic and experimental evidence for the involvement of the CD6 lymphocyte receptor in psoriasis. *Cell Mol Immunol.* 2017.
292. Oliveira VG, Caridade M, Paiva RS, Demengeot J, Graca L. Sub-optimal CD4+ T-cell activation triggers autonomous TGF-β-dependent conversion to Foxp3+ regulatory T cells. *Eur J Immunol.* 2011;41(5):1249-1255.
293. Duarte J, Carrié N, Oliveira VG, et al. T cell apoptosis and induction of Foxp3+ regulatory T cells underlie the therapeutic efficacy of CD4 blockade in experimental autoimmune encephalomyelitis. *J Immunol.* 2012;189(4):1680-1688.
294. Consuegra-Fernandez M, Martinez-Florensa M, Aranda F, et al. Relevance of CD6-Mediated Interactions in the Regulation of Peripheral T-Cell Responses and Tolerance. *Front Immunol.* 2017;8:594.

295. Soiffer RJ, Murray C, Mauch P, et al. Prevention of graft-versus-host disease by selective depletion of CD6-positive T lymphocytes from donor bone marrow. *J Clin Oncol.* 1992;10(7):1191-1200.
296. Soiffer RJ, Ritz J. Selective T cell depletion of donor allogeneic marrow with anti-CD6 monoclonal antibody: rationale and results. *Bone Marrow Transplant.* 1993;12 Suppl 3:S7-10.
297. Soiffer RJ, Fairclough D, Robertson M, et al. CD6-depleted allogeneic bone marrow transplantation for acute leukemia in first complete remission. *Blood.* 1997;89(8):3039-3047.
298. Osorio LM, De Santiago A, Aguilar-Santelises M, Mellstedt H, Jondal M. CD6 ligation modulates the Bcl-2/Bax ratio and protects chronic lymphocytic leukemia B cells from apoptosis induced by anti-IgM. *Blood.* 1997;89(8):2833-2841.
299. Faxas García ME, Guerra Yi ME, Álvarez A, Calderón C. Ensayo clínico fase I del anticuerpo monoclonal IOR-T1 en linfoma T: farmacocinética y respuesta inmune. *Revista Cubana de Medicina.* 2003;42:140-142.
300. Montero E AR, Pérez R., Inventor. Pharmaceutical compositions capable of inducing apoptosis in tumour cells, useful for diagnosis and treatment of B-chronic lymphocytic leukaemia.
301. Izquierdo Cano LM, Espinosa Estrada EE, Padrón CH, et al. *Itolizumab humanized monoclonal antibody (anti-cd6) in patients with cd6+ lymphoproliferative disorders. Preliminary experience.* Vol 302014.
302. Carrasco E, Escoda-Ferran C, Climent N, et al. Human CD6 Down-Modulation following T-Cell Activation Compromises Lymphocyte Survival and Proliferative Responses. *Front Immunol.* 2017;8:769.
303. Escoda-Ferran C, Carrasco E, Caballero-Banos M, et al. Modulation of CD6 function through interaction with Galectin-1 and -3. *FEBS Lett.* 2014;588(17):2805-2813.
304. Weidle UH, Eggle D, Klostermann S, Swart GW. ALCAM/CD166: cancer-related issues. *Cancer Genomics Proteomics.* 2010;7(5):231-243.
305. Spassov DS, Wong CH, Wong SY, Reiter JF, Moasser MM. Trask loss enhances tumorigenic growth by liberating integrin signaling and growth factor receptor cross-talk in unanchored cells. *Cancer Res.* 2013;73(3):1168-1179.
306. Bos PD, Rudensky AY. Treg cells in cancer: a case of multiple personality disorder. *Sci Transl Med.* 2012;4(164):164fs144.
307. Gounaris E, Blatner NR, Dennis K, et al. T-regulatory cells shift from a protective anti-inflammatory to a cancer-promoting proinflammatory phenotype in polyposis. *Cancer Res.* 2009;69(13):5490-5497.
308. Li L, Kim J, Boussiotis VA. IL-1β–Mediated Signals Preferentially Drive Conversion of Regulatory T Cells but Not Conventional T Cells into IL-17–Producing Cells. *Journal of immunology (Baltimore, Md : 1950).* 2010;185(7):4148-4153.
309. Osorio F, LeibundGut-Landmann S, Lochner M, et al. DC activated via dectin-1 convert Treg into IL-17 producers. *Eur J Immunol.* 2008;38(12):3274-3281.
310. Xu L, Kitani A, Fuss I, Strober W. Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta. *J Immunol.* 2007;178(11):6725-6729.
311. Li L, Boussiotis VA. The role of IL-17-producing Foxp3(+) CD4(+) T cells in inflammatory bowel disease and colon cancer. *Clinical immunology (Orlando, Fla).* 2013;148(2):10.1016/j.clim.2013.1005.1003.

312. Ibanez A, Sarrias MR, Farnos M, et al. Mitogen-activated protein kinase pathway activation by the CD6 lymphocyte surface receptor. *J Immunol.* 2006;177(2):1152-1159.
313. Alonso-Ramirez R, Loisel S, Buors C, et al. Rationale for Targeting CD6 as a Treatment for Autoimmune Diseases. *Arthritis.* 2010;2010:130646.
314. Rodriguez PC, Torres-Moya R, Reyes G, et al. A clinical exploratory study with itolizumab, an anti-CD6 monoclonal antibody, in patients with rheumatoid arthritis. *Results Immunol.* 2012;2:204-211.
315. Aira LE, Lopez-Requena A, Fuentes D, et al. Immunological and histological evaluation of clinical samples from psoriasis patients treated with anti-CD6 itolizumab. *MAbs.* 2014;6(3):783-793.
316. Kofler DM, Farkas A, von Bergwelt-Baildon M, Hafler DA. The Link Between CD6 and Autoimmunity: Genetic and Cellular Associations. *Curr Drug Targets.* 2016;17(6):651-665.
317. Consuegra-Fernandez M, Julia M, Martinez-Florensa M, et al. Genetic and experimental evidence for the involvement of the CD6 lymphocyte receptor in psoriasis. *Cell Mol Immunol.* 2017.
318. Brown MH. CD6 as a Cell Surface Receptor and As a Target for Regulating Immune Responses. *Curr Drug Targets.* 2016;17(6):619-629.
319. Pinto M, Carmo AM. CD6 as a therapeutic target in autoimmune diseases: successes and challenges. *BioDrugs.* 2013;27(3):191-202.
320. Aira LE, Hernández P, Prada D, et al. Immunological evaluation of rheumatoid arthritis patients treated with itolizumab. *MAbs.* 2016;8(1):187-195.
321. Bach F, Hirschhorn K. LYMPHOCYTE INTERACTION: A POTENTIAL HISTOCOMPATIBILITY TEST IN VITRO. *Science.* 1964;143(3608):813-814.
322. Bughani U, Saha A, Kuriakose A, et al. T cell activation and differentiation is modulated by a CD6 domain 1 antibody Itolizumab. *PLoS One.* 2017;12(7):e0180088.
323. Gimferrer I, Ibanez A, Farnos M, et al. The lymphocyte receptor CD6 interacts with syntenin-1, a scaffolding protein containing PDZ domains. *J Immunol.* 2005;175(3):1406-1414.

Appendix

EBioMedicine 47 (2019) 427–435

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

EBioMedicine
Published by THE LANCET

Modulation of CD4 T cell function *via* CD6-targeting

Raquel Filipa Freitas [a,b], Afonso Basto [a,b], Silvia C.P. Almeida [a,b,1], Rita F. Santos [c,d,e], Carine M. Gonçalves [c,d], Jesus Corria-Osorio [f], Tânia Carvalho [a], Alexandre M. Carmo [c,d], Vanessa G. Oliveira [a,b], Kalet Leon [f], Luis Graca [a,b,*]

[a] Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal
[b] Instituto Gulbenkian de Ciência, Oeiras, Portugal
[c] i3S – Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Portugal
[d] IBMC – Instituto de Biologia Molecular e Celular, Porto, Portugal
[e] Programa Doutoral em Biologia Molecular e Celular (MCbiology), Instituto de Ciências Biomédicas Abel Salazar, Universidade do Porto, Portugal
[f] Centro de Inmunologia Molecular, Havana, Cuba

ARTICLE INFO

Article history:
Received 21 October 2018
Received in revised form 30 July 2019
Accepted 2 August 2019
Available online 31 August 2019

Keywords:
CD6
CD4 T cells
Treg cells
Foxp3
EAE
T-cell polarization

ABSTRACT

In recent years molecules involved on the immune synapse became successful targets for therapeutic immune modulation. CD6 has been extensively studied, yet, results regarding CD6 biology have been controversial, in spite of the ubiquitous presence of this molecule on virtually all CD4 T cells. We investigated the outcome of murine and human antibodies targeting CD6 domain 1. We found that CD6-targeting had a major impact on the functional specialization of CD4 cells, both human and murine. Differentiation of CD4 T cells towards a Foxp3$^+$ Treg fate was prevented with increasing doses of anti-CD6, while Th1 polarization was favoured. No impact was observed on Th2 or Th17 specialization. These *in vitro* results provided an explanation for the dose-dependent outcome of *in vivo* anti-CD6 administration where the anti-inflammatory action is lost at the highest doses. Our data show that therapeutic targeting of the immune synapse may lead to paradoxical dose-dependent effects due to modification of T cell fate.

