UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA

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SÉRGIO TIAGO DE FREITAS RIBEIRO

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

Orientador: Professor Doutor Bruno Miguel de Carvalho e Silva Santos

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- Doutor Luís Ricardo Simões da Silva Graça, Professor Associado com Agregação da Faculdade de Medicina da Universidade de Lisboa;
- Doutora Ana Cristina Gomas Espada de Sousa, Investigadora Principal, Professora Associada Convidada com Agregação da Faculdade de Medicina da Universidade de Lisboa;
- Doutor João Pedro Monteiro e Louro Machado de Simas, Professor Associado da Faculdade de Medicina da Universidade de Lisboa;
- Doutor João Pedro Taborda Barata, Professor Associado Convidado da Faculdade de Medicina da Universidade de Lisboa
- Doutor Bruno Miguel de Carvalho e Silva Santos, Professor Associado com Agregação da Faculdade de Medicina da Universidade de Lisboa; (orientador)

Instituição Financiadora: Fundação para a Ciência e Tecnologia, IP (SFRH/BD/84123/2012)

2017

PREFACE

This thesis describes the research work under the scope of my PhD project developed between 2013 and 2016 at the Instituto de Medicina Molecular (Lisbon, Portugal) at Silva-Santos lab (T cell Differentiation & Tumor Targeting unit) under the supervision of Professor Bruno Silva Santos, PhD.

Sérgio T Ribeiro was financially supported by a scholarship from "Programa SFRH" (SFRH/BD/84123/2012), Fundação para a Ciência e Tecnologia, Portugal, and is a PhD candidate from Programa Doutoral do Centro Académico de Medicina de Lisboa from Universidade de Lisboa.

This thesis is organized in five chapters, which are preceded of an abstract briefly describing the work developed and a summary written in Portuguese. In chapter I an introductory review, the state of the art and the aims of the work are provided. The chapter II comprises material and methods used to obtain the original results presented in chapter III. The chapter IV comprises the conclusions and the biological implications of the results. The future perspectives are highlighted in chapter V.

JC Ribot and DV Correia developed part of the results presented in section 1 of chapter III as disclosed in Ribot *et al* JI 2014. M Tesio and JC Ribot helped the development of the results presented in section 2 of chapter III as disclosed in Ribeiro *et al* Leukemia 2016

The statements expressed in this thesis are from the exclusive responsibility of the author.

The impression of this thesis was approved by Conselho Científico from Faculdade de Medicina da Universidade de Lisboa at 22 of November 2016.

The following agencies financially support the development of the work presented in this thesis:

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Acknowledgments

Quero expressar um especial agradecimento conjunto às pessoas que de variadas formas ajudaram não só no trabalho desenvolvido nestes últimos 4 anos, mas sobretudo pela transmissão de conhecimentos, inspiração e apoio:

Bruno Silva-Santos
Afonso Almeida
Agostinho Freitas
Alice Melão
Ana Água Doce
Ana Espada Sousa
Ana Pamplona
Ana Sílvia Gonçalves
Ana Soares
Ana Veríssimo
André Simões
Andreia Carneiro
Anita Gomes
Bethania Cassani
Biagio Di Lorenzo
Bruno F Ribeiro
BSS lab members
Daniel Correia
Daniel Ribeiro
David Guéniot
Diogo Pereira
Elizabeth Macintyre
Elvira Leite
Eva Rolo
Fernando M Ribeiro
Francisco Caiado
Gisela Gordino
Guiomar Rosa
Hakan Norell
Hélder Ribeiro
Helena Brigas
Helena Cabaço
Helena Soares
Henrique Girão
Herwig Turk
Hiroshi Kubo
Hugo Gonçalves
Joana Lapa
Joana Martins
Joana Palha
João Ferreira
João T Barata
Joaquim Freitas
Jorge Freitas
José Ramalho
Julie C Ribot
Karine Serre
Luís Graça
Margarida Rei
Maria M Freitas
Melania Tesio
Miguel A Ribeiro
Miguel Muñoz Ruiz
Miguel Ribeiro
Natacha Sousa
Nina Schmolka
Noel de Keyzer
Patrícia Almeida
Paula Romero
Paulo Pereira
Pedro Oliveira
Pedro Papotto
Pedro Providência
Rita Domingues
Saulė Kiaunytė
Sílvia Madeira
Sofia Mensurado
Telma Lança
Tiago Amado
Vanda Póvoa

muito obrigado,
Sérgio

List of abbreviations

7-AAD	7-aminoactinomycin D
ADCC	antibody-dependent cellular cytotoxicity
Ags	antigens
AICD	activation-induced cell death
AKT	Protein kinase B
ALCL	Anaplastic large cell lymphoma
ALK	Anaplastic lymphoma kinase
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AP-1	Activator protein 1
APCs	Antigen presenting cell(s)
ATP	Adenosine triphosphate
B-CLL	B-cell chronic lymphocytic leukemia
BAFT	basic leucine zipper transcription factor ATF-like
BAT3	B-associated transcript 3
Bcl-2	B-Cell Lymphoma 2
BCR	B cell receptor
BM	bone marrow
BTLA	B and T lymphocyte attenuator
BTN3A	Butyrophilin-3A (CD277)
CARs	chimeric antigen receptors
CCL3	Chemokine (C-C motif) ligand 3 (or MIP-1α)
CCL4	Chemokine (C-C motif) ligand 4 (or MIP-1β)
CCL5	Chemokine (C-C motif) ligand 5 (or RANTES)
CCT6A	Chaperonin Containing T-complex protein 1 subunit zeta
CD	Cluster of differentiation
Cdc25	cell division cycle 25
CDR3δ	complementarity-determining regions 3δ
CFSE	Carboxyfluorescein succinimidyl ester
CK2	Casein kinase II
CLL	Chronic lymphocytic leukemia
CML	chronic myeloid leukemia
CMV	Cytomegalovirus
CNS	Central nervous system
Csk	C-terminal Src kinase
cTEC	cortical thymic epithelial cells
CTLA-4	Cytotoxic T lymphocyte antigen 4
CXCL13	C-X-C Motif Chemokine Ligand 13
CXCR5	C-X-C chemokine receptor type 5
DAP10	DNAX-activating protein of 10 kDa
DAP12	DNAX-activating protein of 12 kDa
DC	Dendritic cell
DETCs	Dendritic epidermal T cells

DLBCL Diffuse large B-cell lymphoma
DN double-negative
DNA Deoxyribonucleic Acid
DNAM-1 DNAX accessory molecule-1
DOT Vδ1+ T cell
DP double-positive
DUSPs dual-specificity phosphatases
E# embryonic day #
EDTA Ethylenediaminetetraacetic acid
eGFP enhanced green fluorescence protein
EGIL European group for immunological characterization of leukemias
EGR Early Growth Response
ERK extracellular signal-regulated kinases
ETPs Early T cell precursors
FACS Fluorescence-activated cell sorting
FAF-1 Fas-associated factor-1
FBS Fetal bovine serum
FGF-2 fibroblast growth factor-2
FITC Fluorescein
FPPS farnesyl pyrophosphate synthase
GM-CSF granulocyte–macrophage colony-stimulating factor
Grb2 Growth factor receptor-bound protein 2
GSK3β glycogen synthase kinase-3β
HIV human immunodeficiency virus
HMBPP (E)-4-hydroxy-3-methyl-but-2-enyl pyrophosphate
HRP horseradish peroxidase
ICAM-1 Intercellular Adhesion Molecule 1
ICOS Inducible T-cell co-stimulator
Id3 Inhibitor of DNA binding 3
IFN-γ Interferon-γ
Ig Immunoglobulin
IkB Inhibitor of κB
IKK IkB kinase
IL-2 Interleukin-2
IL-2R Interleukin-2 Receptor
IP3 Inositoltriphosphate
IPP Isopentenyl pyrophosphate
IRES internal ribosomal entry site
ITAM Immunoreceptor tyrosine-based activation motif
ITIM Immunoreceptor tyrosine-based inhibitory motif
ITK interleukin-2-inducible T cell kinase
ITSM Immunoreceptor tyrosine-based inhibitory switch motif
Jak Janus kinase
JNK c-Jun N-terminal kinase
LAT Linker of activated T cells
LCK Lymphocyte-specific protein tyrosine kinase

Lef1 Lymphoid enhancer binding factor 1
LFA-1 Lymphocyte function-associated antigen 1
LMO1/2 Rhombotin-1/2,
LPC lysophosphatidylcholine
LTβR lymphotoxin-β receptor
mAb Monoclonal antibody
MACS Magnetic-activated cell sorting
MAPK mitogen-activated protein kinase
MCL mantle cell lymphoma
MDSCs myeloid-derived suppressor cells
MFI Median fluorescence intensity
MHC Major Histocompatibility Complex
MICA MHC class I polypeptide-related sequence A
MICB MHC class I polypeptide-related sequence B
MM multiple myeloma
MPM mannosyl-β1-phosphomycoketide
mRNA messenger RNA
MTD maximum tolerated dose
mTEC medullary thymic epithelial cells
mTOR mammalian target of rapamycin
mTORC1 mTOR complex I
MULT1 mouse UL16-binding protein-like transcript 1
NCR Natural cytotoxicity receptor
NF-κB Nuclear factor kappa B
NFAT Nuclear Factor of Activated T Cells
NK Natural killer
NKR Natural killer cell–associated receptor
NKT Natural killer T cell
NOD Non-obese diabetic
NPM Nucleophosmin
NRGS NOD-Rag1^{null} IL-2Rγ^{null}
NSG NOD-SCID IL-2Rγ^{null}
PAGE polyacrylamide gel electrophoresis
PBL Peripheral blood lymphocyte
PBMC Peripheral blood mononuclear cell
PBS Phosphate Buffered Saline
PCR Polymerase Chain Reaction
PD-1 Programmed cell death protein 1
PE Phycoerythrin
Pen/Strep Penicillin Streptomycin
PHA Phytohemagglutinin
PI3K Phosphatidylinositol 3-kinase
PKA protein kinase A
PKC protein kinase C
PLC phospholipase
PMA Phorbol 12-myristate 13-acetate

PRR	Pattern recognition receptors
PTEN	Phosphatase and tensin homolog
RAG	recombination activating gene
RNA	Ribonucleic Acid
ROR	retinoic acid-related orphan receptor
RPMI	Roswell Park Memorial Institute cell culture medium
RT-qPCR	Real-time–quantitative polymerase chain reaction
S6K	40S ribosomal protein S6 kinase
SCID	Severe combined immunodeficiency
SDS	sodium dodecyl sulfate
SFKs	Src family kinase
SFKs	Src-family kinases
SH2	Src homology region 2
SHP-1/2	SH2 domain-containing phosphatase-1/2
SKG	Sakaguchi (or Zap70^{m1Saka})
Skip2	S-phase kinase interacting protein 2
SLP-76	Lymphocyte cytosolic protein 2
SOCS3	Suppressor of cytokine signaling 3
SOX13	sex-determining region Y-box 13
SP	Single positive
STAT	Signal transducer and activator of transcription
Syk	Spleen tyrosine kinase
TAL1	T-cell acute lymphocytic leukemia protein 1
TCF1	T cell factor
T_{CM}	Central memory T cell
TCR	T cell receptor
T_{EM}	Effector memory T cell
TGF-β	Transforming growth factor β
Th1	T helper cell type 1 (Interferon γ-producing cell)
Th17	T helper cell type 17 (IL-17-producing cell)
TIL	Tumor infiltrating lymphocyte
TLR	Toll-like receptor
TLX1/3	T-cell leukemia homeobox 1/3
TNF	Tumor necrosis factor
TNFR	Tumor necrosis factor receptor
TRAF	TNF receptor associated factors
TRAV14	TCRα variable region 14
TSC2	tuberous sclerosis complex 2
ULBP	UL16 binding protein
UV	Ultraviolet
ZAP70	zeta-chain (TCR) associated protein kinase 70kDa
β2m	β2-microglobulin
γc	Common cytokine receptor γ chain

Abstract

Among the various leukocyte populations that build up the immune defense against infections and tumors, γδ T lymphocytes constitute an enigmatic lineage whose molecular mechanisms of differentiation and activation are still poorly understood. The key roles played by γδ T cells in immunity critically depend on their survival, activation and differentiation into effectors capable of secreting cytokines and killing infected or transformed cells. These processes are controlled, at the molecular level, by surface receptors that capture key extracellular cues and convey downstream intracellular signals that regulate both lymphocyte physiology and pathology.

In this PhD thesis we evaluated the contribution of cell receptor signaling pathways to human γδ T cell differentiation and activation. Firstly, we showed that human γδ thymocytes are functionally immature and their differentiation program requires additional IL-2 or IL-15 signals to drive their differentiation into IFN-γ and TNF-α producers endowed with potent cytotoxicity against leukemia targets.

We further elucidated the signals involved in normal/ healthy γδ T cell maintenance as well as in γδ T cell tumorigenesis. We identified that the protein kinase CK2 (Casein Kinase 2) is overactivated in the γδ T cell lineage compared to αβ counterparts. We further showed that the clinical grade-inhibitor of CK2, CX-4945, impairs γδ T cell survival by inhibiting the CK2/AKT/mTOR/GSK3β signaling pathway. Moreover, we showed that CK2 is hyperactivated in γδ T acute lymphoblastic leukemia (T-ALL) samples, compared to both normal γδ T cells and αβ T-ALL. Importantly, we demonstrated a high sensitivity of γδ T-ALL cells to CX-4945 treatment in vitro and in vivo, thus supporting the use of CK2 inhibitors as a putative therapy for γδ T-ALL.

Overall, the data presented in this thesis provided new evidences indicating that: (i) human γδ thymocytes are functionally immature and require IL-2 or IL-15 to differentiate into type 1 cytotoxic effector lymphocytes; (ii) the protein kinase CK2

is a novel determinant of both healthy and leukemic γδ T cell survival; and (iii) the CX-4945 chemical inhibitor is a promising therapeutic approach for γδ T-ALL.

Keywords: γδ T lymphocytes; T cell differentiation; acute lymphoblastic leukemia; signaling pathways; protein kinase CK2.

Resumo (Portuguese)

O sistema imunitário consiste num vasto e complexo conjunto de moléculas, células, tecidos e órgãos responsáveis pela proteção de um organismo contra agentes externos ou células transformadas do próprio organismo. Estes mecanismos de proteção são conceptualizados em dois tipos de resposta imunitária, a imunidade inata e a imunidade adquirida ou adaptativa. Estes mecanismos são, em larga media, baseados no principio básico da distinção entre o "próprio" e o "não próprio". Os linfócitos T, ou células T, são responsáveis pela imunidade adaptativa celular, desempenhando um papel fundamental no reconhecimento de organismos invasores ou moléculas "estranhas" ou associadas a stress celular. As células T derivam das células estaminais hematopoiéticas da medula óssea e maturam no timo onde ocorre a recombinação somática V(D)J, que contribui para a diversidade dos seus receptores de células T (TCR). As células T do timo (timócitos), através de sucessivos estádios de seleção, dão origem a células T αβ ou T γδ, que se caracterizam respectivamente pela expressão de TCRαβ ou TCRγδ.

As células T γδ são caracterizadas como células T não convencionais, apresentando características quer das células do sistema inato quer do sistema adquirido. Por exemplo, as células T γδ são independentes do complexo principal de histocompatibilidade (MHC) aquando do reconhecimento do antigénio pelo TCR. As células T γδ têm a capacidade de reconhecer e responder a vários estímulos incluindo bactérias, vírus e células tumorais, tendo recentemente sido as células imunitárias associadas a um prognóstico mais favorável em doentes oncológicos.

No homem, existem maioritariamente dois subtipos de células T γδ, Vδ1 e Vδ2, que reconhecem ligandos distintos e apresentam diferentes tropismos. As células Vδ1 são o subtipo maioritário no timo e tecidos epiteliais, ao passo que as células Vδ2 se encontram maioritariamente no sangue (e gânglios linfáticos). As funções das células T γδ resultam, predominantemente, da sua ativação e diferenciação em

células capazes de produzirem citocinas e induzirem a morte das células alvo. Estas funções são iniciadas por sinais extracelulares, captados pelas células e que desencadeiam diversas vias de sinalização no interior da célula. Em última instância, estas vias de sinalização determinam a resposta das células T ao estímulo, assegurando uma função imunológica normal e uma resposta adequada em situações de stress. Porém, estes mecanismos são amplamente desconhecidos no caso da linhagem T γδ.

Nesta tese de doutoramento, analisamos a contribuição de diferentes vias de sinalização mediadas por diferentes citocinas na diferenciação de timócitos γδ humanos. São ainda analisados, com especial detalhe, os mecanismos moleculares envolvidos na sobrevivência das células T γδ normais ou tumorais. A compreensão e modulação destes mecanismos moleculares é especialmente importante na medida em que estes podem constituir potenciais alvos terapêuticos em contexto de imunoterapia envolvendo células T γδ. Estas vias podem, por exemplo, ser moduladas com o objectivo de promover as funções citotóxicas das células γδ contra células tumorais; ou constituir alvos terapêuticos em situações de leucemia linfoblástica aguda com origem nas células T γδ.

Os resultados descritos nesta tese mostram que os timócitos γδ, ao contrario do que se observa nos linfócitos T γδ periféricos, são funcionalmente imaturos. Isto é, os timócitos γδ (maioritariamente do subtipo Vδ1) não têm a capacidade de produzir citocinas nem a capacidade de induzir a morte celular de células tumorais, o que mostra que o programa de diferenciação das células T γδ humanas não é finalizado no timo. Tal constitui uma diferença importante em relação aos timócitos γδ de murganho. No entanto, a estimulação dos timócitos γδ humanos com interleucina-2 (IL-2) ou IL-15, mas não com IL-4 ou IL-7, promove a diferenciação celular, através da via de sinalização da proteína cinase activada por mitogénios (MAPK)/ proteína cinase activada por sinais extracelulares (ERK), em células citotóxicas produtoras de interferão-γ (IFN-γ) e factor de necrose tumoral-

α (TNF-α). Esta diferenciação dos timócitos necessita apenas das citocinas (IL-2 ou IL-15), mas não de sinais derivados do TCR.

Por outro lado, os nossos resultados mostram que a sobrevivência das células T γδ humanas é dependente da proteína cinase CK2, que tem uma atividade aumentada quando comparada com a das células T αβ. A CK2 é uma enzima pleiotrópica que fosforila resíduos de serina e treonina. Esta cinase apresenta atividade constitutiva que, frequentemente, se encontra hiperativada em células tumorais. Nesta tese, mostramos que a ativação do TCR nas células T γδ leva a um aumento da atividade de CK2 e à ativação das vias de sinalização AKT/ receptor mamífero de rapamicina (mTOR), promovendo a sobrevivência celular. Por outro lado, a inibição especifica de CK2, através do inibidor CX-4945, leva a um bloqueio da via AKT e a um aumenta da morte celular por apoptose das células T γδ, mas não das T αβ saudáveis. Verificamos, ainda, que a hiperativação de CK2 é uma característica adquirida por células malignas de leucemia linfoblástica aguda (LLA) com origem na linhagem T γδ. Os resultados obtidos em amostras provenientes de doentes mostram que as células transformadas são do subtipo Vδ1, e que o tratamento com o inibidor CX-4945 leva à apoptose destas células LLA-T γδ primárias. O conjunto dos resultados obtidos quer in vitro e in vivo (modelo xenotransplante em murganhos imunodeficientes) mostram que as células LLA-T γδ são sensíveis ao inibidor da CK2, CX-4945, abrindo novas oportunidades à sua utilização no tratamento de doentes com este tipo de leucemia.

Globalmente, os resultados apresentados e discutidos nesta tese, demonstram que as células T γδ provenientes do timo humano são funcionalmente imaturas, necessitando de um último estádio de diferenciação dependente de IL-2 ou IL-15, de modo a adquirirem capacidades de produção de IFN-γ e TNF-α e citotoxicidade anti-tumoral. Por outro lado, mostramos que as células T γδ, em comparação com as células T αβ, apresentam uma maior atividade da proteína cinase CK2 e maior suscetibilidade à morte celular após inibição de CK2. As funções de CK2 podem ser exacerbadas por estímulos externos como TCR, quer em células normais quer em

células transformadas. Dada a hiperativação da CK2 em células transformadas LLA-T γδ, e a sua susceptibilidade ao inibidor CX-4945, propomos que a CK2 constitui um importante alvo terapêutico em doentes diagnosticados com este subtipo de leucemia.