1. Introduction

CD6 is a transmembrane glycoprotein (105/130 kDa) expressed mostly on mature T cells, but also in thymocytes, B1a lymphocytes and CD56$^+$ NK cells. Its structure includes three extracellular scavenger receptor cysteine-rich (SRCR) domains and a cytoplasmic tail (244 amino acids) without catalytic activity but with several sites for phosphorylation and recruitment of signal transduction proteins [1–3]. So far, two CD6 ligands have been described: CD318, that binds CD6 domain 1 (d1), being expressed mostly on synovial tissues by epithelial cells but also in some tumors; and the activated leukocyte cell adhesion molecule (ALCAM) or CD166, that binds CD6 d3 and is expressed mainly by monocyte-derived cells and endothelial cells [4–7]. From the two ligands, ALCAM is the best characterized, being established that its ligation to CD6 allows for stable T cell-antigen presenting cell (APC) interactions, essential for maturation of immunological synapse (IS) and consequent optimal T cell proliferation [8–12].

The high degree of conservation of CD6 and ALCAM binding regions suggests an evolutionary relevance for this specific interaction [13–16]. Because CD6 is a SRCR family member, present at the immune synapse during activation, a putative role for CD6 in the pathogenesis of autoimmunity has been investigated [17,18]. Indeed, it was reported that CD6-deficient mice have altered susceptibility to autoimmunity. However, while in experimental autoimmune encephalomyelitis (EAE) and imiquimod-induced psoriasis CD6-deficient mice had disease protection or attenuation, in collagen-induced arthritis (CIA) CD6-deficient mice had more severe disease [19–21]. Different genetic backgrounds, different knockout strategies, and particularities intrinsic to the pathogenesis of each disease model might justify the differences.

In humans, CD6 was also implicated in the pathogenesis of several autoimmune diseases, including rheumatoid arthritis (RA), Sjögren's syndrome and psoriasis [22–25]. Furthermore, genome wide association studies (GWAS) and gene-specific candidate-driven studies also identified *CD6* as a major susceptibility locus for multiple sclerosis (MS), psoriasis and Behcet's disease [26–29].

Given the involvement of CD6 in autoimmunity, there has been an effort to develop therapeutic strategies based on CD6-targeting [30,31].

One of these strategies relates to Itolizumab, a humanized non-depleting mAb targeting CD6 d1, that was shown effective and safe for

* Corresponding author at: Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Avenida Professor Egas Moniz, 1649-028 Lisboa, Portugal.
E-mail address: lgraca@medicina.ulisboa.pt (L. Graca).
[1] Current affiliation: Charles River Laboratories, Ireland, Ltd.

https://doi.org/10.1016/j.ebiom.2019.08.008

Research in context

Evidence before the study

CD6 has been linked to autoimmunity, and CD6-targeting antibodies have been proposed as a promising autoimmune therapy. A key issue regarding the biology of CD6-targeting has remained unaddressed: its impact on the functional polarization of CD4 T cells.

Added value of this study

We found a surprising dose-response effect of anti-CD6 monoclonal antibodies on the functional specialization of murine and human CD4 T cells: High doses of anti-CD6 inhibited polarization towards Foxp3+ Treg cells while favouring Th1 polarization. Th2 and Th17 polarization remained unaffected. The impact of CD6-targeting on T cell specialization was observed in the absence of any major effect on T cell survival or proliferation.

Implications

Our data show that therapeutic antibodies targeting the immune synapse, namely anti-CD6, may lead to paradoxical dose-dependent effects due to modification of T cell fate.

the treatment of psoriasis [32,33]. Clinical trials in RA also showed clinical benefits, with lower doses providing the highest and long-lasting improvements [34,35]. Thus, we investigated how different dosages of CD6 d1-targeting would impact on murine neuroinflammatory disease. We found that high doses of anti-CD6 were not protective and could even promote inflammation. In order to find the mechanism for such high-dose exacerbation of disease, we addressed the impact of CD6 d1-targeting on the functional specialization of activated CD4 T cells. Here we show that CD4 T cells exposed to higher doses of anti-CD6 were prevented from acquiring a regulatory T (Treg) cell phenotype, while preferentially differentiating towards Th1. Our findings were observed with murine and human cells.

2. Materials and methods

2.1. Ethics and in vivo experiments

C57BL/6 and OVA-specific TCR-transgenic mice (OT-II *Rag2*$^{-/-}$) were bred and maintained under specific pathogen-free conditions. Sex-matched mice, between 8 and 10 weeks of age were used in the experiments. All experimental protocols were approved by the Local Ethics Committee and are in compliance with European Union guidelines. EAE was induced in C57BL/6 mice by s.c. immunization with 125 μg $MOG_{35–55}$ (MEVGWYRSPFSRVVHLYRNGK) (AnaSpec, Inc.) emulsified in complete Freund's Adjuvant (CFA) suspension (4 mg/ml mycobacteria in IFA), and i.v. injection of 200 ng pertussis toxin (List Biological Laboratories) on days 0 and 2 following immunization. Disease severity was scored daily: 1, tail atony; 2, hind limb weakness; 3, hind limb paralysis; 3.5- flattening of hind quarters with complete paralysis; 4, quadriplegia; 5, moribund.

2.2. Histopathology

Mice were deeply anesthetized for transcardiac perfusion with PBS, followed with 4% paraformaldehyde. After perfusion, head and spinal cord were further immersed into neutral buffered formalin for 48 h. Brain and spinal cord were then removed from the bone, trimmed and routinely processed for paraffin embedding. Sections with 4 μm were stained with hematoxylin-eosin and Luxol fast blue, and screened by a pathologist blinded to experimental groups, in a Leica DM2500 microscope coupled to a Leica MC170 HD microscope camera. Semi-quantification of inflammation and demyelination were performed using a 5-tier system with 0–4 grading scale: 0, absent; 1, minimal; 2, mild; 3, moderate; 4, marked.

2.3. mAbs and flow cytometry

Anti-mouse CD6 d1 (10F12) and anti-human CD6 d1 (Itolizumab), as well as isotype controls were produced at the CIM (Havana, Cuba). Anti-IL-4 (11B11) and anti-IFNγ mAbs were produced at IMM (Lisbon, Portugal) using Integra CL1000 flasks (IBS Integra Biosciences, Chur, Switzerland), purified by 50% (*w*/*v*) ammonium sulphate precipitation, dialyzed against PBS, and purity was checked by native and SDS gel electrophoresis. Murine single cell suspensions were stained with CD4 PE (GK1.5); CD4 APC-eFluor® 780 (RM4–5); TCRβ APC-eFluor® 780 (H57–597); CD25 PE-Cy7 (PC61.5); IFNγ FITC (XMG1.2); IL-13 PE (eBio13A); IL-17 PE (ebio17B7); Foxp3 APC (FJK-16 s), CD6 PE (BX222 Biolegend), anti-rat IgG Biotin and Streptavidin PE (all from eBioscience). Human single cells suspensions were stained with CD4 PE (RPA-T4); CD4 FITC (OKT4); CD3 PE (OKT3); CD25 PE-Cy7 (BC96); CD45RA APC-eFluor® 780 (HI100); IFNγ PerCP-Cy5.5 (4S.B3); IL-13 PerCP-Cy5.5 (JES10-5A2); IL-17 APC (eBio64DEC17); Foxp3 APC (PCH101), CD6 FITC (BL-CD6) and anti-human IgG APC-Cy7. Cell viability was detected with Live/Dead Fixable Aqua Dead Cell Stain Kit (Life Technologies) and Annexin V Apoptosis Detection Kit (eBioscience). CellTrace™ Violet Cell Proliferation Kit was used for cell proliferation assessment according to the manufacturer's protocol (Thermofisher). In some studies, cytokine production was assessed following 4 h stimulation with 50 ng/ml PMA, 500 ng/ml ionomycin, 10 μg/ml brefeldin (all from Sigma Aldritch) and 0.66/ml Golgistop™ (BD Biosciences). Cells were permeabilized with eBioscience kit (# A25866A).