Palavras-chave: linfócitos T γδ; diferenciação de células T; leucemia linfoblástica aguda; vias de sinalização; proteína cinase CK2.

Chapter I

General Introduction

I. GENERAL INTRODUCTION

1. The immune system

The immune system is responsible for discriminating the cells and tissues that are a legitimate part of the body, from foreign molecules and organisms ("nonself") that might be present in the organism. By complex cellular and molecular events mediated by innate (antigen-nonspecific) and adaptive (antigen-specific) immune response, the immune system eliminates those nonself invaders, which are often dangerous bacteria or viruses. In addition, the immune system can recognize, and usually eliminate, "altered self"–cells or tissues that otherwise could originate malignancies[1].

Leukocytes of the innate immune system serve as sentinels for detecting general signs of danger. These cells are usually equipped with pattern recognition receptors (PRR) leading to a fast and unspecific activity against groups of pathogens. Thus, these innate myeloid cells (Phagocytes, Mast cells, Basophils, Eosinophils) and innate-like lymphocytes (NK cells, NKT cells and γδ T cells) constitute the first line of defense against pathogens and/or transformed cells. Innate responses can also modulate the adaptive branch of the immune system. In particular, dendritic cells act as critical antigen-presenting cells (APCs) to start αβ cell responses.

Additionally, the adaptive system is composed of B cells and αβ T lymphocytes, which react specifically to diverse pathogenic conditions. Adaptive immunity is triggered later and usually generates memory cells that persist in the organism and can be reactivated upon a second encounter with the same antigen. The cells of the adaptive immune system express a comprehensive repertoire of cell surface antigen-specific immunoglobulin receptors - B cell receptors (BCR) for B cells and T cell receptors (TCR) for T cells - that can collectively recognize millions of distinct antigens. These receptors are generated by somatic recombination

mediated by recombination activating gene (RAG) enzymes, an extraordinary process common to all jawed vertebrates which developed primary (like the thymus) and secondary (like spleen and lymph nodes) lymphoid organs[1].

1.1. Thymus and T cell development

The anatomic sites where the major steps in lymphocyte development occur are called primary or generative lymphoid organs. These include the bone marrow, where the precursors of all lymphocytes arise and B cells mature, and the thymus, where T cells mature. Following maturation, the lymphocytes enter the circulation and peripheral lymphoid organs (e.g. the spleen and lymph nodes) where they survey for invading pathogens and/or tumor cells[2].

The thymus is present in all jawed vertebrates, present similar structure and undergoes the same shrinkage with age and plays the same immunological function as in human beings. The lymphocytes in the thymus, also called thymocytes, are T lymphocytes at various stages of maturation. The most immature cells enter the thymus, and their maturation begins in the cortex where thymic cortical epithelial cells (cTEC) produce interleukin-7 (IL-7), a critical factor for survival and differentiation. During maturation, through different steps of cell selection, thymocytes migrate toward the medulla where medullary thymic epithelial cells (mTEC) play a special role in presenting self-antigens to developing T cells and causing their ablation (negative selection). This is one mechanism of ensuring that the immune system remains tolerant to self-antigens, avoiding autoimmunity. It is only after maturation, that positively-selected naïve T cells exit the thymus and enter the blood and peripheral lymphoid tissues.

Due a large extend to the remarkable technical advances, mainly by the creation of genetically altered animals, T cell development is better characterized in mice than in human. However, human T cell development appears to depend on similar mechanisms (as in mice), and the key factors in T cell development may be broadly conserved among jawed vertebrates. Of note, the congenital absence of the

thymus, as occurs in DiGeorge syndrome in humans or in the nude mouse strain (athymic), is characterized by very low numbers of mature T cells in the circulation and peripheral lymphoid tissues and severe deficiencies in T cell–mediated immunity[1].

It is well established that T-cell precursors that arrives in the thymus contain TCR genes in their germline configuration and do not express TCR, CD3, ζ chains, CD4, or CD8 receptors. These double-negative (DN) thymocytes are highly immature progenitors, comprising about 1–2% of thymocytes, that in both murine and human undergo successive stages of cell selection. T cell commitment corresponds, at the molecular level, to productive TCR rearrangements. These occurs at four gene loci, TCRα, β, γ and δ, present in all jawed vertebrates. The protein products of somatic recombination in T cells can pair in two stable complexes, TCRαβ or TCRγδ, which are mutually exclusive and thus define two T cell lineage, αβ and γδ T cells, which diverge in T cell development[3–5].

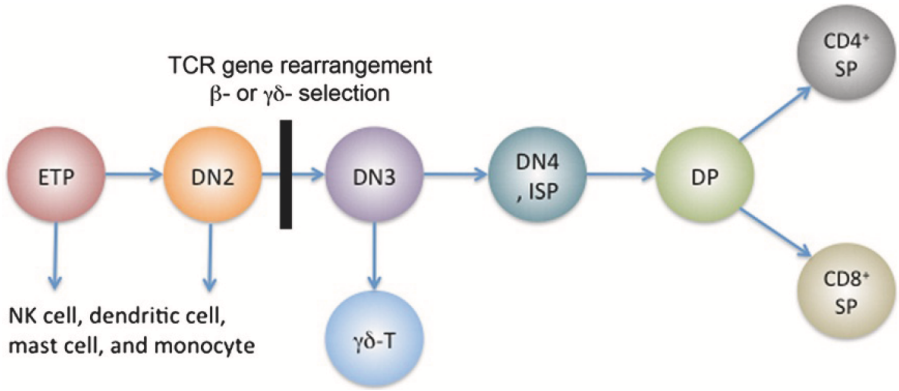

Figure 1: Stages in T cell development.

Early T cell precursors (ETPs) differentiate from double negative (DN) to double positive (DP) to single positive (SP) stages. Arrows indicate cell differentiation. Note that ETP and DN2 thymocytes contain non-T-cell options. β- and γδ- selection occurs during the accumulation of the DN3 T cells (adapted from Peng Li *et al*[6]).

Immature murine DN thymocytes can be separated into four populations (DN 1-4) based on the expression of CD44 and CD25 (Ref. [7]). However, immature human thymocytes do not express the same surface markers and are characterized by the differential expression of CD34, CD38, and CD1a[4].

During T cell maturation, there is a precise order in which *TCR* genes are rearranged and expressed. In the mouse, surface expression of the TCRγδ occurs 3 to 4 days after precursor cells arrived the thymus, and the TCRαβ is expressed 2 or 3 days later. In human fetal thymuses, TCRγδ expression begins at about 9 weeks of gestation, followed by expression of the TCRαβ at 10 weeks[1].

These differentiation steps are controlled by a complex system of several transcriptional factors[3]. The transcription factors Notch-1 and GATA-3 are responsible to commit developing lymphocytes to the T cell lineage. Firstly, the cell surface molecules of Notch family are proteolytically cleaved following interaction with specific ligands on neighboring cells in the thymus. In mammals, two families of Notch ligands, Delta-like (DL) and Jagged, have been identified[8]. The activated intracellular domain of Notch proteins migrate to the nucleus and modulate the expression of specific target genes[1,9]. T cell development depend on several transcription factors, including: GATA-3, c-Myc, members of the Runx family, members of the E2A/HEB family, and members of the Ikaros family[10,11]. In addition the Wnt/TCF signaling cascade has an important role in proliferation coupled with differentiation of T cells[11].

Somatic rearrangement of the genes encoding the TCRβ, TCRγ and TCRδ chains, mediated by Rag-1 and Rag-2 proteins, is essential for TCR expression and diversity. The rearrangement of these *TCR* genes begins in DN2 cells and is mostly completed during the DN3 stage. If a cell succeeds in productively rearranging its TCRγ as well as its TCRδ loci before it makes a productive TCRβ rearrangement, it is selected into the γδ T cell lineage. Otherwise, when a TCRβ chain is expressed from a productively rearranged Tcrβ locus (V-D-J recombination) the cells progress to a αβ T cell lineage. The TCRβ pairing with the invariant pre-TCRα chain

forms a pre-TCR[12–15]. Pre-TCR signaling, coupled to signals from cytokine and possibly other receptors, promotes cell survival, proliferation and differentiation. Following β-selection DN4 cells subsequently become DP $CD4^+CD8^+$ population that comprises 75–88% of thymocytes. DP cells complete Tcrα gene rearrangement (V-J recombination) and present TCRαβ heterodimers at cell surface. The expression of functional TCRαβ provides the substrate for MHC-mediated positive selection into SP $CD4^+$ or $CD8^+$ populations followed by negative selection that eliminates autoreactive αβ thymocytes. Finally, these mature thymocytes proliferate shortly immediately before migrating to the periphery to function as mature αβ T cells[16]. However this conventional model of T cell development does not apply to MHC-unrestricted γδ T cells.

1.1.1. γδ T cell development

Early during thymocyte differentiation γδ T cells diverge from αβ T cells and continue along different developmental paths. The bifurcation of αβ and γδ lineage-commitment is thought to be complete at DN3 and these decisions are influenced by different site-specific signals, derived from cytokines, chemokines, TCRs, Wnt signaling, Notch ligands, and others thymic microenvironment stimuli[17]. Successful in-frame rearrangement of the *TCRγ* and *δ* genes results in the expression of a TCRγδ complex and favos differentiation along the γδ lineage[17–19]. In both mouse and humans, *TCRδ* rearranges first, followed closely by *TCRγ* rearrangement[17].

This recombination joins any one of several variable (*V*) gene segments with any one of several joining (*J*) segments and, in TCRδ, also with diversity (*D*) gene segments. The somatic recombination of multiple gene segments contributes to the diversity of receptor-structure. Following gene recombination, only cells that make productive TCRs are selected to survive[20].

Factors such as IL-7R expression[21] or Notch signaling[22–25] are described to be involved in γδ lineage-commitment[26]. IL-7R controls the recombination at the

TCRγ locus by regulating Rag accessibility[27]. Moreover, murine thymocytes with abundant IL-7R are more likely to give rise to γδ T cell lineage than thymocytes lacking IL-7R[28]. In addition, immature thymocytes with high levels of IL-7R express more of the transcription factor SOX13 (sex-determining region Y-box 13) protein. SOX13 is a putative T cell lineage regulator that promotes the γδ lineage and impairs αβ development. Mice deficient in SOX13 expression can still produce mature αβ T cells, whereas the development of γδ T cells is impaired[17,29].

Notch signaling is also thought to be involved in αβ versus γδ T cell lineage commitment. In mouse, high levels of Notch signaling are required for αβ T cell development compared to γδ T cells. Reduced levels of *Notch1* gene in vivo favors γδ T cell development over αβ T cells[30,31]. However γδ T cell commitment may require different ligands of Notch[32]. In humans, the involvement of Notch signaling in T cell development is less clear and paradoxical to the mouse data. Toribio and Plum's groups showed that increased Notch activation results in increased γδ T cell development at the cost of αβ T cells[33–35]. Thus, the involvement of Notch in γδ T cell commitment remains somewhat controversial.

The γδ T cell lineage fate is not simply instructed by the type of rearranged TCR complex that is found on the surface of a given cell. Instead, the "strength" of the signal that is delivered by the TCR complex seems to be critical[36,37]. Thus, TCRγδ, which appears to signal relatively strongly, directs cells towards the γδ lineage, whereas the preTCR, which generates a weaker signal (than TCRγδ), promotes the development of αβ lineage cells[38–41]. This model suggests that a strong TCRγδ signal would result in higher activation of the extracellular signal-regulated kinases/ mitogen-activated protein kinases (ERK-MAPK) pathway, leading to a higher induction of the Early Growth Response (EGR1, EGR3) transcription factors and their target Inhibitor of DNA binding 3 (Id3) in a mouse model[36,42,43]. Id proteins are direct inhibitors of E2A, a helix-loop-helix protein, which activates the pre-TCRα promoter[44]. The higher accumulation of Id3 results in stronger inhibition of E2A, and thus expression of γδ T-cell hallmark genes. Conversely,

weaker signals through the Notch receptors and pre-TCR would result in more modest accumulation of Id3, and hence weaker inhibition of E2A leading to further development along the αβ lineage[17,41]. By analogy, as a consequence it is also likely that the signals downstream of each TCR results in differential regulation of transcription factors that are essential for the functional maturation of different effector subsets[41]. Thus, the TCR signal strength required for the development of multiple subsets of γδ T cells is likely heterogeneous. Consistent with this hypothesis, in the host lab, Muñoz-Ruiz *et al* elegantly showed that TCR signal strength controls the differentiation of specific subsets of mouse γδ T cells during thymic ontogeny[45].

1.1.2. Mouse γδ T cell subsets

γδ T cells are conserved throughout evolution and across vertebrate species. However, γδ T cell subsets are highly heterogeneous. The different subsets of γδ T cells are typically grouped by the variable (V) segments encoded by rearranged Vγ and/or Vδ genes, with Vγ being the most significant in the mouse, while Vδ is the most relevant in humans[20,46–48].

Murine γδ T cells are generated in the thymus in "developmental waves" that sequentially populate different tissues during embryonic development[20].

Following Vγ gene nomenclature of Heilig and Tonegawa[19] for murine γδ T cells, thymocytes bearing an invariant canonical Vγ5Vδ1 TCR at embryonic day E15-17 are the first to leave the foetal thymus, giving rise to skin-associated dendritic epidermal T cells (DETCs). Thymocytes bearing a Vγ6Jγ1Cγ1 TCR at E16-18 give rise to the γδ T cells in the tongue and reproductive tract, whereas peri-and postnatal thymocytes bearing Vγ1Cγ1 and Vγ4Cγ1 TCRs give rise to systemic γδ T cells (that predominate in lymph nodes and spleen).

Many of the studies elucidating the physiological roles of γδ T cells have been performed in murine models, where the identification of pro-inflammatory subsets naturally producing either IFN-γ or IL-17 was a major recent breakthrough[49–51].

The segregation of the two functional γδ T cell subsets has been greatly facilitated by the identification of cell surface markers: CD27, CD122 and NK1.1 mark IFN-γ-producing γδ cells, whereas their IL-17-expressing counterparts display a $CD27^{-}$ $CCR6^{+}$ phenotype[49–51]. Moreover, the two subsets show distinct Vγ chain usage in their TCR repertoires, with a bias towards Vγ1 among IFN-γ-producing γδ cells, and an enrichment in Vγ4 and Vγ6 in IL-17-producing γδ cells[52]. Moreover, the transcription factors Sox13 and RORγt (*Rorc*) are essential for IL-17 producing γδ T-cells, including $V\gamma6^{+}$ and some $V\gamma4^{+}$ T cells, while Eomes and Tbet are hallmarks of IFN-γ producing subtypes, such as $V\gamma5^{+}$ dendritic epidermal T-cells[41]. The diversity of mouse γδ T cell subsets and associated functions is reviewed elsewhere[20,39,41,48,52–55].

2. Human γδ T cells

Human γδ T cells, like their murine counterparts, are a minor population (1–10% of nucleated cells) in peripheral blood but are abundant in tissues, especially in epithelial layers[46]. For identification purposes, they are usually sub-divided based on the variable regions of TCRδ. Using Lefranc and Rabbits numenclarure[56], the human γδ T cell subsets comprise the Vδ1, Vδ2 and, the less studied, Vδ3 subset. Vδ1 γδ T cells are the predominant subset found at mucosal surfaces (**Table 1**). By contrast, Vδ2 γδ T cells (that are almost exclusively $V\gamma9^{+}$) largely dominate in the peripheral blood[18,57,58]. Indeed, Vδ2 T cells can represent more than 50% of blood leucocytes after certain bacterial or parasitic infections[59]. However the ligands, signaling mechanisms of cell activation and functional development as well as the cell receptors involved are poorly described.

γδ T cells may be considered a component of the adaptive immune system as they somatically rearrange their TCR genes to generate great diversity; and can selective expand particular subpopulations upon infection. But, on the other hand, γδ cells endow the T cell compartment with a rapid, innate-like reaction to insults, which places them in the afferent phase of the immune response. γδ T cells are

responsible for "lymphoid stress surveillance", i.e., sensing and responding immediately to infections or non-microbial stress without the need of clonal expansion or *de novo* differentiation, in synchrony with prototypic innate immune responses[60]. Critically, this implicates γδ T cells in inflammation[61], autoimmunity[62], infectious diseases[63,64], and tumor surveillance[65–67]. This section 2 describes the main characteristic of human γδ T cells including their subsets, functions as well as the mechanisms involved in their differentiation and activation.

2.1. Human γδ T cell subsets

In humans, γδ T cells have a small repertoire of V gene segments to select from when undergoing TCR chain rearrangement in comparison with those available for Vα (43–45 (Ref.[68])) and Vβ (40–48 (Ref.[69])). Three main Vδ gene segments, Vδ1, Vδ2 and Vδ3 (**Table 1**), are most frequently used in rearrangement of the δ chain; less commonly used are the five V segments that have both Vδ and Vα designation (Vδ4/TRAV14, Vδ5/TRAV29, Vδ6/TRAV23, Vδ7/TRAV36 and Vδ8/TRAV38)[70]). Seven functional Vγ gene segments, Vγ2, Vγ3, Vγ4, Vγ5, Vγ8, Vγ9 and Vγ11, located within the γ locus on chromosome 7 in humans, are used for rearrangement of the γ chain[71]. Despite the restricted repertoire of Vδ and Vγ gene segments available for rearrangement, the complementarity-determining regions 3δ (CDR3δ) allows the incorporation of multiple Dδ segments (in forward and reverse direction) and theoretically results in the most diverse of all the rearranged receptors[71], representing about 10^{18} different repertoire possibilities compared to only 10^{16} of αβ T cells[1]. The genetic evolution of TCRγδ associated genes and ligand recognition from non-human primates to humans is reviewed elsewhere[71].

Table 1: Distribution and repertoire of human γδ T cells.

(Adapted from Silva-Santos *et al.*; Bonneville *et al.* and Chien *et al.*[47,54,72])

Subset	Body distribution	Most common VγVδ pairs	V(D)J diversity	TCR-ligand/ reactivity
Vδ1	Thymus, spleen, liver, gut epithelium, dermis	variable	High	CD1d-sulfatide/ α-GalCer; CD1c-sulfatide/ LPC/ MPM[73]; MICA; CMV-infected cells;
Vδ2	Peripheral blood	Vγ9Vδ2	Intermediate	Phosphoantigens; ULBP4; F1-ATPase[53]; CCT6A[74]
Vδ3	Liver, gut epithelium	variable	High	Not defined

2.1.1.

2.1.2. Vδ1 T cells

Vδ1-expressing γδ T cells represent only 10-30% of all γδ T cells in the peripheral blood but is very abundant in healthy epithelial tissues and comprises the major T cell population in the gut epithelium[75]. The Vδ1 T-cell population expands upon infections with bacteria (Mycobacterium tuberculosis, Listeria monocytogenes and Borrelia burgdorferi) and virus (HIV and cytomegalovirus (CMV))[76–78]. Importantly, γδ tumor infiltrating lymphocytes (TILs) were described as being the immune population that gives the most favorable prognostic in several cancer types[79] and the abundance of Vδ1 TILs usually correlate with increased survival[80], with some notable exceptions[81].

γδ TILs isolated from various types of cancer, including colorectal, breast, prostate, ovarian and melanoma[82–84], recognize and kill both the autologous tumor and a broad range of related tumors, presumably via the recognition of shared stress-related ligands, but do not kill nontransformed cells[85]. This, together with the

observation that Vδ1 T cells infiltrate and respond to solid tumors, supports the use of Vδ1 T cells as a promising cancer immunotherapeutic approach[72].

Several studies attempted to improve Vδ1 T cell efficacy to be used in cancer treatments. In the host lab, Correia and collaborators formulated an in vitro protocol that induces the expression of natural cytotoxicity receptors (NCRs; e.g. NKp30, NKp44 and NKp46), on Vδ1 T cells isolated from peripheral blood. These *de novo* NCRs, which had been previously regarded as NK-specific markers, enhance tumor targeting by Vδ1 T cells[86]. Although neither Vδ1$^+$ nor Vδ2$^+$ cells express NCRs constitutively, these can be selectively upregulated in Vδ1$^+$ cells by AKT-dependent signals provided synergistically by γc cytokines (IL-2 or IL-15) and TCR stimulation[86,87]. However, the intracellular signaling mechanisms involved in γδ T cell activation and expansion are still poorly understood. The insufficient γδ T cell numbers was an additional limitation to the use of Vδ1 T cells in the clinic. This limitation was recently overcome by a robust protocol for the selective expansion and differentiation of cytotoxic Vδ1 T cells[88]. These cells selectively target leukemic cells via the combined action of TCR and NCRs and production of IFN-γ and TNF-α, *in vitro* and *in vivo,* but not IL-17[88], which has been implicated in the promotion of tumor cell growth[72,89].

In contrast to Vδ2 T cells, the Vδ1 T cell population is not prone to activation-induced cell death (AICD). As a result, tumor-reactive Vδ1 T cells can persist in the circulation for many years[90–92]. The therapeutic potential of Vδ1 T cells is largely determined by the nature of ligands that bind and activate their receptors. Vδ1 T cells are activated by stress-induced self-antigens that are often constitutively expressed by solid tumors, leukemias and lymphomas[92]. In particular, Vδ1 T cells recognize MIC-A/B[93,94] induced by oxidative stress[95], and often upregulated on malignant cells[96]. The MIC-A/B recognition by NKG2D receptor on Vδ1 (but also on Vδ2) T cells is critical for recognition and subsequent killing of several target cancers via perforin and granzymes[92,97]. Vδ1 T cells have been reported to recognize several ligands presented by different members of the MHC superfamily

receptors[53]. Vδ1 T cells are able to sense glycolipids presented by CD1c[73,98–100]; or CD1d[71,101–103]. Until now, the identified ligands of Vδ1 T cells are cancer and virus-associated molecules, which highlight the tissue-immunosurveillance functions of these cells[54].

2.1.3. Vδ2 T cells

γδ T cells make up approximately 4% of the peripheral blood lymphocytes (PBL) T cells in healthy human adults but can expand up to 60% of blood leucocytes during a variety of infectious diseases. Most of the expanded γδ T cells express Vγ9 and Vδ2 TCR chains, suggesting that some of the specificities within this population may be important in responding to pathogenic challenges. Vδ2 T cells are found only in humans and higher primates and constitute the best studied γδ T cell subset.

Vδ2 T cells are often sub-divided on the basis of surface expression of two receptors: CD45RA and CD27. However, CD27 does not identify a human γδ T subset comparable to the $CD27^+$ γδ T subset in mouse (i.e. pre-committed to robust IFN-γ secretion)[50]. CD27 is a member of the TNF receptor family with known co-stimulatory activity that is expressed by a major proportion of human Vδ2 T cells[104]. CD27 and CD45RA identify four Vδ2 T subsets: (i) 'Naive' (T_{naive}) $CD45RA^+$ $CD27^+$ $V\gamma9V\delta2^+$ cells are highly proliferative, do not secrete IFN-γ, and generally comprise 10–20% of those in peripheral blood (but the major population in lymph nodes)[105]. (ii) T_{naive} cells become largely $CD45RA^-$ $CD27^+$ (and $CD45RO^+$) after TCR stimulation. In healthy individuals, these 'Central Memory' (T_{CM}) cells represent 25% and 50% of Vγ9Vδ2 cells in lymph nodes and peripheral blood, respectively. T_{CM} cells appear to proliferate less than T_{naive} but can secrete low levels of IFN-γ[106]. (iii) Following additional TCR stimulation, T_{CM} cells generate $CD45RA^-$ $CD27^-$ (and $CD45RO^+$) 'Effector Memory' (T_{EM}) cells that lose CCR7 and CD62L, but acquired the tissue-associated chemokine receptors CCR2, CCR5, CCR6 and CXCR3 (Ref.[106]). Although with low capacity for proliferation, T_{EM} cells secrete abundant IFN-γ and

tumor necrosis factor-α (TNF-α) and are present in higher percentages in blood and inflammatory sites. (iv) T_{CM} cells also appear to generate a $CD45RA^+$ ($CD27^-$, $CCR7^-$) effector memory (T_{EMRA}) population when activated with IL-15 (Ref.[107]), however these cells are virtually absent from blood. T_{EMRA} cells express abundant perforin, granulysin and display robust cytolytic activity (but little production of IFN-γ). Additionally, T_{EMRA} cells are unresponsive to further TCR stimulation and have little proliferative capacity, a phenotype consistent with a terminally differentiated state[108].

Vδ2 T cells are unique in their recognition of low-molecular-weight non-peptidic and phospho-containing compounds collectively called phosphoantigens (PAgs). Vδ2 T cells responds robustly to isopentenyl pyrophosphate (IPP), an intermediate in the human mevalonate pathway and to (E)-4-hydroxy-3-methyl-but-2-enyl-pyrophosphate (HMBPP), a microbial isoprenoid intermediate[109]. Importantly, several microbial and plant PAgs produced through the deoxyxylulose pathway, such as HMBPP, show 10,000-fold higher activity than those from mammalian PAgs[54]. Nanomolar concentrations of HMB-PP lead to rapid TCR-dependent activation of Vγ9Vδ2 T cells, enabling them to respond to a diverse range of pathogens, including Mycobacterium tuberculosis[110,111] and Plasmodium falciparum[112]. Indeed, in vitro incubation of PBL with mycobacterial lysates induces an expansion of Vγ9Vδ2-expressing γδ T cells, and the stimulatory components are protease resistant and phosphatase sensitive[54,113]. Importantly, the recognition of PAgs is restricted mainly to higher primates, with mice and other rodents not possessing any T cell subsets that respond to PAgs[114].

The ability to respond to prenyl pyrophosphates has been used to redirect Vδ2 T cells to tumors by manipulating isoprenoid metabolism in the cancer cells. Such manipulation can be achieved using aminobisphosphonates (e.g. zoledronate, pamidronate, risedronate) which inhibit farnesyl pyrophosphate synthase (FPPS) in the mevalonate pathway leading to the accumulation of prenyl pyrophosphate substrates[114–116]. In addition, although some Vγ9Vδ2 T cells respond poorly to

PAgs, they can respond robustly to naturally occurring primary alkylamines, such as iso-butylamine that are secreted by bacteria and found in certain edible plants[117]. Collectively, the reactivity of these Vγ9Vδ2 γδ T cells could allow them to perceive microbial products in diverse infectious contexts.

The mode of PAg recognition has been amply studied. Early findings indicated that recognition of PAgs by human Vγ9Vδ2 T cells required cell surface presentation by species-specific molecules[118]. These have been recently identified as butyrophilin molecules, which are encoded within the MHC class I locus and act as PAg-binding molecules and activate Vγ9Vδ2 T cells[119]. Butyrophilin-3A (BTN3A/CD277) is present in humans in three isoforms (BTN3A1, BTN3A2 and BTN3A3) structurally homologous to the B7 superfamily of proteins[120]. Recent studies have shown that antibodies specific for BTN3A1 could either mimic PAg-mediated activation of the TCR (antibody 20.1) or abrogate this stimulatory effect (antibody 103.2)[121]. These findings raised a series of new questions associated with the occurrence of both stimulatory and inhibitory anti-BTN mAbs that was better understood after the characterization of the 3D structure of BTN3A1 (Ref.[122]). Currently findings indicates that while the inhibitory antibody bounds to the distal part of the V-like domain, the stimulatory antibody bounds to a more membrane proximal region of the V-like domain, preventing or facilitating the BTN3 homodimerization respectively[123]. However, the precise role of BTN3A molecules in PAg induced activation of Vγ9Vδ2 T cells has been controversial and remains to be fully elucidated. Different molecular mechanisms have been proposed in order to clarify the Vγ9Vδ2 T cell activation process by BTN3A1. While Vavassori *et al*.[119] suggest that BTN3A1 as antigen presenting molecule triggers Vγ9Vδ2 T cell activation, data from Sandstrom *et al*.[124] and Harly *et al*.[121] support an inside-out signaling mechanism for BTN3A1, where phosphonatigens bind to its intracellular region and in which model immobilization of BTN3A1 at the cell surface may contribute to an extracellular cue for recognition by Vγ9Vδ2 TCRs. This could be through several means that work individually or in concert to initiate TCR recognition: (i)

immobilization/clustering of BTN3A that increases the avidity for the TCR, (ii) a conformational change of the BTN3A extracellular domains from non-stimulatory to stimulatory, or (iii) the two previous situations resulting in the recruitment of an additional factor that directly engages the Vγ9Vδ2 TCR. The models (i) and (ii) invoke a direct interaction between Vγ9Vδ2 TCRs and the extracellular domains of BTN3A supported by several modulatory molecules (e.g. RhoB[125]) whereas model (iii) involves an unknown accessory protein that is the true Vγ9Vδ2 TCR ligand[71,124–128].

Several approaches have been developed in order to redirect Vδ2 T cells to target different tumors. Zheng *et al*. combined the extracellular domains of TCR from Vγ9Vδ2 TILs and conjugated them with Fc domain of human IgG1 (Ref.[129]). This bispecific construct mediated the killing of a range of ovarian cancer cells via antibody-dependent cellular cytotoxicity (ADCC). ADCC can be mediated by binding of CD16 (FccRIII) to the Fc region of IgGs. A similar approach involves transducing Vδ2 T cells with chimeric antigen receptors (CARs) that recognizes conformational epitopes independently of their TCR (recently reviewed in Maus *et al*.[130]). Recently, Deniger et al. have transduced polyclonal γδ T cells with a CD19-specific CAR, demonstrating their efficacy in killing $CD19^+$ leukemia cells[131]. Additionally, the transduction of TCRαβ into γδ T cells[132] or the transduction of a specific TCRγδ into αβ T cells[133] have been showed to efficiently target specific tumors[114].

Altogether the presence an accumulation of γδ T cells in areas of disease relevance (tissues prone to cancers and/or infections) makes them an obvious target for immunotherapy. Understanding the antigens to which they respond, how they respond to them, as well as the molecular mechanism involved in γδ T-cell differentiation and activation, will provide the first steps in effective management of these cells in the clinic[71].

2.2. Five layers of receptor signaling in γδ T-cell differentiation and activation

γδ T cells functional responses are initiated upon recognition of antigens that are likely induced by stress signals and sensed by either T-cell or natural killer receptors. Some γδ T cell populations are also particularly responsive to cytokines or innate toll-like receptor (TLR) agonists[47,54]. Following proliferation and effector responses, the return to homeostasis is controlled by inhibitory receptors. Altogether, the various layers of T (TCR and costimulatory/inhibitory receptors), NK, and cytokine receptor signals synergistically regulate the activation and differentiation of effector γδ T cell populations (**Figure 2**).

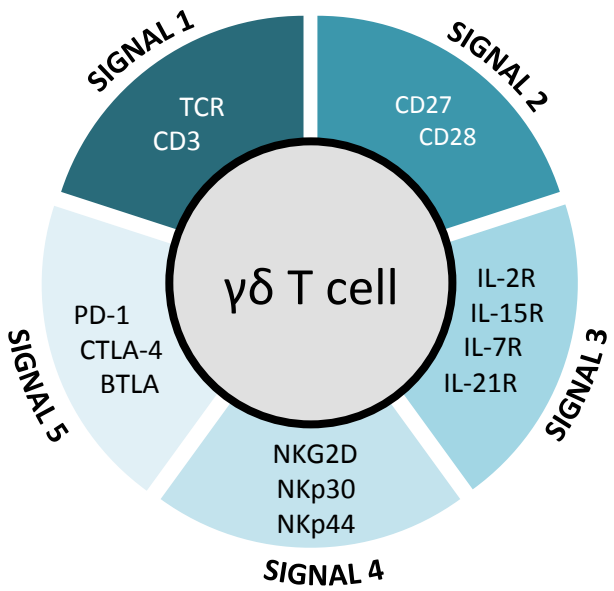

Figure 2: Five layers of cell membrane receptors involved in differentiation, activation and functions of γδ T cells

2.2.1. Signal 1: T cell receptor

The TCRγδ complex is composed by the TCRγδ itself and various CD3 chains following the stoichiometry: TCRγδCD3$\varepsilon_2\gamma\delta\zeta_2$ in humans, and TCRγδCD3$\varepsilon_2\gamma_2\zeta_2$ in mice[134]. The assembly of a TCRγδ complex in thymic progenitors has immediate

consequences for γδ T cell development. The “strong” signals stemming from the TCRγδ (when compared to the “weaker” pre-TCR signalling) drive γδ/αβ common precursors into the γδ lineage[36,37]. These “stronger” TCRγδ signals associate with increased phosphorylation of ERK1/2, abundant calcium release and induction of early growth response (Egr) transcription factors[135,136].

The TCR complex does not present intrinsic kinase activity but the intracellular signalling is initiated after phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the CD3 cytoplasmic domains by the Src-family kinases (SFKs) Lck and Fyn[137]. The recruitment of these SFKs to the TCR complex in γδ T cells remains obscure since these cells do not express the CD4 or CD8 coreceptors, that have been shown, in αβ T cells, to be responsible for recruiting SFKs upon TCRαβ ligation[137]. Nonetheless, the importance of SFKs in γδ T cells is underscored by the substantial phosphorylation of ERK upon inhibition of Csk, a potent inhibitor of SFKs[138].

SFK-mediated phosphorylation of the ITAMs on CD3 chains allows the recruitment, phospholylation and activation of Zap70 that facilitates phosphorylation of the scaffolding proteins SLP-76 and LAT. This lead to the formation of a supramolecular signalosome that recruits the phospholipase PLCγ1 resulting on propagation of downstream signalling events[136]. Here again, γδ T cell signalling is different from αβ T cells, since mutations on the binding site of PLCγ1 on LAT resulted in a severe block in murine αβ thymocyte development while γδ T cell numbers were only modestly reduced in the thymus, intestine and liver, and remained normal in the skin. Unexpectedly, a population of γδ T cells in the secondary lymphoid organs in these mice underwent uncontrolled expansion and caused autoimmune pathology, suggesting distinct functions for LAT/PLCγ1-mediated signalling in subpopulations of γδ T cells[135,139].

In humans, the major γδ T cell subset in the peripheral blood, Vγ9Vδ2 T cells, are uniquely and specifically reactive to self- and foreign non-peptidic phosphorylated

intermediates of isoprenoid synthesis – "phosphoantigens'' or "phosphoagonists" (P-Ags)[109,111,140]. These P-Ags were shown to trigger bona fide Vγ9Vδ2 TCR signalling in various studies. Cipriani and colleagues showed that the activation of Vγ9Vδ2 T cells with the P-Ag isopentenyl pyrophosphate (IPP), induced rapid and persistent PKC-dependent phosphorylation of ERK1/2, p38 MAPK, and JNK, resulting in NF-κB and AP-1 activation as well as the release of CCL-3, CCL-4, IFN-γ and TNF-α [141]. Moreover, P-Ag stimulation and CD3-crosslinking produced identical phosphorylation of the signalling proteins Zap70, PI3K, LAT, ERK1/2 and p38 MAPK[142,143]; and induced highly sustained calcium signalling in Vγ9Vδ2 T cells [144]. Importantly, activation by P-Ags is the basis of current cancer immunotherapy strategies involving Vγ9Vδ2 T cells[145].

Recent work has produced some puzzling results on the role of the TCRγδ in the development of effector subsets of murine γδ T cells[146–148], namely $CD27^+$ $CD122^+$ γδ T cells producing IFN-γ or $CD27^-$ $CCR6^+$ γδ T cells making IL-17[49,149]. First, Chien and co-workers showed that T10/T22-specific γδ T cells required thymic expression of their TCR ligands to differentiate into IFN-γ producers, in contrast with "ligand naïve" IL-17 producers[49]. Consistent with this, TCR-dependent thymic selection was also shown to set the functional potential of DETC progenitors away from IL-17 production[150]. Furthermore, peripheral IL-17-producing $CD27^-$ $CCR6^+$ γδ T cells were shown to expand and produce IL-17 independently of TCR activation[151]. However, a subsequent study by Chien and collaborators demonstrated that a subset of phycoerythrin (PE)-specific γδ T cells produced IL-17 specifically upon TCR ligation[152]. Moreover, a recent study by Hayday and colleagues suggested that an impairment in Zap70 signalling (in SKG mice) mostly affected the development of $IL\text{-}17^+$ rather than $IFN\text{-}\gamma^+$ γδ T cells[153]. The authors further proposed that "innate-like" γδ T cell populations, including IL-17 producers and some subsets of IFN-γ producers, receive strong TCR signals during thymic development to become hyporesponsive to TCR stimulation in the periphery[153]. Future research should thus clarify the developmental effects of

manipulating distinct TCRγδ signalling pathways and their downstream (transcriptional and post-transcriptional) mechanisms on discrete γδ T cell subsets.

Table 2: Co-receptors of γδ T cells – extracellular ligands and intracellular signaling pathways.

Receptor	Ligands	Intracellular signalling initiators/ adaptors	Downstream signalling pathway	Target molecules	Reference
CD28	B7.1 (CD80) B7.2 (CD86)	PI3K ITK Grb2	PI3K/ AKT Grb2/ MEK/ ERK	IL-2, NF-κB, AP-1, Bcl-x_L, NFAT	154–157
CD27	CD70	TRAF2 TRAF5 Siva	IKK/ NF-κB JNK	NF-κB, Ca^{2+}, *cyclinD2, Bcl2a1,* Bcl-x_L	158–161
CD30	CD30L	TRAF2 TRAF5	TRAF/ IKK/ IkB Ca^{2+}	NF-κB, IL-4, IFN-γ, IL-8, CC chemokines	158,162,163
4-1BB (CD137)	CD137L	TRAF2		NF-κB, IFN-γ	164–166
IL-2R IL-15R	IL-2 IL-15	Jak1 Jak3	PI3K/ AKT Jak/ STAT4/ STAT5 MEK/ ERK STAT1	IFN-γ, TNF-α, T-bet, Eomesodermin	167–170
IL-7R	IL-7	Jak1 Jak3	STAT3	IL-17, SOCS3	171
IL-21R	IL-21	Jak1 Jak3	STAT3	CXCL13, CXCR5	172
NKG2D	MIC(A-B) ULBP(1-6) H60 MULT1 RAE1	DAP10	PI3K/ AKT Grb2/ VAV1/ SOS1 PKCθ/ Ca^{2+}	NF-κB, RelB, Bcl-x_L, Bcl-2	144,158,173–175
NKp30	B7-H6 BAT3	CD3ζ	cAMP/ PKA	CC-chemokines: CCL3, CCL4, CCL5	78,176–178
NKp44	NKp44L	DAP12	Zap70/ Syk		177,179–181
DNAM-1 (CD226)	Nectin-like-5 Nectin-2	PKC LFA-1 Fyn	SLP-76/ VAV1/ ERK		182,183
PD-1	PD-L1 (B7-H1) PD-L2 (B7-DC)	SHP-1 SHP-2	CK2/ PTEN/ PI3K/ AKT MEK/ ERK	GSK-3, Bcl-x_L SMAD3, Cdc25A, IFN-γ, IL-2	184–187
BTLA	HVEM	SHP-1 SHP-2	Zap70/ ERK	IL-17, TNF, IL-2	188–190

2.2.1. Signal 2: costimulatory receptors

A series of T cell costimulatory receptors are known to induce qualitative and quantitative changes that lower activation thresholds, prevent "anergy" and enhance T cell functions. Typical costimulatory receptors are type I transmembrane proteins that can be divided into two groups, based on their structural characteristics: Immunoglobulin (Ig) or Tumour Necrosis Factor Receptor (TNFR) superfamilies. Ig superfamily members have a variable Ig-like extracellular domain and a short cytoplasmic tail, whereas TNFR family members present extracellular domains rich in six cysteine repeats (which form disulphide bridges) and a more complex cytoplasmic tail (reviewed in[191]). These two main types of costimulatory receptors display different modes of intracellular signalling: whereas the CD28 family members associate directly with protein kinases (like PI3K or ITK), TNFR superfamily coreceptors require the adaptor proteins TRAF (TNFR-associated factor), namely TRAF2 and TRAF5, to link to downstream signalling mediators (**Table 2**). Here, based on their specific roles in γδ T cells, we shall discuss CD28 (of the Ig superfamily) and the TNFR superfamily members, CD27, CD30 and CD137 (4-1BB).