2.4. Recombinant mouse extracellular CD6 protein

Murine soluble CD6 (Gly17-Thr398) was provided by INVIGATE GmbH, Jena, Germany (www.invigate.com). The recombinant protein is derived from HEK 293 cells and comprises C-terminally fused HA-Tag (YPYDVPDYA), BirA-Tag (GLNDIFEAQKIEWH) and His-Tag (HHHHHH).

2.5. T-cell activation and polarization (murine cells)

OVA-specific $CD4^+$ T cells were magnetically sorted with CD4 (L3 T4) microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) from OTII-$Rag2^{-/-}$ mice, with a purity >90%. T cells were cultured for 4 days and activated with bone marrow-derived dendritic cells (bmDCs) (at 2:1 ratio) [44] and 10 μM $OVA_{323–339}$ (Eurogentec) or with 3 μg/ml plate bound anti-CD3 and 2 μg/ml soluble anti-CD28 (OKT3 and 37.51, eBioscience). For Th1 polarization the medium was supplemented with 5 ng/ml IL-2 and 10 ng/ml IL-12 (both from Peprotech), and 0.5 mg/ml anti-IL-4 (11B11). For Treg polarization, we added 5 ng/ml IL-2 and 5 ng/m TGF-β (R&D). For Th17 polarization, the medium included 10 ng/ml IL-1β and 20 ng/ml IL-6 (both from Peprotech), 1 ng/m TGF-β (R&D), and 0.5 mg/ml anti-IFNγ (R46A2). Finally, for Th2 polarization we added 5 ng/ml IL-2, 10 ng/ml IL-4 (Peprotech), and 0.5 mg/ml anti-IFNγ.

2.6. T-cell activation and polarization (human cells)

Peripheral blood mononuclear cells (PBMCs) were isolated from blood or buffy-coats from healthy volunteers provided by Instituto Português do Sangue e Transplantação (IPST), following informed consent, by Ficoll gradient (Sigma Aldritch) using SepMate™ (STEMCELL Technologies). Naïve $CD4^+$ T cells ($CD4^+CD3^+CD25^-CD45RA^+$) were

then sorted with FACS Aria III (BD Biosciences). Irradiated (25 Gy) PBMCs were used as APCs (iPBMCs). $CD4^+$ cells were cultured with iPBMCs (at 1:2 ratio) and 1 μg/ml of soluble anti-CD3 (OKT3); or with 3 μg/ml plate bound anti-CD3 (OKT3) and 2 μg/ml soluble anti-CD28 (CD28.2). For Th1 polarization the medium was supplemented with 10 ng/ml IL-2, 2.5 ng/ml IL-12 (both from Peprotech), and 5 μg/ml anti-IL-4 (11B11) mAb. For Treg polarization we added 10 ng/ml IL-2 and 10 ng/ml TGF-β (R&D).

2.7. Statistical analysis

Statistical significance was calculated using nonparametric Mann-Whitney *U* test, and Kruskal-Wallis one-way analysis of variance, *p* values of <0.05 were considered significant ($^*p < .05$, $^{**}p < .01$, $^{***}p < .001$). Results are presented as mean ± SEM.

3. Results

3.1. Targeting CD6 d1 leads to anti-inflammatory effects exclusively at low doses

Given prior reports on the importance of CD6 alleles for MS susceptibility [26,27], and studies showing CD6-deficient mice resist induction of EAE [19], we investigated whether antibodies targeting CD6 d1 can prevent neuroinflammation.

We used an established model of EAE, induced following MOG-CFA and pertussis toxin administration to C57BL/6 mice (Fig. 1a). It is possible to prevent the onset of EAE in this experimental system using antibodies that promote peripheral induction of Treg cells, such as neutralizing anti-CD4 antibodies that do not induce cell lysis [36]. We used YTS177 (a non-depleting pro-tolerogenic anti-CD4) as positive control (Fig. 1b and Supplementary Table 1).

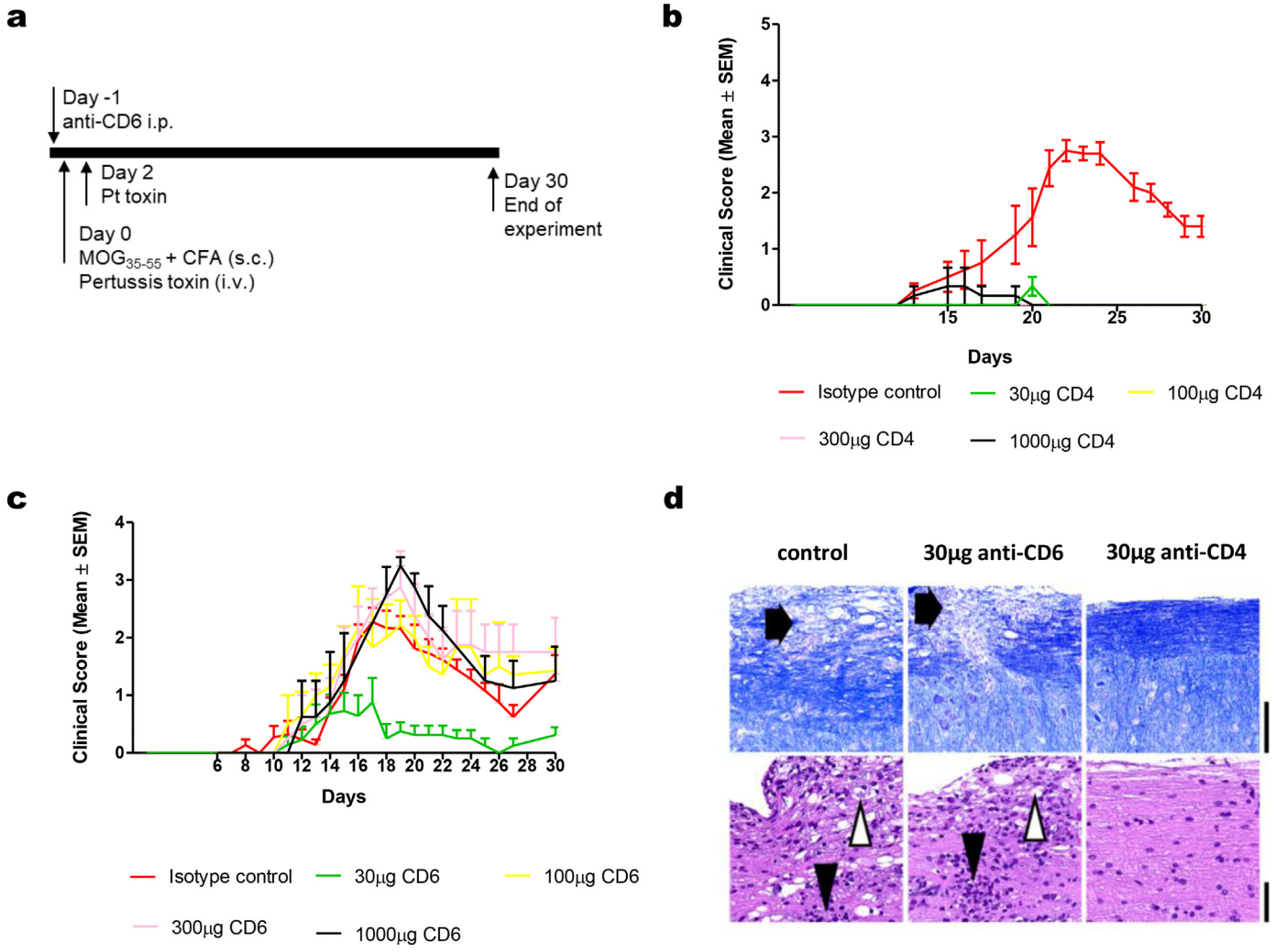
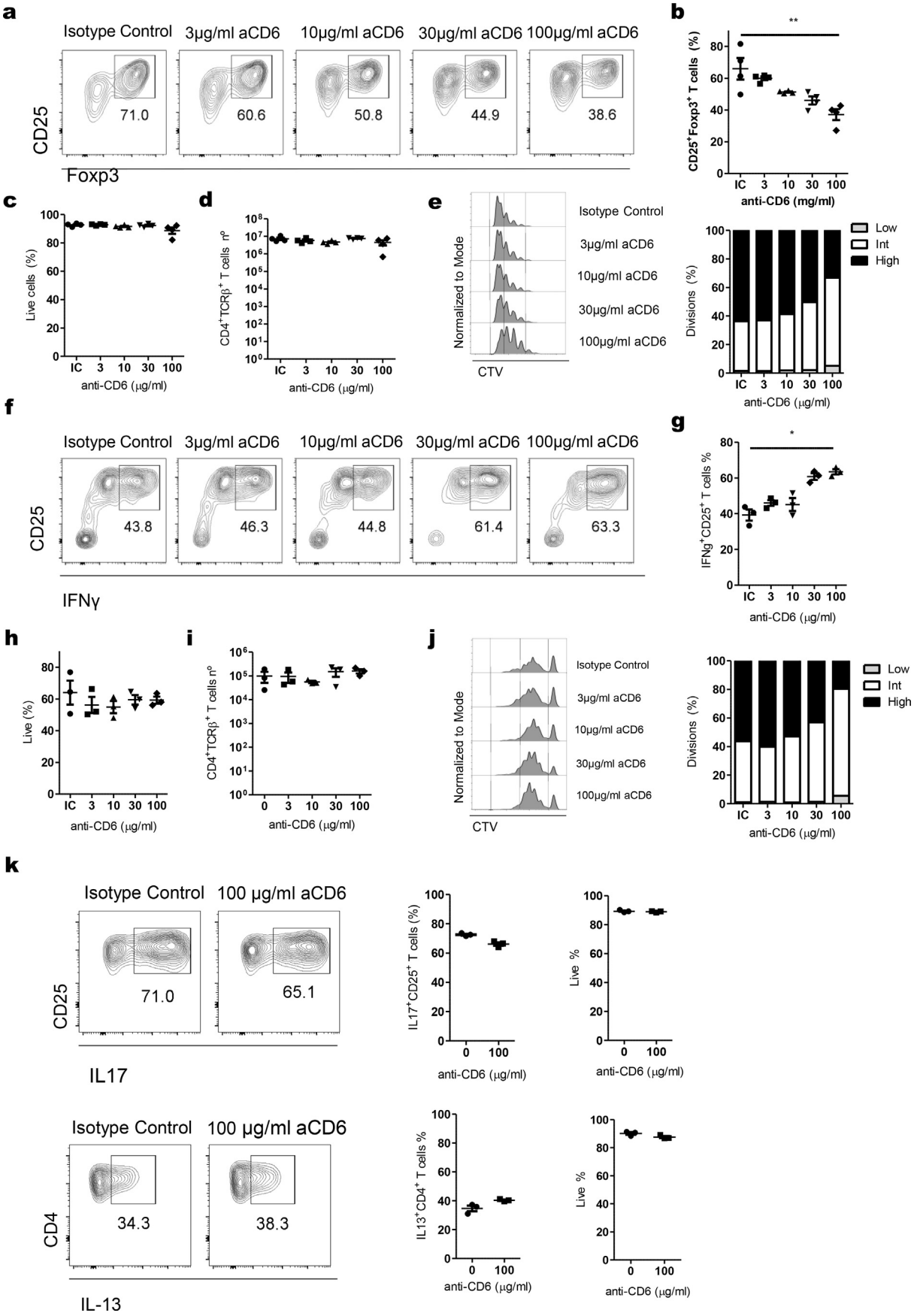


Fig. 1. *Low dose of anti-CD6 mAb (10F12) prevents the onset of EAE.* (a) C57BL/6 mice were immunized with MOG and treated with different doses of anti-CD6, or an isotype control at day 0. (b) Clinical score of mice treated with different doses of non-depleting anti-CD4 (YTS177), on the day before MOG_{35-55} immunization. All mice treated with anti-CD4 were protected from EAE ($n = 5$ per group). (c) Clinical score for each concentration of anti-CD6 and control group of mice immunized with MOG_{35-55} peptide are shown as mean values ± SEM, pooled data from three independent experiments. Mice treated with 30 μg anti-CD6 ($n = 11$) were protected from EAE. However, mice treated with 100 μg anti-CD6 ($n = 8$), or greater doses ($n = 4$ per group), developed EAE with disease severity and incidence similar to the control group ($n = 15$). (d) Longitudinal sections of spinal cord from mice 22 days after MOG_{35-55} immunization. In the Luxol fast blue stained section (upper panel), mice treated with anti-CD6 but not anti-CD4 show demyelination of the peripheral spinal cord white matter (black arrow), similar to control mice (original magnification 10×, bar 250 μm). In the hematoxylin-eosin-stained section (lower panel), mice treated with anti-CD6 but not anti-CD4 also show an intense mononuclear inflammatory infiltration of the peripheral white matter, with macrophage-rich areas that include numerous myelin-containing phagocytes (white arrowhead), and with fewer lymphocytes (black arrowhead), similar to control (original magnification 40×, bar 50 μ).


a
Isotype Control
3μg/ml aCD6
10μg/ml aCD6
30μg/ml aCD6
100μg/ml aCD6
CD25
Foxp3
71.0
60.6
50.8
44.9
38.6
b
CD25+Foxp3+ T cells (%)
anti-CD6 (mg/ml)
c
Live cells (%)
anti-CD6 (μg/ml)
d
CD4+TCRβ+ T cells nº
anti-CD6 (μg/ml)
e
Normalized to Mode
CTV
Isotype Control
3μg/ml aCD6
10μg/ml aCD6
30μg/ml aCD6
100μg/ml aCD6
Divisions (%)
Low
Int
High
anti-CD6 (μg/ml)
f
Isotype Control
3μg/ml aCD6
10μg/ml aCD6
30μg/ml aCD6
100μg/ml aCD6
CD25
IFNγ
43.8
46.3
44.8
61.4
63.3
g
IFNg+CD25+ T cells %
anti-CD6 (μg/ml)
h
Live (%)
anti-CD6 (μg/ml)
i
CD4+TCRβ+ T cells nº
anti-CD6 (μg/ml)
j
Normalized to Mode
CTV
Isotype Control
3μg/ml aCD6
10μg/ml aCD6
30μg/ml aCD6
100μg/ml aCD6
Divisions (%)
Low
Int
High
anti-CD6 (μg/ml)
k
Isotype Control
100 μg/ml aCD6
CD25
IL17
71.0
65.1
IL17+CD25+ T cells (%)
anti-CD6 (μg/ml)
Live %
anti-CD6 (μg/ml)
Isotype Control
100 μg/ml aCD6
CD4
IL-13
34.3
38.3
IL13+CD4+ T cells %
anti-CD6 (μg/ml)
Live %
anti-CD6 (μg/ml)