The best studied costimulatory receptor, CD28, has historically yielded paradoxical results on γδ T cells[158]. We have recently readdressed this issue for both human and mouse γδ T cells. We described that CD28 is constitutively expressed on lymphoid γδ T cells and promotes survival and proliferation via IL-2 production. CD28 receptor agonists enhanced γδ T cell expansion, which was conversely inhibited by blocking antibodies against its B7 ligands[154]. Importantly, CD28-deficient mice displayed lower (relative to controls) numbers of total or activated γδ T cells upon *Plasmodium berghei* infection, and failed to expand both their IFN-γ^+ and IL-17^+ subsets[154]. By contrast, Hayes and colleagues reported that both functional γδ T cell subsets differentiated and expanded normally in a *Listeria* model[192]. It would be interesting to determine how variable is the dependence on CD28 costimulation for γδ T cell responses to distinct infectious agents.

In naïve mice, while CD28 is not required for the development of either IFN-γ$^+$ or IL-17$^+$ γδ T cell subsets[192], the TNFR superfamily member CD27 is selectively implicated in the generation of IFN-γ$^+$ γδ T cells[50]. In fact, we showed that CD27 expression segregates IFN-γ$^+$ (CD27$^+$) and IL-17$^+$ (CD27$^-$) γδ T cells. Most interestingly, these phenotypes are established in the thymus, and since embryonic stages. Based on the results from our[149] and Chien's[49] teams, the development of IFN-γ-producing γδ T cells seemingly requires strong TCR signalling and CD27 costimulation in the thymus.

Beyond its role in thymic differentiation, CD27 is critical for the expansion of peripheral IFN-γ-producing γδ T cells upon infection with herpes viruses or malaria parasites in mice[193]. We showed that, in the context of TCR stimulation and upon ligation to CD70, CD27 signalling activates the non-canonical NF-κB pathway and enhances the expression of anti-apoptotic and cell cycle-related genes, thus promoting murine γδ T cell survival and proliferation[193].

We have also addressed the impact of CD27 costimulation on the activation of human γδ T cells. Administration of soluble recombinant CD70 enhanced, whereas anti-CD27 (or anti-CD70) antibodies reduced, Vγ9Vδ2 T cell expansion *in vitro*[194]. Moreover, CD27 signals induced calcium fluxes and upregulated the expression of *Cyclin D2* and the anti-apoptotic gene *Bcl2a1*. Given the typical IFN-γ secretion and cytotoxicity of activated Vγ9Vδ2 T cells[142], our work suggests that the modulation of CD70-CD27 signals may be beneficial in the context of γδ T cell-based cancer immunotherapy.

Upon activation, human γδ T cells can also express another TNFR superfamily member, CD30 (Ref.[195]). CD30 signalling, which potentiated calcium fluxes induced by TCR activation, also enhanced pro-inflammatory cytokine production[162]. Recently, Yoshikai and colleagues compared γδ T cell homeostasis and response to *Listeria monocytogenes* in CD30-sufficient versus deficient mice. They demonstrated a selective depletion of IL-17-producing Vγ6$^+$ T cells in mucosal tissues in the steady-

state and upon infection[196]. This associated with reduced bacterial clearance, which could be rescued, alongside the IL-17$^+$ Vγ6$^+$ T cell pool, by agonistic anti-CD30 antibody administration. By contrast, Lee et al. reported that agonistic anti-CD137 (4-1BB) antibodies promoted the expansion of IFN-γ$^+$ Vγ1$^+$ T cells, which protected (in an IFN-γ-dependent manner) also from *Listeria* infection[166]. This study also showed that 4-1BB was expressed and functional on activated human γδ T cells, and its ligation upon cell transfer protected NOD/ SCID mice against *Listeria* infection.

Interestingly, activated Vγ9Vδ2 T cells also express high levels of 4-1BBL (CD137L)[197], which besides acting as a ligand for 4-1BB on T and NK cells, may also participate in Vγ9Vδ2 T cell activation due to its known reverse signalling ability[198]. This may in fact also apply to CD70 (CD27-ligand), which is highly induced upon phosphoantigen-mediated stimulation of Vγ9Vδ2 T cells[194,199]. These possibilities deserve further investigation.

2.2.2. Signal 3: cytokine receptors

Interleukins are key determinants of T cell survival, proliferation and differentiation. IL-7, IL-15 and IL-2 are essential for lymphocyte development and homeostasis; upon inflammation, other cytokines, namely IL-1β, IL-12, IL-18, IL-21 and IL-23, take a central role in determining T cell functions. Here we review the main contributions of homeostatic and inflammatory cytokines specifically to γδ T cell physiology.

IL-7 and IL-15 are seemingly the key determinants of murine γδ T cell development[200–202] and homeostasis[203]. A recent study that depleted IL-7 specifically from (Foxn1$^+$) thymic epithelial cells showed that γδ T cells were significantly reduced in the adult thymus and in the gut, whereas they were completely absent in the foetal thymus and epidermis[201]. In the dermis, it was also IL-7, but not IL-15, that supported the development and survival of the resident γδ

T cell population[204]. Conversely, in the gut, IL-15 seems to play the primordial role in sustaining the local intraepithelial γδ T cell compartment[205].

Unexpectedly, IL-7 was recently reported to promote the selective expansion of murine IL-17-producing γδ T cells[171]. STAT3-dependent IL-7 signals allowed CD27$^-$ γδ T cells to resist AICD and undergo proliferative responses to TCR agonists. Such an IL-7/ IL-17 axis was also reported to be required for the γδ T cell response to viral hepatitis infection *in vivo*[206]. Moreover, IL-7 also seems to support the expansion of human IL-17-producing γδ T cells[171].

We recently assessed the functional differentiation of human γδ thymocytes, which are >80% of the Vδ1 subtype. We observed that IL-15 and IL-2, but not IL-7, induced the cytotoxic type 1 (IFN-γ-producing) programme in functionally immature γδ thymocytes[169]. This was consistent with previous data on peripheral γδ T cells isolated from cancer patients[207]. However, additional reports on peripheral Vγ9Vδ2 T cell cultures showed that IL-15 or IL-2 stimulation, despite efficient ERK and AKT activation, were not sufficient to induce effector responses; these required phosphoantigen-dependent TCR activation and downstream calcium mobilization[170,208]. Unexpectedly, in our cultures of γδ (mostly Vδ1) thymocytes, TCR stimulation was not required for neither ERK activation nor T-bet and Eomesodermin induction and the acquisition of effector functions[169].

IL-2 and IL-15 play key roles in the peripheral expansion of Vγ9Vδ2 T cells in response to microbial phosphoantigens or synthetic drugs like bisphosphonates[170,209]. This notwithstanding, it is important to note, towards the therapeutic application of Vγ9Vδ2 T cells, that optimal effector responses seemingly require the combination of these cytokines with TCR agonists. Thus, recent work from Chen and colleagues demonstrated that the differentiation of cytotoxic type 1 Vγ9Vδ2 T cells capable of controlling *Mycobacterium tuberculosis* infection in macaques required a phosphoantigen/ IL-2 combination[210].

Effector γδ T cell differentiation is also greatly impacted by inflammatory cytokines, particularly IL-12 and IL-18 that typically promote IFN-γ production; and IL-1β and IL-23 that mostly drive IL-17 production.

High expression of IL-12Rβ expression on activated murine γδ T cells guarantees a dominance of type 1 (IFN-γ^+) over type 2 (IL-4^+) effector fates [211]. Type 1 differentiation is also predominant in human γδ T cells, and can be further enhanced by IL-18 (Ref.[212,213]) or IL-21 (Ref[214]). The induction of a type 17 programme in human γδ T cells requires persistent stimulation with IL-23 for neonatal Vγ9Vδ2 T cells[215]; and IL-23 and IL-1β in the presence of TGF-β for adult Vγ9Vδ2 T cells[216,217]. In mice, IL-1β and IL-23 are also the main drivers of abundant IL-17 production by peripheral γδ T cells[62,64,193,218–220], although recent data surprisingly suggest that IL-18 can replace IL-1β in combining with IL-23 to induce IL-17 expression[221]. By contrast, IL-1β upstream of IL-1R seems essential for GM-CSF production by γδ T cells[222].

Finally, IL-21 was recently suggested to endow human Vγ9Vδ2 T cells with B cell helper activity associated with a T follicular helper cell-like phenotype[172,223], which may impact on the generation of high affinity antibodies against microbial infections.

2.2.3. Signal 4: natural killer receptors

An important key characteristic that allows the recognition of transformed cells by γδ T cells is the expression of a wide set of germline-encoded receptors that were initially described in NK cells and hence are collectively known as NK receptors (NKRs), including natural cytotoxicity receptors (NCRs).

The C-type lectin-like NKG2D (NK receptor group 2 member D) is the best studied NKR in γδ T cells. NKG2D binds extracellularly to multiple ligands of the MIC(A-B) and ULBP(1-6) families in humans; and to H60, MULT1 and various RAE1 molecules in mice[224]. NKG2D ligands are induced upon cellular stress, for example,

downstream of the DNA-damage response pathway in tumour cells[225,226]. The biological significance of this recognition system is underlined by the increased susceptibility of NKG2D-deficient mice to tumour development[227].

Intracellularly, NKG2D binds to DNAX-activating protein of 10 kDa (DAP10), which carries an YXNM motif that after tyrosine phosphorylation recruits PI3K or a Grb2–Vav1–SOS1 signalling complex (**Table 2**). This motif is similar to that in CD28, and thus, NKG2D/ DAP10 may provide T cells with costimulatory signals that synergise with the ITAM-based TCR/CD3 complex[173]. However, unlike αβ T cells but similarly to NK cells, γδ T cells can express both DAP10 and DAP12 (Ref.[174]). The latter contains an ITAM motif, which after tyrosine phosphorylation recruits and activates Syk and ZAP70. Interestingly, only murine but not human NKG2D is able to associate with DAP12 (in addition to DAP10).

The controversy on a primary stimulatory versus costimulatory role of NKG2D in γδ T cells has been discussed elsewhere[87,228]. Briefly, the costimulatory function of NKG2D in human Vγ9Vδ2 T cells was supported by additive effects on TCR-mediated activation: an upregulation of cytokine production upon MICA-NKG2D interactions[229]; and an increase in intracellular calcium mobilization and cytotoxic activity[144]. However, other lines of evidence have suggested that NKG2D signals can activate γδ T cells in the absence of TCR engagement: NKG2D ligation can upregulate CD69 expression in Vγ9Vδ2 T cells to similar extent as TCR stimulation[230]; NKG2D but not TCR blockade can inhibit Vγ9Vδ2 T cell cytotoxicity against various haematological tumours[231]; and murine DETC can target tumours upon recognition of NKG2D ligands[65,232].

Another NKR implicated in tumor cell recognition by Vγ9Vδ2 T cells is DNAX accessory molecule-1 (DNAM-1). DNAM-1 is an Ig-like family glycoprotein composed of a cytoplasmic domain containing three putative sites of phosphorylation by intracellular kinases. The phosphorylation of the Ser329 by protein kinase C (PKC) was shown to be critical for the association between

DNAM-1 and LFA-1 which recruits the Fyn Src kinase to phosphorylate the Tyr322 of DNAM-1, thus initiating downstream signalling leading to SLP-76 and Vav1 phosphorylation (**Table 2**)[233]. Antibody-mediated DNAM-1 blockade impaired Vγ9Vδ2 T cell cytotoxicity and IFN-γ production against hepatocellular carcinoma lines expressing Nectin-like-5[182].

Recently, we characterized a Vδ1$^+$ T cell population capable of targeting hematological tumors resistant to fully activated Vγ9Vδ2 T cells[86]. Unexpectedly, the enhanced killer function resulted from induced NCR expression, namely NKp30 and NKp44, which had been previously regarded as NK-specific markers. Although neither Vδ1$^+$ nor Vδ2$^+$ cells express NCRs constitutively, these can be upregulated selectively in Vδ1$^+$ cells by PI3K/AKT-dependent signals provided by γc cytokines (IL-2 or IL-15) and TCR stimulation. Once expressed on the cell surface, NKp30 and NKp44 can signal via CD3ζ and DAP12, respectively[177]. We further showed that NKp30 and NKp44 are both functional in NCR$^+$ Vδ1$^+$ T cells, and synergise with NKG2D to target lymphocytic leukaemia cells[86].

In sum, NKRs seem critical for tumour recognition and deployment of the cytotoxic program that is endowed by TCR/ γc cytokine-dependent differentiation, thus defining distinct mechanisms to be integrated in γδ T cell-mediated cancer immunotherapy.

2.2.4. Signal 5: inhibitory receptors

Beyond efficient activation and deployment of effector functions, it is necessary to negatively regulate the T cell response in order to return to the homeostatic baseline. Inhibitory receptors like PD-1 or CTLA-4 are known to be critical for this contracting phase of the T cell response and have become major clinical targets in cancer immunotherapy. Although γδ T cells rarely express CTLA-4, they can upregulate PD-1 upon activation, while they constitutively express BTLA, and thus these two receptors may be the key to control γδ T cell responses.

Programmed Death-1 (PD-1) is absent or low expressed on circulating Vγ9Vδ2 T cells but is rapidly induced upon activation[234]. The cytoplasmic tail of PD-1 contains conserved immunoreceptor tyrosine-based inhibitory motif (ITIM) and switch motif (ITSM), both of which are phosphorylated to recruit negative regulators that block Lck activity downstream of the TCR complex[235]. Moreover, PD-1 ligation can augment the activity of the protein phosphatase and tensin homolog (PTEN), a cellular phosphatase that inhibits PI3K/AKT signalling and thus leads to impaired survival, proliferation and IL-2 release[236]. The expression of the ligand PD-L1 on tumour cells inhibited Vγ9Vδ2 T cell cytotoxicity and IFN-γ production[234]. However, zoledronate-induced accumulation of P-Ags in tumour cells and consequent Vγ9Vδ2 TCR activation seemed to overcome the inhibitory effect of PD-1/ PD-L1 interactions. More research is required to understand the full extent to what PD-1 may control γδ T cell functions and homeostasis.

B- and T-lymphocyte attenuator (BTLA) is another inhibitory receptor, member of the CD28 family and structurally related to PD-1 and CTLA-4. Binding to its ligand, herpesvirus entry mediator (HVEM), induces phosphorylation of the ITIM domain and association with SH2 domain-containing protein tyrosine phosphatase 1 (SHP-1) and SHP-2, which leads to attenuation of cellular activation and growth[237]. Recent data showed that BTLA engagement with HVEM reduced P-Ag/TCR-mediated signalling and inhibited Vγ9Vδ2 T cell proliferation, including in response to lymphoma cells[188]. Conversely, BTLA-HVEM blockade using monoclonal antibodies enhanced Vγ9Vδ2 TCR signalling and may thus have therapeutic potential for the positive manipulation of γδ T cells.

A detailed study on BTLA function in murine γδ T cells has revealed a selective involvement in the homeostasis of the IL-17-producing $CD27^-$ γδ T cell subset[190]. Although these cells constitutively express low levels of BTLA, it is upregulated by IL-7 stimulation and thereby limits γδ T cell numbers. Consequently, BTLA-deficient mice accumulated $IL\text{-}17^+$ $CD27^-$ γδ T cells and were more susceptible

(than wild-type controls) to dermatitis, which could be reversed by agonist BTLA antibodies. Thus, BTLA may be an important target for controlling pathogenic γδ T cells in inflammatory and autoimmune diseases.

3. T cell malignancy

In the current understanding, normal cells can evolve progressively to a neoplastic state, undergoing a multistep framework of alterations that enable them to become tumorigenic and ultimately malignant[238]. The rapid advances in cancer research have generated a rich and complex body of knowledge. These advances are being translated into more selective and effective treatment of cancers and, although there are still considerable challenges, particularly with drug resistance and metastatic disease, many patients with otherwise lethal malignancies now experience protracted remissions or cure. One largely unheralded theme of this story is the extent to which new biological insights and novel clinical applications have their origins with leukaemia and related blood cell cancers[239]. Nevertheless, the deep characterization of cancer biology, including the cellular origin, the molecular changes and the clonal evolution of the cancer cell populations will originate crucial biological insights with translational therapeutic potential[239,240].

The self-renewal and differentiation processes, which occur in hematopoiesis through life, can become deregulated and result in leukemia. This hematological cancer is generally characterized by an accumulation of immature blasts that fail to differentiate into functional cells. Leukemia is a greek-derived word meaning white blood (leukos “white”; aima “blood”) and is classified according to the lineage of the transformed cells (lymphoid *versus* myeloid) and to the proliferation state of the cells (chronic *versus* acute). Chronic leukemias refers to malignant cells relatively well differentiated with slower proliferation rates, whereas acute is used to characterize leukemias displaying higher proliferation rates of usually immature blast cells and are the most common forms of leukemia in children[241].

3.1. T Cell Acute lymphoblastic leukemia

Acute lymphoblastic leukemia (ALL) is the most common cancer in children, accounting for approximately 25% of all the pediatric cancers[242]. Due to the

remarkable advances in molecular characterization of ALL cells and new therapies in clinical trials, the treatment of ALL patients dramatically improved in the last decades and the overall survival rate of ALL patients is about 85%[242–244].

The majority symptoms and signs associated with ALL at diagnosis derive from the collapse of normal hematopoiesis. These symptoms include fever, anemia and bone and joint pain. Other manisfestations such as fatigue, shortness of breath and dizziness are associated with anemia as a result of the decrease in red blood cell count. Enlargement of organs such as spleen, liver, lymph nodes and appearance of mediastinal masses are manifestations that occur upon the progression of the disease. The involvement of the central nervous system (CNS) is also a common feature, resulting in the appearance of symptoms like headache, nausea, vomiting, lethargy and cranial nerve dysfunction. The diagnostic of ALL is achieved when the presence of these symptoms is associated with the molecular, cytogenetic and immunophenotypic characterization of the leukemic blasts[245].

ALL is a very heterogeneous disease that presents several variations on cellular morphology, immunological markers and cytogenetic abnormalities. The malignant ALL cells can be broadly characterized into B-ALL or T-ALL with BM or thymic origin, respectively[246,247] and subsequently divided according to the stage of differentiation. Uckun and collegues[248] defines three stages of T-ALL maturation: pro-thymocytes ($CD7^+$ only), immature thymocytes ($CD7^+$, $CD2^+$ and/or $CD5^+$, $CD3^-$) and mature thymocytes ($CD7^+$, $CD2^+$, $CD5^+$, $CD3^+$). Additionally the European Group for Immunological Characterization of Leukemias (EGIL)[249] uses a further marker, CD1 and consider the following stages: pro-T-ALL ($CD7^+$ only), pre-T-ALL ($CD7^+$, $CD1^-$, $CD2^+$ and/or CD5 and/or $CD8^+$, $CD3^-$), cortical T-ALL ($CD1^+$, independently of the presence of other markers) and mature T-ALL ($CD1^-$, $CD3^+$). It is known that patients with more immature T-ALL blasts achieve remission less often[250] and have a worse prognosis[251,252] than more differentiated subtypes. However the molecular mechanism involved in T-ALL tumorigenesis are poorly understood.

3.2. Aberrant signaling in T-ALL

The leukemic transformation of immature thymocytes is believed to be caused by multistep pathogenesis involving proliferative advantage and differentiation impairment. It is currently accepted that T-cell leukemogenesis is a stepwise process that culminates in the acquisition of a fully malignant phenotype. These events include defects in the control of cell cycle machinery, gene mutations that confer self-renewal capacity to thymic progenitors, deregulated expression of pivotal transcription factors and also aberrant activation of protein kinases[253–255].