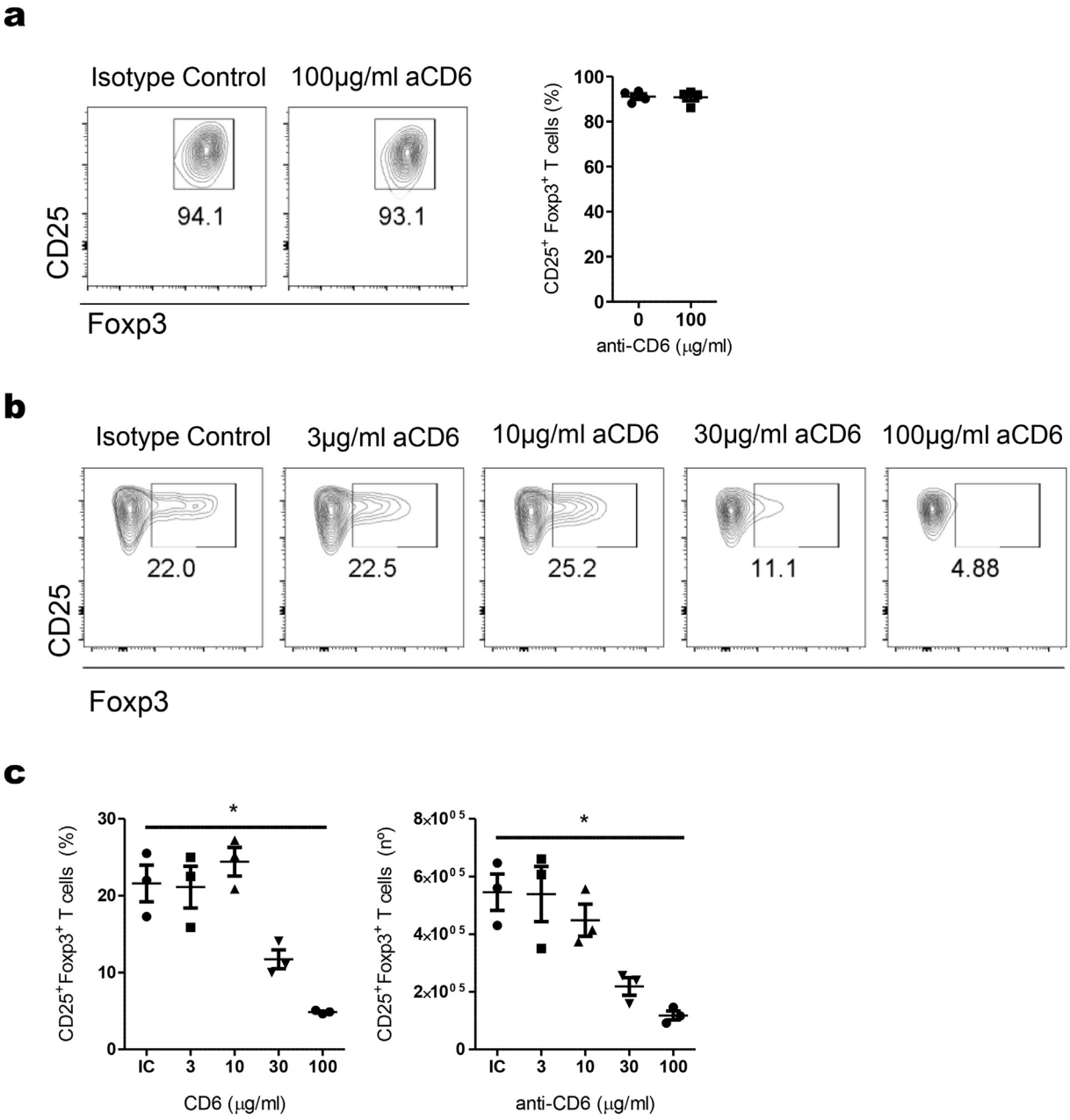


Fig. 3. *CD4$^+$T cell polarization is dependent on CD166 binding.* (a) CD25$^+$Foxp3$^+$ T cells within CD4$^+$TCRβ$^+$ T cells at the end of 4-days culture of OVA-specific TCR-transgenic OT-II.Rag$^{-/-}$ CD4 T cells with plate-bound anti-CD3 and anti-CD28 under Treg polarizing conditions. Anti-CD6 did not alter the frequency of induced Foxp3$^+$ T cells. (b) OT-II.Rag$^{-/-}$ CD4 T cells were cultured in a 2:1 ratio with BMDC for 4 days under Treg polarizing conditions with increasing concentrations of soluble CD6. Representative dot plots and (c) graphs showing the frequency and number of CD25$^+$Foxp3$^+$ T cells within CD4$^+$TCRβ$^+$ T cells. Data are representative of two independent experiments ($n = 4$).

In mice treated with mAbs targeting CD6 d1 we could only find a partial protection from EAE with the lowest dose (Fig. 1c and Supplementary Table 2). On the contrary, the highest doses of anti-CD6 d1 did not show any beneficial effect (Fig. 1c).

The partial suppression afforded by low-dose anti-CD6 was not sufficient to completely abrogate inflammatory changes observed by histopathology, or to completely abolish infiltration of the CNS with lymphocytes (Fig. 1d and Supplementary Table 3).

3.2. CD6 d1-targeting favours Th1 differentiation while suppressing Treg cell induction

Given our previous results showing that protection from EAE following CD4-blockade was due to peripheral expansion of Treg cells at the expense of effector T cell (Th1 and Th17) polarization [36], we investigated how CD6 d1 targeting impacted on T cell polarization.

Fig. 2. *CD6-targeting increases Th1 polarization while inhibiting Treg differentiation.* OVA-specific TCR-transgenic OT-II.Rag$^{-/-}$ CD4 T cells were cultured for 4 days in a 2:1 ratio with bone marrow derived dendritic cells (BMDC) in Th1 and Treg polarizing conditions. (a, b) Representative flow cytometry dot plots and scatter plots showing the percentage of CD25$^+$Foxp3$^+$ T cells within CD4$^+$TCRβ$^+$ T cells at the end of Treg polarizing cultures with different doses of anti-CD6 (10F12) or 100 μg/ml isotype control (IC). (c) Survival of CD4 T cells at the end of culture. (d) Number of CD4 T cells recovered at the end of the culture. (e) Representative histograms showing CTV dilution of T cells following culture and bar graph displaying the frequency of cells within gates representing low, intermediate and high proliferation as displayed in the histograms. (f, g) Representative flow cytometry dot plots and scatter plots showing the percentage of CD25$^+$IFNγ$^+$ T cells within CD4$^+$TCRβ$^+$ T cells in Th1-polarizing cultures. (h) Viability of CD4 T cells under Th1 polarizing conditions. (i) Number of CD4 cells recovered at the end of culture. (j) T cell proliferation under Th1 polarizing conditions. (k) Representative dot plots and scatter plots showing the percentage of T cells producing IL-17 (top) or IL-13 (bottom) following culture under, respectively, Th17 and Th2 polarizing conditions as well as their viability (right). Statistical tests: Kruskal-Wallis and Mann-Whitney. Data are representative of three independent experiments, each with $n = 3$. *$p < .05$ **$p < .01$.

To do that, we sorted $CD4^+$ OVA-specific TCR-transgenic cells from OT-II.$Rag^{-/-}$ mice. Because virtually all murine CD4 T cells constitutively bear CD6 on their surface, it was unnecessary to sort subsets of CD4 cells based on CD6 levels (Supplementary Fig. 1). The sorted OVA-specific CD4 T cells were then stimulated with OVA-loaded DCs under the appropriate cytokine environment to promote functional polarization of uncommitted CD4 cells towards Treg, Th1, Th2, or Th17 effector phenotype. We found that CD6 d1 targeting showed a dose-dependent suppression of Treg polarization, as assessed by the decrease of Foxp3 expression (Fig. 2a,b), without an impact on T cell viability (Fig. 2c). Cell proliferation also remained largely unaffected as the number of cells retrieved at the end of the culture remained unchanged (Fig. 2d), although a trend in slower progression through intermediate classes of cell division was observed at the highest dose of anti-CD6 (Fig. 2e). We confirmed that the number of Treg cells were consistently decreased, and CD6 was not downmodulated/internalized during *in vitro* cultures or *in vivo*, in mice treated with anti-CD6 (Supplementary Fig. 2).

We also found that polarization towards a Th1 phenotype responded to CD6 d1-targeting in an opposite way, with higher doses of anti-CD6 d1 leading to greater frequency of Th1 cells (Fig. 2f,g), again without significant impact on T cell survival or proliferation (Fig. 2h–j).

The polarization of uncommitted CD4 T cells towards Th2 and Th17 phenotypes remained unaffected by anti-CD6 d1, even at the highest doses (Fig. 2k).

3.3. Alteration of T cell functional specialization by anti-CD6 d1 is a consequence of abrogation of CD6-CD166 interactions

We then investigated whether the observed impact of anti-CD6 d1 on T cell polarization was a consequence of steric hindrance of CD166 binding to CD6.

First, we stimulated T cells, under cytokine conditions favouring Treg polarization, by providing anti-CD3 and anti-CD28 in the absence of APCs (and, consequently, without CD166 provision at the T cell – APC

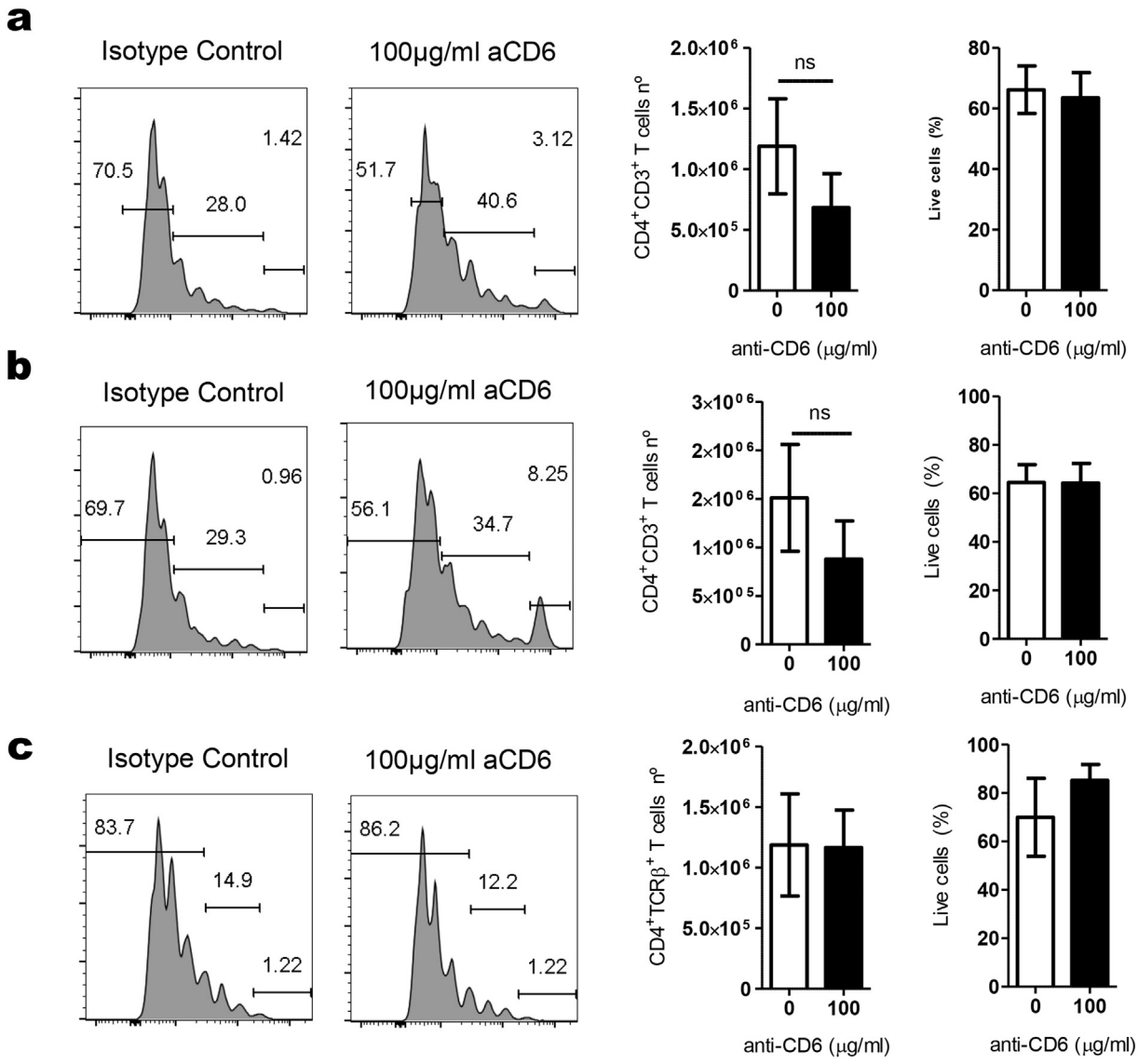


Fig. 4. *Impact of itolizumab on human CD4 T cell proliferation and survival.* Peripheral blood mononuclear cells (PBMCs) from healthy donors were used as source of naïve T cells (responders). Those naïve T cells were labelled with CTV and stimulated with syngeneic irradiated PBMC plus anti-CD3 (a); with allogeneic irradiated PBMCs (b); or with anti-CD3 and anti-CD28 in the absence of stimulating cells (c). Responder naïve CD4 T cells were cultured for 5 days in a ratio of 1:2 with the irradiated PBMCs (except in C) in non-polarizing conditions with IL-2. (a–c) Representative histograms of cultures with and without itolizumab and bar graphs showing the number of recovered CD4 T cells (left) and their viability (right). The graphs represent the pooled data from seven independent experiments, each with triplicates. Statistical test: Mann-Whitney.

interface). We found that addition of anti-CD6 d1 to those conditions did not change the polarization of uncommitted CD4 T cells (Fig. 3a).

However, the different stimulation regime (APCs *vs.* anti-CD3/anti-CD28) leads in itself to a different polarization efficiency. As a consequence, we addressed this issue with a more comparable stimulatory regime. We stimulated uncommitted CD4 T cells under the same conditions as described in Fig. 2, but now using soluble CD6 to prevent CD6 interactions with CD166 on APCs. We found that the addition of soluble CD6 led to a dose-dependent impact on Treg polarization similar to what we observed with anti-CD6 (Fig. 3b,c). Therefore, anti-CD6

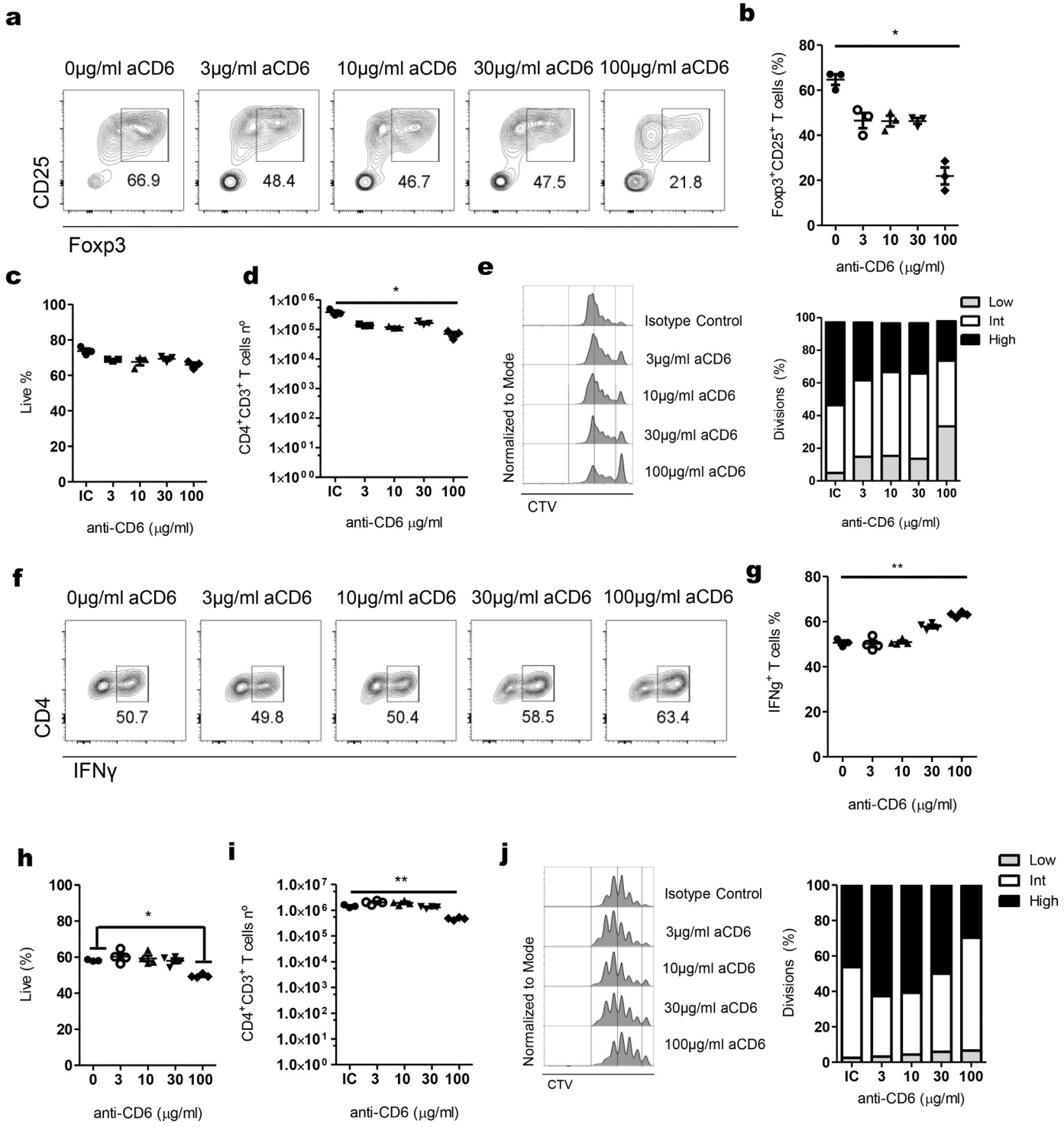


Fig. 5. *Itolizumab inhibits Treg polarization while enhancing acquisition of Th1 phenotype.* (a–e) PBMCs from healthy donors were used as source of naïve CD4 T cells (responders), labelled with CTV and stimulated 5 days with syngeneic irradiated PBMC (at a 1:2 ratio) with added anti-CD3, TGF-β, and IL-2 for Treg polarization; or (f–j) with IL-12, IL-2 and anti-IL4 for Th1 polarization. (a) Representative contour plots of $CD25^{+}Foxp3^{+}$ Treg cells and (b) graph with pooled data. (c) Viability of CD4 T cells at the end of culture in Treg polarizing conditions and (d) number of recovered cells. (e) Representative histograms showing T cell proliferation (CTV dilution) under Treg polarizing conditions and pooled data with the frequency of T cells within the represented low, intermediate and high cell division gate. (f) Representative contour plots of IFNγ producing CD4 T cells and (g) graph with pooled data. (h–j) Viability, number of recovered CD4 T cells, and T cell proliferation at the end of cultures under Th1-polarizing conditions. Data are representative of two independent experiments, each of them run with triplicates. Statistical test: Kruskal-Wallis. $^{*}p < .05$ $^{**}p < .01$.

modulation of T cell functional specialization upon activation appears to be a consequence of displacement of CD6-CD166 interactions.