The genetic abnormalities in T-ALL can be categorized in mutually exclusive chromosome translocations that arrest T-cell development at a specific maturational stage and are associated with a distinct gene-expression signature. Frequently, these translocations are combined with deletions or point mutations in genes that affect cell cycle or signaling pathways. The knowledge of these molecular abnormalities may have prognostic significance and provide targets for novel therapies. The chimeric oncogene BCR-ABL1 results from the translocation t(9;22)(q34;q11.2), well known as Philadelphia Chromosome, and express a constitutively active Bcr-Abl tyrosine kinase that is implicated in the pathogenesis and poor prognosis. This non-random chromosomal aberration was discovered in chronic myeloid leukemia (CML) but it is also found in 1% of T-ALL patients[253,256]. Thus the BRC-ABL1 discovery allowed the development of ABL1-directed tyrosine kinase inhibitor therapies. Chromosomal translocations are the hallmark of ALL[257], where deregulated activity of RAG proteins appears to be responsible for the formation of chimeric proteins with aberrant expression, activity or functions. *Notch1* was first discovered as a partner gene in the t(7;9)(q34;q34) chromosomal translocation[258] and was later implicated in the pathogenesis of up to 60% of T-ALL cases, harboring activating mutations, involving negative regulatory domains responsible for the control of initiation and termination of Notch signaling[259,260] (**Figure 3**). Mutations in the *FBXW7* gene are present in 15% of T-ALL cases, and deregulate the proteasomal degradation of activated Notch1 protein and

consequently the Notch1 signaling pathway[261]. The transcriptional repressor Hes1 is one of the best-characterized direct target genes of Notch1 signaling involved in normal T cell development[262]. Although the mechanism through which Hes1 exerts its function in T-ALL is not fully understood, Hes1 has been shown to inhibit NF-κB and PI3K-AKT signaling[263,264]. In addition, Notch1 directly activates c-Myc and these two molecules together activate common target genes required for growth of leukemic cells[265,266]. In addition to *Notch1* mutations, T-ALLs frequently acquired abnormal chromosomal translocations that result in aberrant expression of transcription factor oncogenes, such as TAL1, LMO1, LMO2, TLX1 and TLX3 (reviewed in Van Vlierberghe *et al.*[259]).

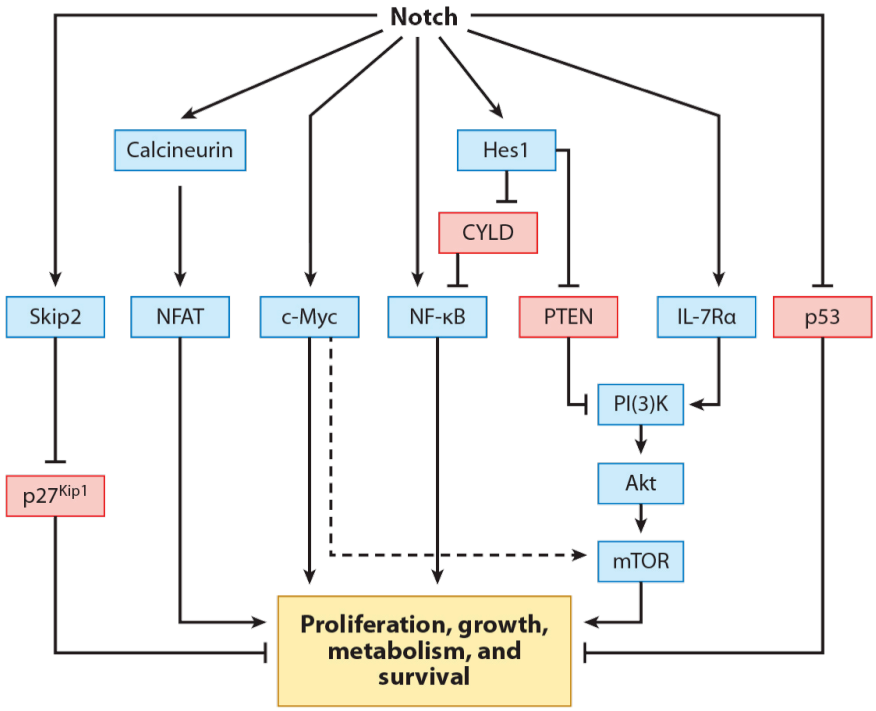

Figure 3: Downstream signaling pathways and molecules initiated in Notch-mediated acute T cell lymphoblastic leukemia (T-ALL).

Arrows indicate positive interactions, and inhibition lines show negative regulatory interactions contributing to the development and/or maintenance of T-ALL. Growth-promoting molecules or pathways are in blue, whereas growth-inhibitory molecules are in red. (adapted from Koch *et al.*[266]).

Besides genetic lesions in transcription factors and chromatin regulators, genes encoding critical components of signaling pathways controlling the growth, proliferation and survival of T cell are frequently mutated in T-ALL. The IL-7R signals through the JAK/STAT pathway and is strictly required to support the growth, proliferation, and survival of early T cell progenitor cells[267] (as previously introduced in this thesis). In addition to the activating mutations in JAK1 and JAK3 that have been reported in T-ALL[268–270], somatic gain-of-function mutations in the *IL7R* gene, resulting in constitutive activation of JAK/STAT signaling, have recently been identified in approximately 10% of pediatric T-ALL[271,272] although they are absent in adult T-ALL[273]. In addition, Barata and collegues showed that IL-4, IL-9 and IL-15 also induce T-ALL cell proliferation and this effect was dependent on the maturation status of the T-ALL cells[274]. In contrast, other cytokines can suppress the growth and induce apoptosis of leukemic cells: IL-6 was shown to suppress the growth of T-ALL cells[275] and TNF-α was shown to induce their apoptosis[276,277].

Both altered forms of AKT with abnormal constitutively phosphorylation independently of external growth factors, as well as NF-κB, a downstream target of PI3K/AKT with constitutive activation, have been associated with T-ALL[278,279]. Interestingly, the presence of activating mutations of PI3K and/or AKT has been poorly described in T-ALL patients. Thus, it has been suggested that PI3K/AKT pathway over-activation results from alterations in upstream mechanisms that remains to be fully understood[254,279]. PTEN, the main negative regulator of the PI3K/AKT pathway, is frequently inactivated in human cancer as result of various genetic lesions[280,281]. These alterations result in decreased or absent PTEN protein expression and activity, with consequent hyperactivation of the PI3K/AKT pathway. Deletion mutations in PTEN occur in 5%–10% of T-ALL cases, and overall 17% of T-ALL cases lack PTEN protein expression[259].

Gutierrez and collaborators identified that PTEN deletion has more adverse therapeutic consequences than mutational disruptions that preserve the

phosphatase domain[282]. However, most primary T-ALL cells did not harbor PTEN gene alterations and displayed normal PTEN mRNA levels. Thus PTEN inactivation and consequent PI3K/AKT pathway aberrant activation may arise from upstream mechanisms other than those targeting PTEN gene integrity[283,284]. Altogether, the understandings of the molecular mechanism altered in PTEN-PI3K-AKT pathway in each patient and subsequently development of new and personalized therapies are important aspects in T-ALL treatments.

4. Protein kinase CK2

4.1. Molecular features of CK2

Protein kinase CK2 (formerly casein kinase II) is a pleiotropic and ubiquitous enzyme generally present in the cell as a tetramer consisting of two catalytic (α and/ or α') and two regulatory non-catalytic (β) subunits[285]. The α-subunits are composed of two major folding domains (N- and C-terminal) harboring the active site in between. The C-terminal regions of the β-subunits bind the α-subunits and enhance the catalytic activity and stability of CK2[286], although there are evidences supporting that the subunits can also be present and active in their free form[287,288]. CK2 phosphorylates serine or threonine residues proximal to acidic amino acids in more than 300 protein substrates[289,290]. Of note, despite the many substrates of CK2, it seems very unlikely that CK2 has any role in the *in vivo* phosphorylation of casein, the protein from which it originally derived its name[285,291]. Several line of evidences indicate that CK2 is constitutively active, as it is not subject to the strict on/off regulation of other kinases, such as MAP kinases and Cdks. However, there are several mechanisms by which CK2 activity can be regulated or focused on one or more substrates while excluding other substrates, including localization, phosphorylation, and protein-protein interactions[287,292,293].

The two catalytic subunits are products of separate genes but demonstrate greater than 90% sequence identity over their N-terminal 330 amino acids, with

completely unrelated C-termini. A third catalytic and less known isoform of CK2 (α'') was reported in human hepatocyte that is nearly identical to α but with diferente 32 amino acids at the end of the protein[287,294]. Both CK2α' and CK2α'' had no obvious catalytic differences, however some data suggest functional specialization mediated by specific binding partners[295,296]. CK2β presents close similarity of the catalytic subunits but it does not share extensive identity with any other known protein[285]. The CK2β subunit interacts with CK2α subunits and is responsible for docking and/or recruitment of CK2 substrates or potential regulators[297]. In this respect, potential CK2 targets, such as Nopp140, p53, Fas-associated factor-1 (FAF-1), topoisomerase II and CD5, as well as potential CK2 regulators such as fibroblast growth factor-2 (FGF-2), interact with CK2 via interactions with CK2β[298–305].

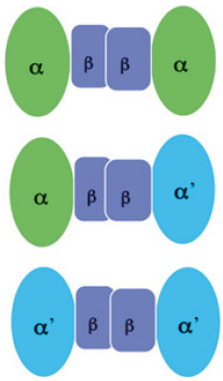

Figure 4: Schematic representation of CK2α, α' and β subunit tetrameric complexes.

Mammalian CK2, tetrameric CK2 complexes contain identical (i.e. two CK2α or two CK2α) or non-identical (i.e. one CK2α and one CK2α') catalytic subunits couple with two regulatory, CK2β, subunits.

4.2. Biological functions of CK2

Based on the broad cellular mechanism controlled by CK2, it is not surprising that CK2 provides survival and proliferative advantage to tumor cells. CK2 functions seem to encompass most of, if not all, the 'hallmarks of cancer'[238,286]. CK2 protects cell apoptosis through several mechanisms from the blockade of protein degradation, stimulation of antiapoptotic responses upon different stresses, enhancement of oncogenes' transformation properties and stabilization of oncokinases[286].

Table 3: Cancer cell types whose survival has been demonstrated to rely on CK2.
(Adapted from Ruzzene *et al*.[306])

Neoplasia	Cell types
T-cell lymphoblastic leukemia (T-ALL)	Jurkat, CEM[a], HPB-ALL, TAIL-7, MOLT-4, primary cells[b]
Chronic lymphocytic leukemia (CLL)	primary cells[b]
Burkitt lymphoma	Raji
Multiple myeloma (MM)	OPM2, U266, RPMI 8226, primary cells[b]
B-cell lymphoblastic leukemia (B-ALL)	PLC1, B1, B2, KOPN-8, RS4;11, NALM-6, primary cells[b]
Acute myeloid leukemia (AML)	NB4, HL60, ML-2, KASUMI-1, primary cells[b]
NPM/Alk-positive Anaplastic large cell lymphoma (ALCL)	Karpas299, SR786, SUDHL 1
Murine leukemia	P388[a]
Osteosarcoma	U2OS[a]
Ovarian carcinoma	2008[a]
Prostate carcinoma	PC-3, LNCaP, DU-145, ALVA-41
Colon carcinoma	HCT8[c], HCT116[c], HT29[c], DLD-1, SW-480
Hepatocellular carcinoma	HepG2[c], Hep3B[c]
Endometrial cancer	IK[c], RL95[c], primary cells[c]
Rabdomiosarcoma	JR1[c], Rh30[c], RD[c]
Pancreatic cancer	MiaPaCa2, DanG
Cervical cancer	HeLa
Breast cancer	NF639, ZR-75, SKBr-3[c], Hs578T[c], MDA231
Squamous cell carcinoma	SCC-15
Lung carcinoma	A549, H1299
Glioma	A172, U87MG, T98G

[a] Apoptosis resistance of the multiple drug resistance (MDR) variants of these lines can be overcome by treatment with CK2 inhibitors. [b] Cell death induced by CK2 blockage is more evident in cancer cells than in normal counterparts. [c] CK2 inhibition sensitizes these cells to receptor-mediated apoptosis.

CK2 genes are not classified as oncogenes. However CK2 is overactivated in several solid tumors, multiple myeloma (MM) and in many lymphoid malignancies, such as mantle cell lymphoma (MCL), diffuse large B-cell lymphoma (DLBCL), B-CLL, B-ALL and T-ALL (**Table 3**) and has been widely associated with increased proliferation, angiogenesis, secretion of growth factors, invasiveness and

resistance to chemotherapy[286,307]. It has been described a non-oncogene addiction of malignant cells to CK2: they depend on CK2 overexpression for survival and are more sensitive to CK2 downregulation than normal counterparts[286]. Given the generalized increased activity of CK2 in cancers, the central role of CK2 in multiple pathways that support the cancer phenotype and the evidence that CK2 is an essential protein for cancer cell survival, the development of selective CK2 inhibitors has emerged as an attractive targeted approach for the treatment of cancers[308].

4.3. CK2 inhibitors

Different studies indicate that CK2 targeting with selective inhibitors is feasible and highly effective in causing tumor cell death. Over the past two decades, different approaches have been followed to discover and develop inhibitors of CK2. Most of them are ATP competitors[288]. CK2 inhibitors can be divided into two main classes: i) the ATP-competitive inhibitors (also called Type I inhibitors), able to interact directly with the ATP-binding site, thus blocking the ATP access and the phosphotransferring reaction and ii) the non-competitive ATP inhibitors (allosteric inhibitors or substrate competitors), which bind to structural elements unrelated with the ATP-binding regions[288]. For instance, relatively small organic compounds such as TBB, DRB, DMAT, IQA, emodin, CX-4945, and the flavonoids apigenin, luteolin, and quercetin have been shown to target and block the ATP-binding site of the CK2 catalytic subunit[308–315]. Other examples are Pc, a cyclic peptide that antagonizes the interaction between CK2 subunits[316], and antisense oligonucleotides that target transcription of the CK2 alpha gene, thereby inducing cell death and antitumoral activity[317]. CIGB-300 is a new anti-CK2 peptide that explores a novel concept to impair CK2 activity: targeting its substrates, rather than the enzyme *per se*[318].

These CK2 inhibitors have resulted in a number of successful proof of concepts exhibiting antiapoptotic effect both in vitro and in vivo tumor models. Among the

CK2 experimental inhibitors, only the small molecule CX-4945 and the cell-permeable peptide CIGB-300 have already reached the clinical stage.

CX-4945 (Silmitasertib) is an orally administered CK2 inhibitor developed by Cylene Pharmaceuticals Inc.[308,315]. CX-4945 has shown tumor growth inhibition effects of 76–97% in breast, prostate, pancreatic, and glioblastoma tumors. CX-4945 has been studied in different human cancer and is currently in Phase I and II clinical trials. The Phase I trial addresses the safety and tolerability of increasing doses of CX-4945 to determine the maximum tolerated dose (MTD). The subsequent Phase II trial is a randomized study of antitumor activity in cholangiocarcinoma patients treated with the standard-of-care protocol of gemcitabine plus cisplatin in combination with CX-4945 (Ref.[288]). In addition, the hematological and solid tumors, in which CK2 has been described to be upregulated and instrumental for growth, are particularly suitable to be treated with CK2 inhibitors.

Collectively, CK2 is a component of regulatory protein kinase networks that seems to be tightly regulated in normal cells, but hyperactive in cellular transformation and cancer. In this work, CK2 emerge as an attractive candidate to control the survival of both normal and malignant γδ T cells.

5. Main objectives of the study

γδ T cells play a key role in immunosurveillance. However, the molecular determinants involved in the process remain largely unknown. The main goal of this thesis was to contribute to the characterization of the molecular determinants of human γδ T cell survival, differentiation and activation. We aimed at elucidating the main molecular players and signaling pathways involved in the activation and differentiation of γδ T cells into effectors capable of secreting cytokines and killing infected or transformed cells. A major goal of this thesis involved the identification of the molecular hallmarks that regulate both healthy γδ T lymphocyte physiology and pathology. Ultimately the data presented here is likely to open new avenues to improve γδ T cell-associated immunotherapies. By elucidating the signaling pathways that sustain the survival of transformed γδ T cells, this thesis further revealed new therapeutic targets for $\gamma\delta^+$ acute lymphoblastic leukemias.

Chapter II

Material and methods

II. MATERIAL AND METHODS

Statement of Ethics

Human thymic specimens from newborn to 15 year-old children were obtained during pediatric corrective cardiac surgery after parents' written informed consent. The surgeries and sample collection were performed by Dr. Miguel Abecasis and Dr. Rui Anjos at "Hospital Santa Cruz" (Lisbon). The used of thymic samples was approved by the ethical board of the Faculty of Medicine of University of Lisbon and the ethics board of Western Lisbon Hospital Center.

Human blood samples were collected from healthy donors and buffy coats were obtained from "Instituto Português do Sangue" (Lisbon) and the use of blood samples was approved by the ethical board of the Faculty of Medicine of University of Lisbon.

Primary human T-ALL blasts derived from diagnostic samples (peripheral blood or bone marrow) were obtained after inform content of the patient. The study was conducted in accordance with the Declaration of Helsinki and approved by local ethical committees from "Hôpital Necker Enfants-Malades", Paris Descartes University and "Institut national de recherche médicale" (Paris).

All experimental procedures in mouse animal models were performed in strict accordance with the recommendations of the European Commission (Directive 2010/63/UE), French National Committee (87/848) and Portuguese authorities (Decreto-Lei 113/2013) for the care and use of laboratory animals. The study was approved by both ethical boards of the Faculty of Medicine of University of Lisbon and Paris Descartes University.

Isolation and *in vitro* cell culture

Thymic T cells were collected after thymus tissue manual dispersion and separation by Histopaque-1077 (Sigma-Aldrich) density gradient separation by centrifugation during 30 minutes at room temperature (RT). TCRγδ-positive T cells were isolated (to >97% purity) by magnetic positive selection; TCRαβ-positive T-cells were isolated (to >96% purity) by magnetic positive selection from the TCRγδ-negative fraction (Miltenyi Biotec). During magnetic cell sorting all procedures were performed at 4ºC following the manufacture's instructions. Cells were used as fresh or, when indicted, cells were cultured at 37°C with 5% CO_2 in complete RPMI-1640 supplemented with 10% FBS, 1mM sodium pyruvate, 10U/ml penicillin, 10µg/ml streptomycin on indicated conditions. For long-term in-vitro cell culture of thymocytes (7 days) recombinant human IL-2 was added to the medium.

The PEER (DSMZ, ACC 6), MOLT-4 (ATCC® CRL-1582™) and MCF-7 (ATCC® HTB-22™) cell lines were cultured in RPMI-1640 supplemented with 10% FBS, 1mM sodium pyruvate, 10U/ml penicillin, 10µg/ml streptomycin following the manufacture instructions. The Hel (DSMZ, ACC 11) cell line was cultured in IMDM supplemented with 10% FBS, 1mM sodium pyruvate, non-essential amino acids, 10U/ml penicillin, 10µg/ml streptomycin following the manufacture instructions.

Viral transduction of PEER cell line

Bicistronic retroviral expression vectors (LZRS) containing the gene of interest were used. The used vectors contained the internal ribosomal entry site IRES followed by enhanced green fluorescence protein (eGFP), (LZRS-IRES-eGFP) empty vector used as negative control; or the gene of interest, expressing myrPKB/AKT (constitutively activated AKT) and eGFP (LZRS-myrPKB/AKT-IRES-eGFP) as previously described[319]. Retrovirus was generated by transient transfection of TAT cells co-transfected with helper vectors pCL-Eco and pCMV-VSV-G. Supernatant

viral particles were isolated by high-speed centrifugation and used to stable transfect the PEER cell line.

To increase the percentage of transduced cells for the following experiments, GFP^+ cells were sorted (≈100% purity) using a FACSAria high-speed cell sorter (BD Biosciences).

Chemicals and Antibodies

Anti-human monoclonal antibodies were used against: CD3 (UCHT1), CD27 (LG.7F9), CD4 (RPA-T4), CD8 (HIT8a), CD7 (4H9), panTCRαβ (IP26) and IFNγ (4S.B3) from eBioscience; CD28 (CD28.2), CD8 (SK1), CD45 (HI30), Vδ2 (B6), CD3 (OKT3), CD45RA (HI100) CD69 (FN50), TNF-α (MAb11), CD107a (H4A3) Annexin-V and 7-AAD from Biolegend; panTCRγδ (5A6.E9) from ThermoFisher; Vδ1 (REA173 or TS8.2) from Miltenyi Biotech; p-S129-AKT, p-S473-AKT, AKT, p-S9-GSK3β, GSK3β, p-S380-PTEN, PTEN, p-S235/236-S6 and S6, p-T202/Y204-ERK1/2, ERK1, from Cell Signaling; p-Y694/699-STAT5 from Merck/Millipore; Calnexin and GAPDH from Sicgen; 7-AAD from Invitrogen and Bcl-2 from Dako. Recombinant human sCD27 ligand and recombinant human IL-2, IL-1, IL-4, IL-6, IL-7, IL-12, IL-15, IL-21, IL-23 and TGF-β were purchased from Peprotech; UO126, LY294002 and Ssi from Calbiochem; TBB from Sigma; TG-003 and Harmine from Focus Biomolecules; CX-4945 (Silmitasertib) from Adooq Bioscience and Biorbyt.