3.4. CD6-targeting in human T cells with itolizumab reduces proliferation and Treg cell induction

We then investigated whether itolizumab, a humanized monoclonal antibody targeting human CD6 d1, can also influence the acquisition of effector functions by activated human CD4 T cells. With human experiments, due to the inability to use populations of T cells with a defined TCR, we sort-purified naïve CD4 T cell that were then stimulated with soluble anti-CD3 in the presence of antigen presenting cells (APCs), or by direct stimulation with allogeneic APCs. In addition, we also used plate-bound anti-CD3 as a strategy to activate T cells in the absence of APCs. We also confirmed that virtually all human CD4 cells constitutively display CD6 on their surface (Supplementary Fig. 1b).

First, we investigated the impact of CD6-targeting with itolizumab following T cell stimulation in presence of APCs. We found that when T cell stimulation was provided with soluble anti-CD3 added to syngeneic irradiated peripheral blood mononuclear cells (PBMCs, Fig. 4a), or when allogeneic irradiated PBMCs were used as stimulators (Fig. 4b), itolizumab tended to reduce T cell proliferation, as assessed by CTV dilution, leading to a reduced number of T cells at the end of the culture. The figure shows data from seven independent experiments, each with triplicates. A statistically significant impact on proliferation was observed in approximately half the experiments, but when all data were pooled, the reduction in proliferation did not reach statistical significance. No significant impact on T cell survival, assessed as the percentage of live cells, was observed (Fig. 4a,b).

We also performed *in vitro* assays providing T cell stimulation in conditions where APCs were absent (Fig. 4c). Under those conditions T cell proliferation was not affected by itolizumab, as assessed by CTV dilution, with similar numbers of T cells recovered at the end of the cultures and with no significant impact on T cell viability.

We also found that anti-CD6 had a small impact on the activation of CD4 T cells, as upregulation of CD69 (used as a surrogate marker for T cell activation) was affected in presence of anti-CD6 (Supplementary Fig. 3).

Finally, we investigated whether CD6 targeting with Itolizumab would have a similar impact on T cell functional specialization as we have observed with murine antibodies targeting the same CD6 d1. We cultured sorted human naïve CD4 T cells in conditions favouring Treg or Th1 polarization. We found a dose-dependent reduction on the frequency of induced Treg cells when itolizumab was added to the cultures (Fig. 5a,b). There was no significant impact on T cell viability (Fig. 5c), and only a small, albeit significant, impairment in T cell proliferation at the highest doses of itolizumab (Fig. 5d,e). Conversely, addition of itolizumab to Th1-polarizing cultures led to a dose-dependent increase of Th1 cells emerging at the end of the culture (Fig. 5f,g). A similar impact on proliferation and survival was observed (Fig. 5h–j). Overall, we found a similar impact of CD6 d1-targeting in human and murine cells.

4. Discussion

Taken together, our data show that a monoclonal antibody targeting CD6 d1 can have a strong impact on the functional specialization of T cells, affecting different lineages in a distinct way: while increasing concentration of anti-CD6 d1 impair Treg differentiation, it favours Th1. Importantly, both mice and human T cells, presented similar results.

Ongoing clinical trials of itolizumab for the treatment of RA reported, in initial dose finding studies, that patients treated with the highest dose of itolizumab responded worse than patients treated with lower doses [35]. Such observations are consistent with the *in vivo* outcome of animals treated with anti-CD6 d1 at the time of EAE induction, where high doses of the therapeutic antibody appear to be less effective. These *in vivo* results contrast with the outcome of anti-CD4 administration. Indeed, neutralizing anti-CD4 antibodies (devoid of lytic function) can prevent EAE at all tested doses – what has been interpreted as partial disruption of the immune synapse. Such protective effect was shown to be dependent on Treg induction [36], although the same approach of CD4-blockade can rely on Foxp3-independent tolerance for soluble proteins [37,38].

In order to address whether disruption of CD6-CD166 interactions could explain the effect of anti-CD6 d1, and if such disruption had an impact on Treg induction, we used soluble CD6 to directly disrupt CD6-CD166 binding. We found that soluble CD6 could recapitulate the dose-dependent outcome of anti-CD6 d1. However, contact-independent strong stimulation with anti-CD3/CD28 was not affected with addition of anti-CD6. Our observations are in line with a recent report that showed the absence of difference for *in vitro* Treg induction, between $CD6^{-/-}$ and $CD6^{+/+}$ T cells, when under contact-independent and supra-physiological conventional conditions [39]. It was previously reported that Treg cells from CD6-deficient mice have reduced suppressive function [39]. We did not assess the function of Treg cells polarized under the presence of anti-CD6. However, irrespective of a possible Treg functional impact of CD6-targeting we found a major quantitative effect on the number of polarized Treg cells.

It has been previously reported that the physical binding of antibodies to CD6 expressed in Jurkat cells, induces inhibitory signals decreasing cell proliferation [40]. We found that anti-CD6 d1 antibodies appear to directly modulate T cell activation independently of CD166 ligation, inferred from CD69 upregulation. These data suggest that the overall impact of anti-CD6 may combine blockade of CD6-CD166 interactions with a small direct effect on signalling.

It should be noted, however, that a putative interference on T cell activation signalling by anti-CD6 d1 cannot be major, but rather a subtle impact. Major interferences in T cell activation would be expected to result in changes in cell proliferation and possibly viability, if that was the case. Although in human studies we observed some response diversity regarding impact on T cell proliferation following T cell activation in presence of anti-CD6 d1, in both human and murine cells that trend is present (especially a delay in proliferation) but without reaching statistical significance. By contrast we observed a major impact on T cell polarization, restricted towards specific functional subsets. While Tregs and Th1 cells were very significantly affected by anti-CD6 d1, Th2 and Th17 were not. Such specificity towards distinct functional subsets are probably related to different levels of activation favouring alternative polarization phenotypes [41].

Other studies have also shown a protective effect of CD6 manipulation in EAE, as well as a suppression of Th1 and Th17 responses by anti-CD6 or CD6 gene deletion [19,42]. A direct comparison between the published reports is difficult given the diversity of experimental protocols, namely distinct genetic backgrounds, CD6-gene ablation *versus* antibody-targeting of CD6, different antibodies targeting distinct domains, and different dosages and treatment schedules. It should be stressed that distinct outcomes may be a consequence of different affinities, avidities and binding specificities of distinct anti-CD6 antibodies [43]. However, our observation highlights the importance of antibody dose in modulating T cell functional specialization. This observation is novel and may be important for other therapeutic targets, in particular molecules important for T cell activation.

Overall, our results show that therapeutic antibodies, such as anti-CD6 d1, may have paradoxical effects at different doses due to distinct impact on CD4 T cell functional specialization: while a low dose anti-CD6 d1 favours regulation, a higher dose may lead to opposite outcome by preventing Treg induction while favouring a Th1 fate. As such, dose selection is important, and the same compound may be therapeutically useful for different indications depending on its dose.

Funding sources

Funded by UID/BIM/50005/2019, project funded by Fundação para a Ciência e a Tecnologia (FCT) / Ministério da Ciência, Tecnologia e Ensino

Superior (MCTES) throught Fundos do Orçamento do Estado, pela Fundação para a Ciência e a Tecnologia (FCT) (PTDC/DTP-FTO/3080/2014); and by the project SRecognite Infect-ERA/0003/2015 using national funds through FCT. Funders did not have a role in study design, data collection, analysis, and interpretation, or in the writing of the manuscript.

Author contributions

RFF designed the research, performed experiments, analysed data, and wrote the manuscript. AB, SCPA, RFS, CMG, TC, and JCO performed experiments and reviewed the manuscript. AMC, VGO, and KL designed the research and reviewed the manuscript. LG designed the research and wrote the manuscript.