Cell surface phenotype analysis, cell viability, cell cycle and proliferation analysis by flow cytometry

Cells were stained using fluorochrome-conjugated mAbs against indicated surface molecules using standard methodology[50]. For intracellular staining the cells were previously fixed and permeabilized using fixation/permeabilization and permeabilization buffers (both from eBioscience), following the manufacturer's instructions.

Quantitative determination of cell viability was performed by using an Annexin V-based apoptosis detection kit as previously reported[320]. Briefly cells were washed with cold PBS, and then resuspend in Annexin V Binding Buffer. The cells were stained with fluorochrome-conjugated Annexin V during 20 min at room temperature in the dark. 7-AAD was added to the cells just before the flow cytometry analysis.

For cell cycle analysis, cells were stained for 1h at 37°C with 30µL of 7-AAD (BD Pharmingen) resuspended in permeabilization buffer (eBioscience) and then washed tree times with RPMI and fluorescence intensity was immediately measured by flow cytometry analysis in linear mode.

CFSE-based proliferation assays (CellTrace CFSE kit, Invitrogen, final concentration 0.5mM) were performed as described[50].

flow cytometry analysis were performed using LSRFortessa or Accuri C6 (both from BD Biosciences). Data were analyzed using FlowJo software (TreeStar).

In vitro tumor-killing assays

The MOLT-4 leukemia cell line was stained with CellTrace Far Red 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one–succinimidyl ester (1mM; Molecular Probes/Invitrogen) and each batch of $3x10^4$ tumor cells was incubated with $3x10^5$ γδ T cells in RPMI-1640 for 3h in a round-bottom plate with 96 wells. Cells were stained with annexin V–FITC (BD Biosciences) and analyzed by flow cytometry.

CK2 kinase activity assay

CK2 activity was measured in cell lysates (from equal cell numbers) using the Casein Kinase-2 Assay kit (17-132) from Upstate Biotechnology, following the manufacturer's instructions. Briefly, total protein lysate were incubated for 10 minutes at 30°C in a reaction mixture containing: CK2α-specific peptide, [γ-32P]ATP and PKA inhibitor cocktail. The radioactivity incorporated into the substrate was determined in P81 phosphocellulose paper-squares by scintillation

counting as previously reported[321]. CK2 activity in γδ+ and αβ+ xenograft-derived blasts was measured on samples, which showed comparable percentages of human engraftment (defined by FACS analysis based on the expression of CD45 and CD7 antigens).

Immunobloting

Cell lysates were used for immunobloting as previously described[321]. Briefly, the cells were lysed, at 4ºC, in cold lysis buffer (50 mM Tris [pH 7.6], 150 mM EDTA, 1% Nonidet P-40 in PBS) enriched with protease and phosphatase inhibitor cocktails (Roche). The total proteins were quantified using a Bradford assay (Bio-Rad), following the manufacturer's instructions. Equal amounts of total protein was denatured in Laemmli buffer (Bio-Rad), boiled for 5 min at 95ºC, and loaded in a 10% SDS-PAGE. After electrophoretic separation, the proteins were transferred to nitrocellulose blotting paper (Amersham Biosciences). The membranes were blocked with 5% BSA and 0.5% Tween 20 (Sigma-Aldrich) in PBS and probed with the indicated primary Abs overnight at 4ºC. After rinse, the membranes were probed using appropriate HRP-conjugated secondary Abs and developed by chemiluminescence using the ChemiDoc XRS+ imaging system (Bio-Rad).

RNA isolation, cDNA synthesis and quantative real time-PCR

Total RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Concentration and purity were determined by spectrophotometry analysis using the NanoDrop ND-1000 spectrophotometer (Thermo Scientific). Total RNA was reverse transcribed into cDNA using a M-MLV RT kit (Promega). Briefly, purified RNA samples and random primers were denatured by heat shock at 70ºC during 10 minutes followed by reverse transcription in described reaction mix with dNTPs and enzyme M-MLV during 60 minutes at 42ºC. The enzyme was inactivated at 90ºC during 10 minutes. Quantitative real-time PCR and analysis was performed using primers from **Table**

4 on ViiA 7 Real-Time PCR system using the ViiA 7 software v1.2 (Applied Biosystems; Life Technologies) using SYBR Green detection system (Applied Biosystems; Life Technologies). *GAPDH* or *Beta-2-Microglobulin* genes were used as internal control for normalization and all samples were run in duplicates or triplicates (dependent on sample availability).

Table 4: List of primers used in this study for RT-qPCR analysis.

Gene	Primer forward (5´-3´)	Primer reverse (5´-3´)
B2m	CTATCCAGCGTACTCCAAAGATTC	CTTGCTGAAAGACAAGTCTGAATG
Clk2	AATATTTTTACCGGGGTCGC	AGCCGCTTAGCTGGTTCATA
CSNK2A1 (CK2α)	AAGACCCTGTGTCACGAACC	GCCAAACCCCAGTCTATTAGTC
CSNK2A2 (CK2α')	AAAAGCTGCGACTGATAGATTGG	GAGGCTACACGAACATTGTACTC
CSNK2B (CK2β)	CCAGGCTCTACGGTTTCAAG	CCCACCACAATAACGACTCC
Dyrk1a	GGAGGAGAGACTTCAGCA	AACCCATTCTTGCTCCACA
GAPDH	CGAGATCCCTCCAAAATCAA	GTCTTCTGGGTGGCAGTGAT
Pfn	GCAATGTGCATGTGTCTGTG	GGGAGTGTGTACCACATGGA
Tbx21	CACCTGTTGTGGTCCAAGTTT	AACATCCTGTAGTGGCTGGTG

In vivo mouse experiments

TCRαβ or TCRγδ-positive T-ALL cells obtained from patient diagnostic were injected into 8-12 weeks old NSG (NOD-SCID IL-2Rγ^{null}) mice (1x10^6 cells/mouse, tail vein injections). Mice were monitored weekly by flow cytometry for human leukemic load (hCD7$^+$, hCD45$^+$ cells) in peripheral blood. Mice were euthanized when terminally ill and blast cells from bone marrow were collected. For the *in vivo* experiment of γδ T-ALL treatment, 10-12-weeks-old NRGS (NOD-Rag1^{null} IL2Rγ^{null}) mice were injected subcutaneously in the right flank with 2x10^6 PEER cells resuspended in 100µL of PBS. At day 20, all mice presented palpable tumors (100-150mm^3) and were randomly distributed into two groups (n≥3). The animals

were treated with CX-4945 (75 mg/kg), by oral gavage twice daily (BID) or vehicle control (25mM sodium bisphosphate buffer) as described[308]. Mice were monitored daily and weighed frequently. Tumors were measured every two days with a caliper and tumor volume was calculated (volume = (length x width)^2/2). At day 13, after starting the treatment, all mice were sacrificed (an ethical requirement for the control group, when tumor reached 2000mm^3).

Statistical analysis

Statistical significance of differences between indicated conditions was assessed using Student's t-test with Welch's correction and is indicated when significant as * (P<.05); ** (P<.01); *** (P<.001). All statistical analysis was performed using GraphPad Prism software.

Chapter III

Results

III. RESULTS

1. Human γδ thymocytes are functionally immature and differentiate into cytotoxic type 1 effector T cells upon IL-2/IL-15 signaling

Introductory background:

All jawed and jawless vertebrates have evolved three lineages of lymphocytes based on somatic gene diversification of Ag receptors[322]. Within the lymphocyte trilogy, γδ T cells clearly remain the most poorly understood lineage, both in terms of Ag recognition and differentiation into effector cell subsets[60]. This notwithstanding, γδ T cells are already being targeted in cancer immunotherapy[85,323] based on multiple promising preclinical studies in both mice[65,66,324,325] and humans[84,231,323,326].

Human γδ PBLs are endowed with potent cytotoxicity against hematological[86,231,323] and epithelial[84,327] malignant cells. Moreover, γδ PBLs are highly polarized toward IFN-γ production since early life, as preterm babies harbor significant proportions of IFN-γ^+ γδ (but not αβ) T cells in the blood[328], and CMV infection in utero promotes the differentiation of IFN-γ^+ and perforin$^+$ γδ T cells[329]. γδ T cells thus constitute the first functional population of circulating T cells[328].

Given an individual's history of infections, circulating γδ PBLs can display very heterogeneous phenotypes ranging from naive to effector/memory and terminally differentiated effector cells[106]. It is therefore difficult to inquire where and how human γδ T cells acquire their effector functions. In healthy individuals, these are tightly linked to IFN-γ production, as alternative functional states, such as IL-17 or IL-22 secretion, are very rare [217,330].

Interestingly, we[50] and others[150,331] have shown that murine γδ T cells acquire their effector properties during thymic development, in a process regulated by TCRγδ (and coreceptor) signaling[146]. For example, IFN-γ–producing γδ T cells

require TCR and CD27 signals for differentiation in the mouse thymus[50,150,331]. This raises the question whether human γδ thymocytes can also complete their functional differentiation before being exported to the periphery. Although thymic commitment to the γδ T cell lineage is controlled by Notch signaling[33,332], much less is known about the subsequent steps of functional differentiation of human γδ T cells[47,48,330]. This will likely have major implications for their manipulation in cancer immunotherapy.

Building on these considerations, in this study we have used pediatric thymic tissue to address the molecular mechanisms of human γδ T cell differentiation toward antitumor lymphocytes. Our results reveal an NK-like mode of differentiation that is de- pendent on IL-2/IL-15 signals but surprisingly not on TCR activation. Interestingly, this process must take place in the periphery, because, unlike their murine counterparts, human γδ thymocytes are devoid of cytotoxic type 1 effector functions. Finally, our data disclose an MAPK/ERK-mediated differentiation pathway that may constitute an important target for future modulation of γδ T cell activity in the clinic.

1.1 Human γδ thymocytes are devoid of cytotoxicity and IFN-γ production

Inspired by the recent identification of fully differentiated effector γδ T cell subsets in the murine thymus[48–50,150], we started this study by analyzing the surface phenotype and functional potential of γδ T cells isolated from human pediatric thymic samples. Based on the differentiation markers CD1a[333], CD27, and CD45RA[106], the vast majority of γδ thymocytes showed an immature and naive phenotype, which contrasted with the dominant effector/memory phenotype[106] of γδ PBLs (**Figure 5A**). Also unlike these, γδ thymocytes produced negligible proinflammatory cytokines, particularly IFN-γ and TNF-α (**Figure 5B**). Moreover, γδ thymocytes lacked cytolytic activity (**Figure 5C**), namely against leukemia

target cells, which were promptly killed by their PBL counterparts (**Figure 5D**). These data clearly demonstrate that, unlike murine γδ thymocytes[48,50,150,331], human γδ T cells do not complete their functional differentiation in the thymus, and they thus lack the cytotoxic type 1 characteristics of γδ PBLs.

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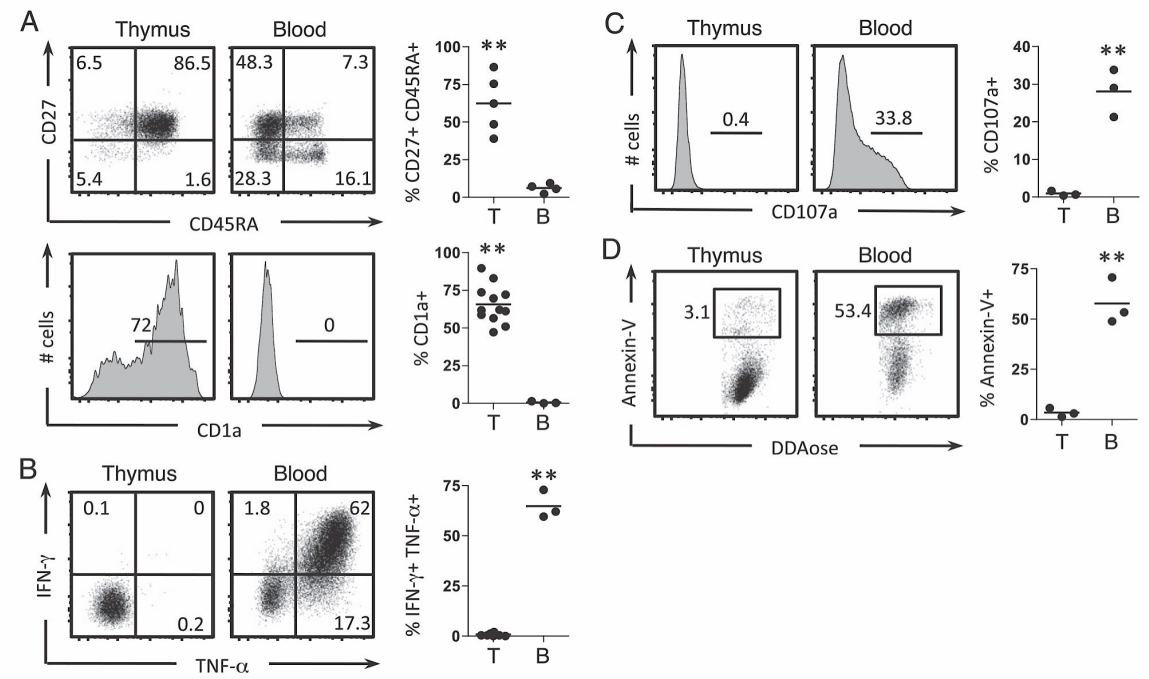

Figure 5: Human γδ thymocytes are devoid of IFN-γ production and cytotoxic functions.

TCRγδ⁺CD3⁺ cells were isolated from pediatric thymic biopsies (Thymus/T) or from the peripheral blood of healthy donors (Blood/B) and analyzed ex vivo by flow cytometry. Dead cells were excluded from the analysis using LIVE/DEAD Fixable Dead Cell Stain Kits (Molecular Probes). (**A**) Surface expression of CD27, CD45RA, and CD1a. (**B** and **C**) Intracellular staining for IFN- γ and TNF-α (**B**) or the degranulation marker CD107a (**C**) following 4 h of stimulation with PMA and ionomycin. (**D**) Cytotoxic activity against MOLT-4 leukemia cells. Percentage of apoptotic annexin V⁺ within 7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one– succinimidyl ester (DDAO-SE⁺) (prelabeled) tumor cells after 3 h of coincubation with γδ T cells at a 10:1 E:T ratio. Results in this figure are representative of 3–12 independent experiments; each dot represents an individual sample. **p , 0.005.

1.2 IL-2 and IL-15 signals drive human γδ cytotoxic type 1 cell differentiation

The functional immaturity of human γδ thymocytes made them an ideal system to investigate the molecular cues required for acquisition of antitumor effector properties. Focusing first on IFN-γ production, we considered that naive $CD4^+$ T cells typically require TCR/CD3 and CD28 ligation in the presence of IL-2 and IL-12 for differentiation along the "T helper 1" pathway[334]. Although such a "Th1 mix" was indeed capable of generating IFN-γ^+ γδ T cells, we surprisingly found that IL-2 alone was also sufficient (**Figure 6A**, upper panel). This effect was potentiated by IL-12, whereas TGF-β abrogated the process. Unexpectedly, the addition of TCR stimulation via anti-CD3 mAb did not enhance, but rather reduced, the IL-2–mediated differentiation of human IFN-γ^+ γδ T cells (**Figure 6A**, upper panel).

We next tested a large panel of individual cytokines and observed that besides IL-2, only IL-15 (but notably not IL-4 or IL-7) was able to induce IFN-γ production in γδ thymocyte cultures (**Figure 6A**, middle panel). Both IL-2 and IL-15 treatments also promoted TNF-α expression (**Figure 6A**, lower panel), upregulated the activation marker CD69 (**Figure 6B**), and drove thymocytes along the effector/memory differentiation pathway, toward a $CD45RA^+$ $CD27^-$ T effector memory stage (**Figure 6C**).

Concerning γδ T cell cytotoxicity, IL-2 and IL-15 (but not IL-7) induced the expression of the degranulation marker CD107a on γδ thymocytes (**Figure 6D**) and endowed them with potent killing capacity against leukemia target cells (**Figure 6E**). Of note, exogenous IL-2 and IL-15 also enhanced the effector functions of γδ PBLs, especially their degranulation/cytotoxic potential (data not shown).

The acquisition of IFN-γ/TNF-α production and cytolytic capacity by γδ thymocytes were positively correlated, suggesting a common pathway of cytotoxic type 1 differentiation (**Figure 6F**). Moreover, IL-2/IL-15 signals induced de novo expression of the type 1 master transcription factors T-bet and eomesodermin, as

well as the cytolytic molecule perforin (**Figure 6G**). These data firmly demonstrate that IL-2 and IL-15 are key functional differentiation factors for human γδ T cells. Importantly, they also show that IL-2 and IL-15 signals are sufficient, in the absence of TCR activation, to generate fully functional γδ T cells from immature thymocytes.

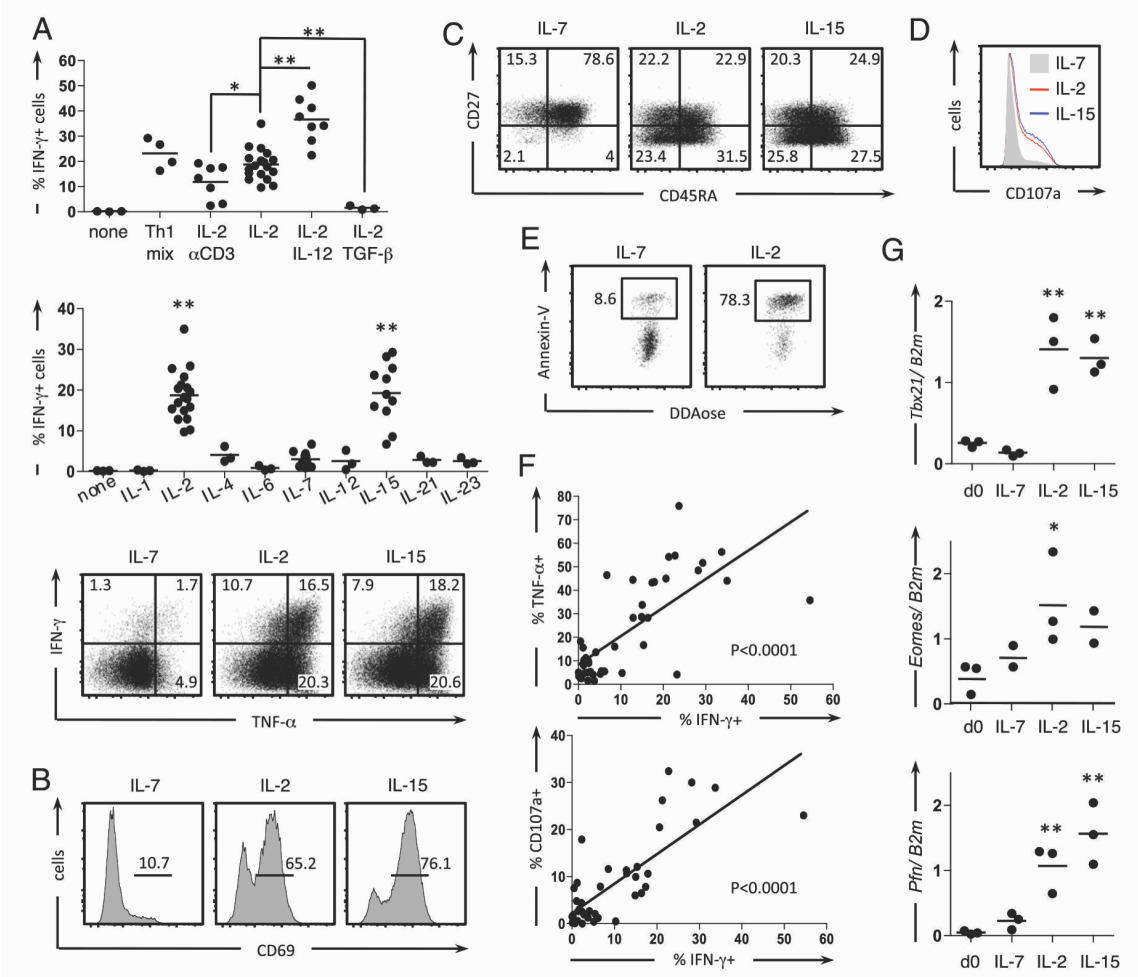

Figure 6: IL-2 and IL-15 signals differentiate γδ thymocytes into cytotoxic type 1 effector T cells.

MACS-purified γδ thymocytes were cultured for 7 d in the presence of 10 ng/ml of the indicated cytokines; anti-CD3 mAb (1 mg/ml) was added when noted, and as part of the Th1 mix also contained anti-CD28 mAb (5 mg/ ml), IL-2, and IL-12. (**A**) Intracellular staining for IFN-γ following 4 h of stimulation with PMA and ionomycin. (**B**) Surface expression of the activation marker CD69. (**C**) Surface staining for CD27 and CD45RA. (**D**) Intracellular staining for CD107a. (**E**) Cytotoxic

activity against MOLT-4 leukemia cells (as in FIGURE 1D). (**F**) Correlations between fractions of cells expressing IFN-γ, TNF-α, or CD107a. Each dot represents a specific culture condition from the experiments in FIGURE (A) and (D). (**G**) Real-time PCR data for the expression of T-bet (Tbx21, upper panel), eomesodermin (Eomes, middle panel), and perforin (Pfn, lower panel), normalized to housekeeping b2-microglobulin (B2m), on γδ thymocytes either freshly isolated or cultured for 7 d with the indicated cytokines. Results in this figure are representative of 3–18 independent experiments; each dot represents an individual sample. *p , 0.05, **p , 0.005.

1.3 Vδ1 and Vδ2 T cell subsets follow similar rules of functional differentiation

Given that human γδ T cells comprise two major subsets, Vδ1$^+$ cells (5–30% of γδ PBLs but more abundant in tissues) and Vδ2$^+$ cells (60–95% of γδ PBLs), both strongly biased toward cytotoxic type 1 functions[323,335], we next assessed whether they followed similar rules of differentiation. Consistent with the literature[47], the thymic γδ repertoire was largely biased for Vδ1$^+$ thymocytes, with an average Vδ1/Vδ2 ratio of 25, which was maintained in vitro upon IL-7, IL-2, or IL-15 treatment (data not shown). In all thymic samples analyzed, Vδ1$^+$ T cells behaved as expected: they were functionally immature ex vivo and differentiated into type 1 effectors in response to IL-2 or IL-15 stimuli. However, the results obtained by gating on the Vδ2$^+$ population were affected by an important intersample variation. We considered that this could be due to blood contamination or recirculation (back to the thymus) of mature Vγ9Vδ2 cells, which are much more abundant in the blood than in the thymus (3% of total γδ thymocytes). To overcome these problems, we purified CD1a$^+$ γδ T cells, which are exclusive to the thymus (**Figure 5A**, lower panel) and cultured them for 7 days with IL-7, IL-2, or IL-15. We found that both Vδ1$^+$ and Vδ2$^+$ cells similarly acquired type 1 effector properties in response to IL-2 and IL-15, but not IL-7 (**Figure 7A**).

As an alternative developmental strategy, we differentiated γδ T cells from sorted CD3$^-$TCRγδ$^-$CD4$^-$CD8$^-$ thymic precursors cultured on OP9-DL1 monolayers, as previously described[336]. In the presence of IL-7 alone, Vδ1 and Vδ2 T cells

developed normally (**Figure 7B**) to a Vδ1/Vδ2 ratio similar to that observed ex vivo (data not shown). We therefore think this is an elegant model to characterize γδ T cell differentiation from very early developmental stages. Most importantly, the further addition of IL-2 was necessary to generate IFN-γ– (Fi**gure 7C**) and TNF-α– (**Figure 7D**) producing γδ T cells, and this occurred similarly for Vδ1 and Vδ2 T cell subsets (**Figure 7C**, **7D**). These data demonstrate that IL-2 (or IL-15) signals drive the functional differentiation of both major subsets of human γδ T cells.

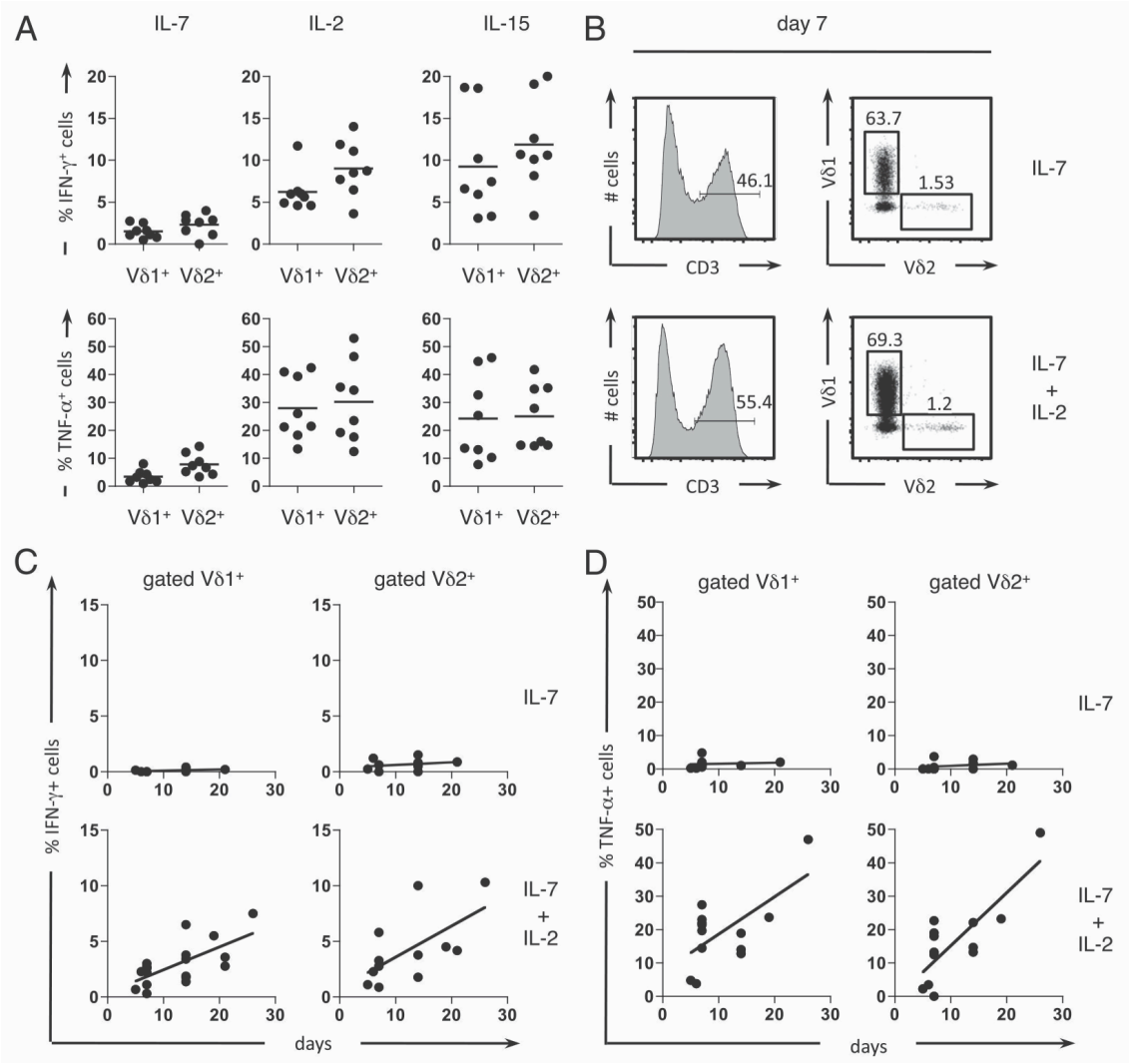

Figure 7: Vδ1 and Vδ2 subsets of γδ T cells follow similar rules of functional differentiation.

(**A**) CD1a$^+$γδ thymocytes were FACS sorted and cultured for 7 d with the indicated cytokines (at 10 ng/ml). Cells were restimulated for 4 h with PMA and ionomycin and stained for TCRVδ1, TCRVδ2, and intracellular IFN-γ and TNF-α. Histograms depict percentages of IFN-γ$^+$ cells (upper panels) or TNF-α$^+$ cells (lower panels) within pregated Vδ1$^+$ or Vδ2$^+$ cells. (B and D) CD3$^-$CD4$^-$CD8$^-$TCRγδ$^-$ thymic progenitors were FACS sorted and cultured on OP9-DL1 monolayers in the presence of IL-7 with or without IL-2. At indicated time points, cells were harvested and stained for surface CD3, TCRVδ1, TCRVδ2 (**B**), and intracellular IFN-γ (**C**) and TNF-α (**D**). Graphs represent percentages of IFN-γ$^+$ cells (**C**) or TNF-α$^+$ cells (**D**) within pregated Vδ1$^+$ or Vδ2$^+$ cells. Dead cells were excluded from the analysis using LIVE/DEAD Fixable Dead Cell Stain Kits. Data are representative of six to eight independent experiments with similar results; each dot represents an individual sample.

1.4 IL-2/IL-15 signals induce γδ type 1 cell differentiation via the MAPK/ERK pathway

To gain further mechanistic insight into the type 1 differentiation pathway of human γδ T cells, we probed the three major signaling pathways downstream of common γ-chain cytokine receptors and observed that IL-2 stimulation hyperphosphorylated ERK1/2 (MAPK pathway), STAT5 (JAK/STAT pathway), and AKT (PI3K pathway) (**Figure 8A**). To determine which of these signaling pathways was critical for functional differentiation of γδ T cells, we analyzed the effect of specific chemical inhibitors on γδ thymocyte cultures. When added at the start of the cultures, all of these drugs interfered with γδ T cell proliferation and prevented their functional differentiation (data not shown). Of note, although proliferation was necessary for IFN-γ induction, this specifically required IL-2 or IL-15 signals, as IL-7 failed to do so even after five cell divisions (**Figure 8B**). To dissociate proliferation from differentiation, we added the inhibitors at day 4 of culture, when cells had already undergone five rounds of division (**Figure 8B**). Whereas blocking STAT5 or PI3K/AKT had no detectable effect, the MAPK/ERK inhibitor UO126[337,338] completely abrogated the differentiation of IFN- γ$^+$ or TNF-α$^+$ γδ T cells (under IL-2 or IL-15 treatment) (**Figure 8B**).

Interestingly, IL-2 or IL-15 signals also enhanced IL-2 production by γδ T cells, thus providing an autocrine mechanism to sustain their functional differentiation. The production of IL-2 (as well as TNF-α) by γδ T cells was also completely abolished by the addition of UO126, but not LY294002 or Ssi (data not shown). These data demonstrate that γδ T cell cytotoxic type 1 differentiation can be manipulated by drugs specifically targeting the MAPK/ERK pathway[339].

Collectively, our results identify MAPK-mediated IL-2/IL-15 signaling as the major functional differentiation pathway of human γδ T cells toward antitumor (cytotoxic type 1) effector lymphocytes.

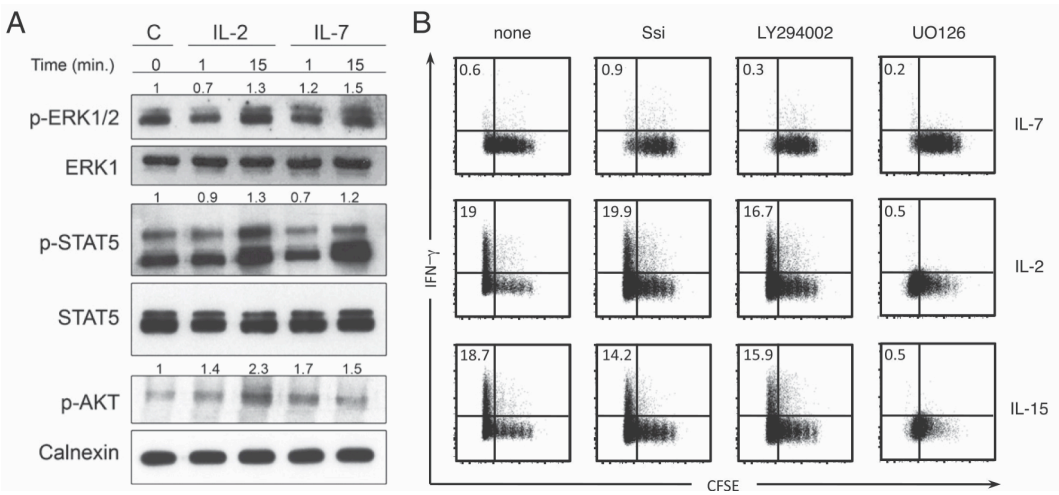

Figure 8: The MAPK/ERK signaling pathway is required for IL-2–mediated type 1 differentiation of human γδ T cells.

MACS-purified γδ thymocytes were cultured in the presence of 10 ng/ml IL-2, IL-7, or IL-15. (**A**) Western blot analysis of p-ERK1/2 (Thr^{202}/Try^{204}), ERK1, p-AKT (Ser^{473}), p-STAT5 ($Tyr^{694/699}$), and STAT5a in cell lysates obtained at the indicated timepoints. C, control medium without cytokines. The numbers above the blots correspond to desitometric analysis of the corresponding bands. (**B**) Effect of chemical inhibitors on the production of IFN-γ in γδ thymocyte cultures. At day 4 (after five cell divisions, based on CFSE dilution), specific inhibitors of STAT5 (Ssi), PI3K (LY294002), or MEK phosphorylation (UO126) were added (20mM each), and at day 7 cells were restimulated for 4 h with PMA and ionomycin and stained intracellularly for IFN-γ. Dead cells were excluded from the analysis using LIVE/DEAD Fixable Dead Cell Stain Kits. Data are representative of three to five independent experiments.

2. Casein Kinase 2 controls the survival of normal thymic and leukemic γδ T cells via promotion of AKT signaling

Introductory background:

T cells develop in the thymus. The dissection of the cell-intrinsic and extrinsic signals that regulate thymocyte survival, proliferation and differentiation is critical to understand their potential for transformation and to devise new therapies for T-cell acute lymphoblastic leukemia (T-ALL).

T-cell commitment is coupled to somatic T cell receptor (TCR) rearrangements, which generates thymocytes bearing either an αβ or a γδ TCR[17]. The expression of a pre-TCR composed of TCRβ and the invariant pTα chain in αβ thymocyte progenitors results in a massive proliferative burst ("β-selection") which dictates that αβ T cells largely outnumber their γδ counterparts. Likely a consequence, while significant progress has been made in our understanding of human αβ T-cell development, the molecular determinants of γδ thymocytes remain poorly characterized[17].

Most of what we know about thymic γδ T cell differentiation comes from studies performed in mice, showing how various receptors (namely, TCRγδ, CD27 and LTβR) and downstream transcription factors (such as Id3, Sox13, TCF1 and Lef1) control various maturation steps, from divergence from the αβ lineage to the acquisition of effector functions such as pro-inflammatory cytokine production[29,43,50,340–342]. By contrast, much less is known about human thymic γδ T cell differentiation. Notwithstanding, we recently showed that IL-2 or IL-15 differentiate human γδ thymocytes into cytotoxic type 1 effector T cells, rendering them highly efficacious against leukemic cells *in vitro* and *in vivo*[88,169].

γδ thymocytes can themselves transform into leukemic cells, causing a rare (<10% of all cases) form of T-ALL with distinctive clinical features[343–345]. Given that malignant γδ T cells have been very poorly studied and lack defined molecular targets, we have here addressed the potential role of Casein Kinase 2 (CK2), a

signaling effector molecule previously implicated in chronic lymphocytic leukemia[321,346], multiple myeloma[347], B-ALL[348,349], T-ALL[283,350] and other hematological disorders (reviewed in[351]).

CK2 is a ubiquitous and constitutively activated serine-threonine protein kinase that regulates multiple pathways including PI3K/AKT and WNT signaling, NF-κB transcription, and the DNA damage response[351]. CK2 displays pro-survival and anti-apoptotic functions that were described in several cancer cell types. CK2 is frequently overexpressed or hyperactivated in both solid tumors and in hematological malignancies, thus making it a promising target for cancer treatment[351]. By contrast, the physiological function of CK2 in non-transformed cells is less established. Recent studies in mice have demonstrated that CK2 activity is necessary for peripheral T cell activation and function: interference with CK2 signaling impaired $CD4^+$ T cell activation and differentiation into Th2 or Th17 cells[352], while the genetic deletion of CK2 in $CD4^+$ $Foxp3^+$ regulatory T cells abolished their suppressive activity against allergy-promoting Th2 cells[353]. However, no functional role has yet been attributed to CK2 in the thymus.

In this study we identified a novel role for CK2 in controlling the survival of normal γδ thymocytes and γδ T-ALL cells. We analyzed CK2 activity in γδ versus αβ thymocytes and T-ALL cells, its regulation by cell-extrinsic signals, the downstream signaling mechanisms, and the effect of its inhibition *in vitro* and *in vivo*, in a xenograft model of γδ T-ALL.

2.1 Human γδ thymocytes have enhanced CK2 activity and are highly sensitive to its inhibition

This study initiated with the analysis of CK2 activity in normal thymocyte subsets obtained from pediatric thymic biopsies. We measured CK2 activity using a substrate-specific kinase assay in freshly-isolated $TCR\gamma\delta^+$ or $TCR\alpha\beta^+$ cells, and unexpectedly found 2-fold higher activity in γδ thymocytes relative to their αβ

counterparts (**Figure 9A**). To address its physiological relevance, we treated thymocytes for 24 hours with a highly specific ATP-competitive inhibitor of CK2, CX-4945[288]. Flow cytometry analysis of Annexin V/ 7-AAD-stained cells revealed increased apoptosis of γδ compared to CD4+ and CD8+ αβ thymocytes (**Figure 9B**). To assess the longer-term impact of CX-4945 treatment, thymocytes were cultured in the presence of TCR plus costimulation for 7 days. γδ thymocytes were highly susceptible to apoptosis upon CK2 inhibition in a dose dependent-manner (**Figure 9C**). By contrast, as previously reported[283], CD4+ or CD8+ αβ thymocyte survival was not significantly affected following CK2 inhibition (**Figure 9C**). These data revealed that healthy γδ thymocytes are exquisitely dependent on their high basal CK2 activity for survival.

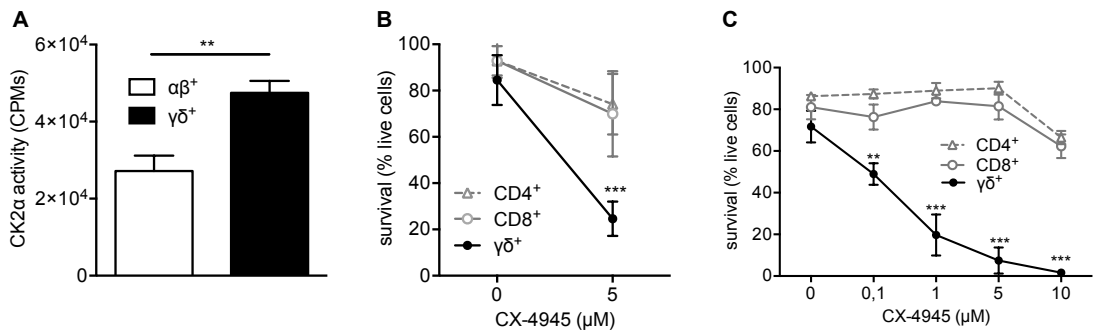

Figure 9: Human γδ thymocytes have enhanced CK2 activity and are highly sensitive to CX-4945

(**A**) *In vitro* CK2α activity (kinase assay) in freshly-isolated human thymic γδ and αβ T-cells (2x10^6 cells per assay). CPM, counts per minute. **(B)** Survival (% of live cells) of human thymic γδ, CD4+ and CD8+ T-cells following 24h of incubation with 5μM of the CK2 inhibitor, CX-4945, analyzed by flow cytometry using Annexin-V/ 7-AAD staining. **(C)** Survival (% of live cells) of human thymic γδ, CD4+ and CD8+ T-cells to different concentrations of CX-4945 (or vehicle), analyzed by Annexin-V/ 7-AAD staining following 7 days in culture with rhIL-2 plus CD3+CD27 or CD3+CD28 stimulation of sorted thymic γδ or αβ T-cells, respectively. Data in this figure are representative of at least three independent experiments; $^{**}p < 0.01$, $^{***}p < 0.001$ (T-test).