Declaration of Competing Interest

Dr. Graca reports grants from Fundação para a Ciência e Tecnologia, during the conduct of the study. All other authors have nothing to disclose.

Acknowledgments

Antibodies targeting CD4 were generously provided by Herman Waldmann (Oxford, UK). We thank Joana Duarte for her contribution to anti-CD4 experiments and Instituto Português do Sangue (IPST) for providing buffy coats.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ebiom.2019.08.008.

References

[1] Kamoun M, et al. A novel human T cell antigen preferentially expressed on mature T cells and shared by both well and poorly differentiated B cell leukemias and lymphomas. J Immunol 1981;127(3):987.
[2] Braun M, et al. The CD6 scavenger receptor is differentially expressed on a CD56 natural killer cell subpopulation and contributes to natural killer-derived cytokine and chemokine secretion. J Innate Immun 2011;3(4):420–34.
[3] Enyindah-Asonye G, et al. CD6 receptor regulates intestinal ischemia/reperfusion-induced injury by modulating natural IgM-producing B1a cell self-renewal. J Biol Chem 2017;292(2):661–71.
[4] Joo YS, et al. Evidence for the expression of a second CD6 ligand by synovial fibroblasts. Arthritis Rheum 2000;43(2):329–35.
[5] Bowen MA, et al. Cloning, mapping, and characterization of activated leukocyte-cell adhesion molecule (ALCAM), a CD6 ligand. J Exp Med 1995;181(6):2213–20.
[6] Enyindah-Asonye G, et al. CD318 is a ligand for CD6. Proc Natl Acad Sci U S A 2017;114(33):E6912–21.
[7] Bowen MA, et al. The amino-terminal immunoglobulin-like domain of activated leukocyte cell adhesion molecule binds specifically to the membrane-proximal scavenger receptor cysteine-rich domain of CD6 with a 1:1 stoichiometry. J Biol Chem 1996;271(29):17390–6.
[8] Zimmerman AW, et al. Long-term engagement of CD6 and ALCAM is essential for T-cell proliferation induced by dendritic cells. Blood 2006;107(8):3212–20.
[9] Te Riet J, et al. Dynamic coupling of ALCAM to the actin cortex strengthens cell adhesion to CD6. J Cell Sci 2014;127(Pt 7):1595–606.
[10] Gimferrer I, et al. Relevance of CD6-mediated interactions in T cell activation and proliferation. J Immunol 2004;173(4):2262–70.
[11] Hassan NJ, Barclay AN, Brown MH. Frontline: optimal T cell activation requires the engagement of CD6 and CD166. Eur J Immunol 2004;34(4):930–40.
[12] Whitney GS, et al. The membrane-proximal scavenger receptor cysteine-rich domain of CD6 contains the activated leukocyte cell adhesion molecule binding site. J Biol Chem 1995;270(31):18187–90.
[13] Bowen MA, et al. Characterization of mouse ALCAM (CD166): the CD6-binding domain is conserved in different homologs and mediates cross-species binding. Eur J Immunol 1997;27(6):1469–78.
[14] Chappell PE, et al. Structures of CD6 and its ligand CD166 give insight into their interaction. Structure 2015;23(8):1426–36.
[15] Skonier JE, et al. Mutational analysis of the CD6 ligand binding domain. Protein Eng 1997;10(8):943–7.
[16] Bodian DL, et al. Identification of residues in CD6 which are critical for ligand binding. Biochemistry 1997;36(9):2637–41.
[17] Resnick D, Pearson A, Krieger M. The SRCR superfamily: a family reminiscent of the Ig superfamily. Trends Biochem Sci 1994;19(1):5–8.
[18] Aruffo A, et al. CD6-ligand interactions: a paradigm for SRCR domain function? Immunol Today 1997;18(10):498–504.
[19] Li Y, et al. CD6 as a potential target for treating multiple sclerosis. Proc Natl Acad Sci U S A 2017;114(10):2687–92.
[20] Orta-Mascaro M, et al. CD6 modulates thymocyte selection and peripheral T cell homeostasis. J Exp Med 2016;213(8):1387–97.
[21] Consuegra-Fernández M, et al. Genetic and experimental evidence for the involvement of the CD6 lymphocyte receptor in psoriasis. Cell Mol Immunol 2017;15(2):92–5.
[22] Krintel SB, et al. CD6 and syntaxin binding protein 6 variants and response to tumor necrosis factor alpha inhibitors in Danish patients with rheumatoid arthritis. PLoS One 2012;7(6):e38539.
[23] Alonso R, et al. Aberrant expression of CD6 on B-cell subsets from patients with Sjogren's syndrome. J Autoimmun 2010;35(4):336–41.
[24] Ramos-Casals M, et al. High circulating levels of soluble scavenger receptors (sCD5 and sCD6) in patients with primary Sjogren's syndrome. Rheumatology (Oxford) 2001;40(9):1056–9.
[25] Montero E, et al. Immunodiagnosis and therapeutic immunosuppression in rheumatoid arthritis with ior t1 (anti-CD6) monoclonal antibody. Arthritis Res 2002;4 (Suppl. 1) (114–114).
[26] De Jager PL, et al. Meta-analysis of genome scans and replication identify CD6, IRF8 and TNFRSF1A as new multiple sclerosis susceptibility loci. Nat Genet 2009;41(7):776–82.
[27] Kofler DM, et al. The CD6 multiple sclerosis susceptibility allele is associated with alterations in CD4+ T cell proliferation. J Immunol 2011;187(6):3286–91.
[28] Kofler DM, et al. The link between CD6 and autoimmunity: genetic and cellular associations. Curr Drug Targets 2016;17(6):651–65.
[29] Zheng M, et al. Genetic polymorphisms of cell adhesion molecules in Behcet's disease in a Chinese Han population. Sci Rep 2016;6:24974.
[30] Brown MH. CD6 as a cell surface receptor and as a target for regulating immune responses. Curr Drug Targets 2016;17(6):619–29.
[31] Pinto M, Carmo AM. CD6 as a therapeutic target in autoimmune diseases: successes and challenges. BioDrugs 2013;27(3):191–202.
[32] Krupashankar DS, et al. Efficacy and safety of itolizumab, a novel anti-CD6 monoclonal antibody, in patients with moderate to severe chronic plaque psoriasis: results of a double-blind, randomized, placebo-controlled, phase-III study. J Am Acad Dermatol 2014;71(3):484–92.
[33] Jayaraman K. Biocon's first-in-class anti-CD6 mAb reaches the market. Nat Biotechnol 2013;31(12):1062–3.
[34] Aira LE, et al. Immunological evaluation of rheumatoid arthritis patients treated with itolizumab. MAbs 2016;8(1):187–95.
[35] Hernandez P, et al. Therapeutic targeting of CD6 in autoimmune diseases: a review of cuban clinical studies with the antibodies IOR-T1 and Itolizumab. Curr Drug Targets 2016;17(6):666–77.
[36] Duarte J, et al. T cell apoptosis and induction of Foxp3+ regulatory T cells underlie the therapeutic efficacy of CD4 blockade in experimental autoimmune encephalomyelitis. J Immunol 2012;189(4):1680–8.
[37] Agua-Doce A, et al. Route of antigen presentation can determine the selection of Foxp3-dependent or Foxp3-independent dominant immune tolerance. J Immunol 2018;200(1):101–9.
[38] Oliveira VG, et al. Adjuvant facilitates tolerance induction to factor VIII in hemophilic mice through a Foxp3-independent mechanism that relies on IL-10. Blood 2013;121 [19): p. 3936-45, S1].
[39] Consuegra-Fernandez M, et al. Relevance of CD6-mediated interactions in the regulation of peripheral T-cell responses and tolerance. Front Immunol 2017;8:594.
[40] Oliveira MI, et al. CD6 attenuates early and late signaling events, setting thresholds for T-cell activation. Eur J Immunol 2012;42(1):195–205.
[41] van Panhuys N. TCR signal strength alters T-DC activation and interaction times and directs the outcome of differentiation. Front Immunol 2016;7:6.
[42] Bughani U, et al. T cell activation and differentiation is modulated by a CD6 domain 1 antibody Itolizumab. PLoS One 2017;12(7):e0180088.
[43] Garner LI, et al. CD6 monoclonal antibodies differ in epitope, kinetics and mechanism of action. Immunology 2018;155(2):273–82.
[44] Oliveira VG, et al. Sub-optimal CD4+ T-cell activation triggers autonomous TGF-β-dependent conversion to Foxp3+ regulatory T cells. Eur J Immunol 2011;41(5):1249–55.