2.2 CK2 activity in γδ thymocytes is modulated by TCR stimulation and promotes AKT signaling

We next asked which signals regulated CK2 activity in γδ thymocytes. Very few studies have documented CK2 modulation by physiological stimuli in T cells[352,354]. When we stimulated (for 6 hours) isolated thymocyte subsets via the TCR complex using agonist anti-CD3ε antibodies, we observed ~3-fold enhancement of CK2 activity selectively in γδ thymocytes (**Figure 10A**). We also tested the impact of costimulation, particularly through CD27 which we have shown to play a major role in γδ T-cell development and expansion[50,104,355]. However, the addition of soluble recombinant CD27-ligand/ CD70 (sCD70) had no additive effect on CK2 activity (**Figure 10A**). Thus, our data suggest that CK2 activity in healthy γδ thymocytes is modulated primarily by TCR signals.

To gain insight on the downstream effects of CK2 signaling and its inhibition in γδ thymocytes, we focused on the AKT signaling pathway, which is involved in cell survival and proliferation and known to be regulated by CK2 in both normal and malignant αβ T lymphocytes[186,283]. We observed that TCR/ CD27 stimulation inhibited PTEN, as measured by the increase in its phosphorylated form, and potentiated the AKT signaling pathway in γδ but not αβ thymocytes, as shown by the phosphorylation of AKT and its downstream targets GSK3β and S6 (**Figure 2C**). The GSK3β phosphorylation at S9 offers an intracellular readout for AKT activity[356]. These effects were completely reversed by CX-4945 (**Figure 10C**). As functional outcomes of CK2 inhibition, we observed decreased γδ thymocyte proliferation (**Figure 10D**) and survival (**Figure 10E**). Moreover, in agreement with the implication of AKT signaling downstream of CK2, we found a similarly striking effect on γδ thymocyte survival upon treatment with the specific AKT inhibitor, MK-2206[357] (**Figure 10E**).

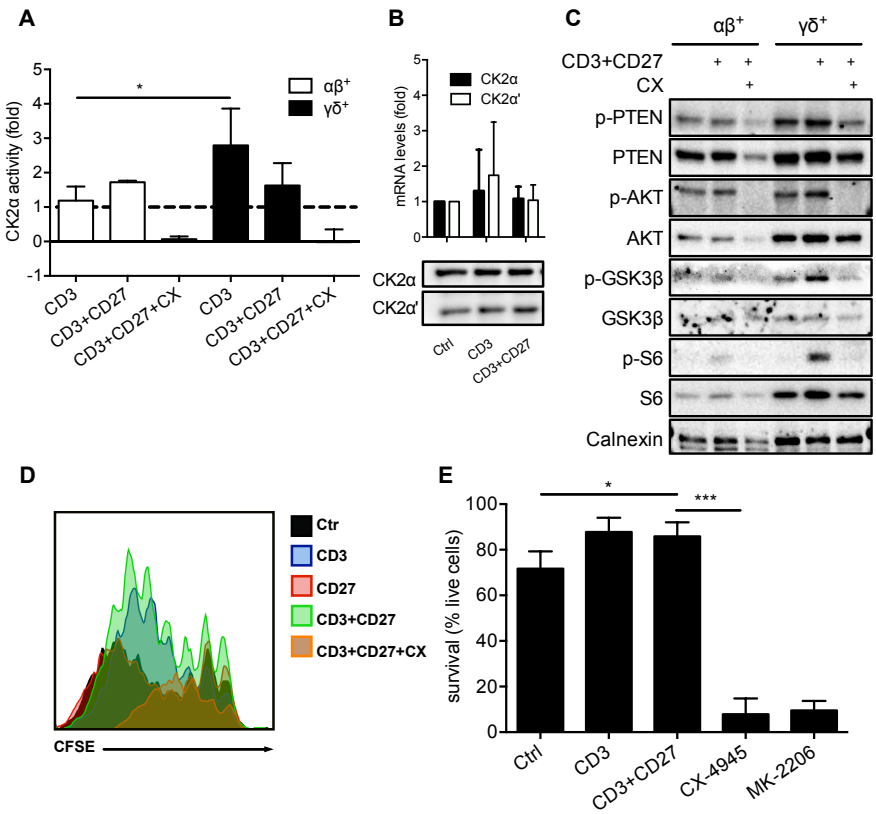

Figure 10: CK2 activity in γδ thymocytes is modulated by TCR stimulation and activates AKT signaling

(**A**) *In vitro* CK2α activity in sorted γδ and αβ thymocytes (2x10^6 cells per sample) after 6 hours of stimulation with anti-CD3 antibodies (CD3), plus soluble CD27-ligand (CD3+CD27) or plus 5μM CX-4945 (CD3+CD27+CX); values were normalized to unstimulated control (dashed line). (**B**) mRNA (top) and protein (bottom; western blot) levels of CK2α and CK2α' of γδ thymocytes stimulated as in (A). (**C**) Western blot analysis of (phospho)proteins implicated in AKT signaling, in γδ and αβ thymocytes (1x10^6 cells per sample) stimulated as in (A). (**D**) Proliferation (CFSE dilution assay) of γδ thymocytes after 7 days in culture with rhIL-2 under the indicated conditions: medium only (Ctrl); anti-CD3 antibody stimulation (CD3); soluble CD27-ligand (CD27); their combination (CD3+CD27) and with 5μM CX-4945 (CD3+CD27+CX). (**E**) Survival (% of live cells) of γδ thymocytes after 7 days of stimulation (or not, Ctrl for control) with anti-CD3 antibodies (CD3), plus soluble CD27-ligand (CD3+CD27), plus 5μM of CX-4945 or 10μM of MK-2206. Data in this figure are representative of at least three independent experiments; *p <0.05, ***p <0.001 (T-test).

2.3 CD27-dependent upregulation of CK2 activity and downstream AKT signaling in γδ T-ALL

We next asked how CK2 activity would impact on γδ T-ALL. First, we compared CK2 activity in normal γδ and αβ thymocytes versus γδ and αβ T-cell blasts obtained from T-ALL patients (and expanded in NSG mice, with similar engraftment, as detailed in the Methods section). As expected, αβ T-ALL cells displayed higher levels of CK2 activity than αβ thymocytes (**Figure 11**). Notably, we detected markedly higher CK2 activity in γδ T-ALL cells as compared to healthy thymocytes and αβ T-ALL blasts (**Figure 11**). Moreover, the γδ T-ALL cell line PEER reproduced the very high CK2 activity observed in γδ T-ALL blasts (**Figure 11**), making it a good model for further biochemical and functional CK2 tests in γδ T-ALL. Likewise, both γδ T-ALL samples and PEER cell line present similar immunephenotypes (CD3$^+$ and TCR Vδ1$^+$, data not shown).

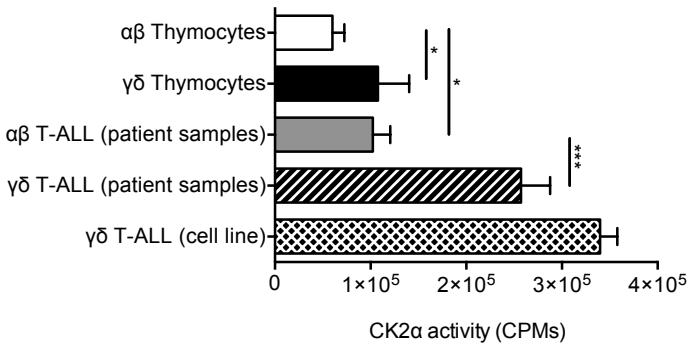

Figure 11: γδ T-ALL cells display higher CK2 activity than αβ counterparts

In vitro CK2α activity (kinase assay; 6.6x10^6 cells per assay) in freshly-isolated γδ (n=4) and αβ (n=4) thymocyte samples; γδ (n=6) and αβ (n=14) T-cell samples obtained from T-ALL patients and expanded in NSG mice (as described in the Methods); and the γδ T-ALL cell line, PEER (n=4). (T-test, *p <0.05, ***p <0.001)

The specific chemical inhibitor, CX-4945, inhibited CK2 activity in γδ T-ALL cells in a dose-dependent manner (**Figure 12A**). On the other hand, CK2 activity could be enhanced upon activation, but CD27 costimulation had a synergistic contribution in γδ T-ALL cells (**Figure 12B**), in contrast with healthy γδ thymocytes (**Figure 10A**). Of note, PEER cells are CD27⁺ Vδ1⁺ γδ T-ALL cells (data not shown). A CD27-dependent effect was also observed on AKT signaling (**Figure 12C**), and was completely abrogated upon CX-4945 treatment, also in a dose dependent manner (data not shown).

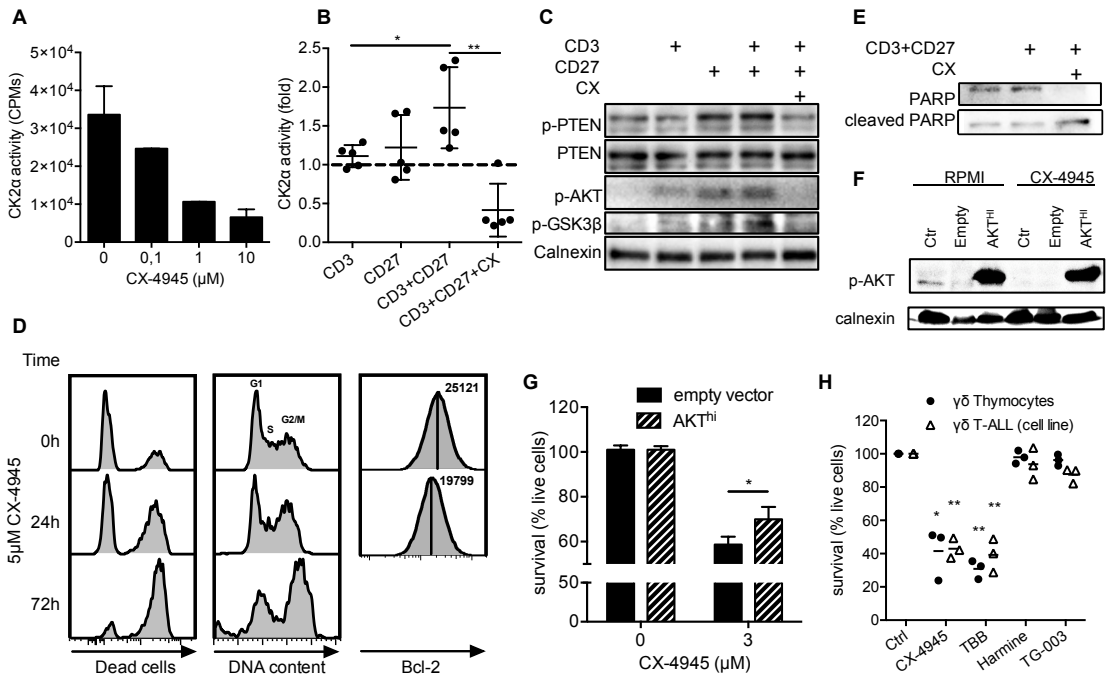

Figure 12: CK2 activity in γδ T-ALL cells is potentiated by CD27 costimulation and promotes AKT signaling

(**A**) CK2α activity in the γδ T-ALL cell line, PEER (2x10⁶ cells per condition), after 6h of treatment with indicated concentrations of CX-4945. (**B**) CK2α activity in lysates from γδ T-ALL (PEER) cells (2x10⁶ cells per condition) after 6h of stimulation under the indicated conditions (T-test, **p* <0.05; ***p* <0.01). (**C**) Western blot analysis of (phospho)proteins implicated in AKT signaling, in γδ T-ALL (PEER) cells treated for 6h with anti-CD3 antibodies (CD3), plus soluble CD27-ligand (CD3+CD27) or plus 5μM CX-4945 (CD3+CD27+CX). Data are representative of five independent experiments

(**D**) Flow cytometry analysis of apoptosis (Annexin-V$^+$; *left panel*), cell cycle/ DNA staining (*middle panel*), and intracellular Bcl-2 protein staining (*right panel*; values indicate MFI) of γδ T-ALL (PEER) cells treated with CX-4945 (5mM) during the indicated times. Western blot analysis of (**E**) Poly-(ADP-ribose) polymerase (PARP) and cleaved PARP of PEER cells treated for 6h as in (C); and (**F**) phospho-AKT (S129; and calnexin loading control) and (**G**) cell survival after 48h of PEER cells transduced with a bicistronic retroviral DNA construct: either empty vector (LZRS) expressing only IRES followed by eGFP (LZRS-IRES-eGFP); or vector co-expressing myrPKB/AKT and eGFP (LZRS-myrPKB/AKT-IRES-eGFP) (AKT^{hi}); and treated with 3μM CX-4945 or vehicle. (**H**) Survival (percentage of live cells) of human γδ thymocytes or γδ T-ALL PEER cells following 24h of incubation with CX-4945 (5μM), TBB (50μM), Harmine (10μM) or TG-003 (10μM) analyzed by flow cytometry using Annexin-V/ 7-AAD staining. Values were normalized to controls (Ctrl), i.e., in the absence of chemical inhibitors. Each dot represents an independent sample. (T-test, **p* <0.05; ** p<0.01).

Functionally, CK2 inhibition led to γδ T-ALL cell cycle arrest at G2/M phase, cell apoptosis and decreased Bcl-2 protein levels and increased cleavage of PARP (Poly-(ADP-ribose) polymerase) (**Figure 12D-E**). To further examine the functional impact of AKT activation downstream from CK2, we tried to rescue this apoptotic phenotype by expressing a myristoylated, constitutively active form of AKT[358]. Cells expressing myristoylated AKT displayed high levels of AKT phosphorylation that were insensitive to CK2 inhibition (**Figure 12F**). Importantly, AKT hyperactivation partially rescued γδ T-ALL survival under CX-4945 treatment (**Figure 12G**). On the other hand, CK2 activity was not affected by AKT hyperactivation (data not shown), collectively suggesting that AKT phosphorylation is downstream rather than upstream of CK2, and that AKT is a key mediator of CK2 functions.

To verify that CX-4945 effects were mediated by inhibition of CK2 rather than other kinases, CLK2 and DYRK1A, recently reported to be sensitive to CX-4945 treatment[359,360], we also tested another CK2 inhibitor, 4,5,6,7-tetrabromobenzotriazole (TBB); TG-003, which specifically targets CLK2[359,361]; and Harmine, which selectively inhibits DYRK1A[362]. Importantly, only the other CK2

inhibitor, TBB, reproduced the effects of CX-4945, whereas TG-003 and Harmine failed to impact on both γδ thymocytes and PEER cell line survival (**Figure 12H**). These results strongly suggest that the physiological target of CX-4945 in γδ thymocytes is CK2.

2.4 γδ T-ALL cells are highly sensitive to CK2 inhibition in vitro and in vivo

To further evaluate the functional impact of CK2 inhibition on γδ T-ALL survival, we compared the effect of CX-4945 treatment on primary γδ versus αβ T-ALL cells, as well as representative cell lines, PEER and MOLT-4, respectively.

Upon 48 hours hours of *in vitro* treatment with CX-4945, we observed increased cell apoptosis in primary γδ T-ALL samples compared to αβ T-ALL cells (**Figure 13A**). We performed a more detailed test with the T-ALL cell lines, at various time points of incubation with CX-4945, and found a profound and dose-dependent effect on PEER (γδ T-ALL) that were significantly more susceptible to apoptosis that MOLT-4 (αβ T-ALL) cells (**Figure 13B**). These data suggest that γδ T-ALL cells, like healthy γδ thymocytes (**Figure 9B-C**), are considerably more sensitive to CK2 inhibition than αβ T-ALL cells.

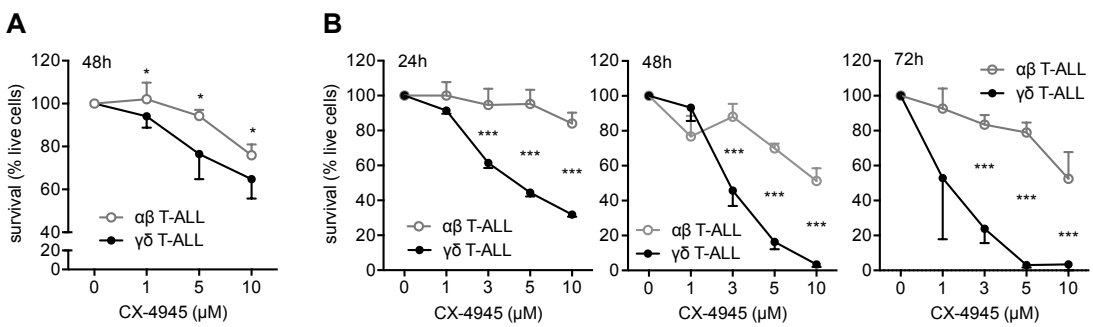

Figure 13: γδ T-ALL cells are more susceptible than αβ T-ALL to apoptosis induced by CX-4945

Flow cytometry analysis of the survival (Annexin-V/ 7-AAD staining) of (**A**) γδ (n=5) and αβ (n=5) T-cell blast samples (obtained from T-ALL patients and expanded in NSG mice) or (**B**) γδ (PEER) or

αβ (MOLT-4) T-ALL cell lines, cultured for the indicated times with increasing concentrations of CX-4945 (T-test, *p<0.05; ***p<0.001).

Finally, the increased sensitivity of γδ T-ALL cells to CX-4945 treatment *in vitro*, led us to explore its therapeutic potential *in vivo*. For this purpose, we established a xenograft model of human γδ T-ALL by injecting $2x10^6$ PEER cells subcutaneously in immune deficient NRGS (NOD-Rag1^{null} IL2Rγ^{null}) mice. After the detection of palpable tumor, mice were equally distributed according to tumor burden into two groups to receive CX-4945 (orally, twice a day) or vehicle control. We observed a striking impact of CX-4945 treatment on tumor growth (**Figure 14A**). Upon sacrificing the mice at day 18 (an ethical requirement for the control group), we scored great reductions in the CX-4945-treated group concerning the tumor weight (**Figure 14B**), as well its dissemination to the blood, bone marrow and spleen (**Figures 14C-E**). Of note, this therapeutic effect was dose-dependent, as it was only observed with 75 mg/kg (**Figures 14A-E**) but not with 25 mg/kg (data not shown) of CX-4945. These data collectively demonstrate the potential of CK2 inhibition for treatment of γδ T-ALL.

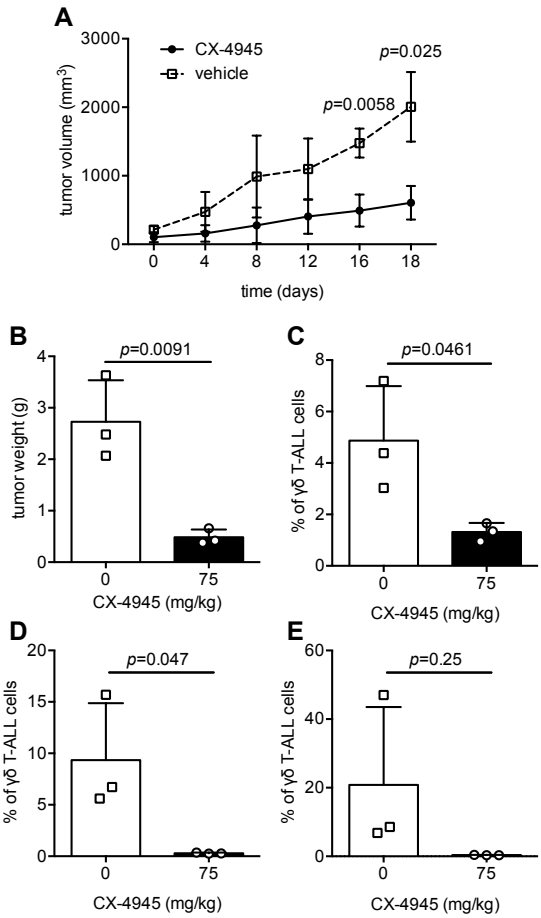

Figure 14: CX-4945 treatment inhibits γδ T-ALL growth *in vivo*

(**A**) Tumor volume following injection of 2x10^6 PEER γδ T-ALL cells subcutaneously into NRGS mice, treated with 75 mg/kg CX-4945 or vehicle (T-test, **P*<0.05, ** *P*<0.01). Day 0 refers to the start of treatment of mice bearing palpable tumors. (**B-E**) Tumor weight (**B**) or percentage of CD45$^+$ CD7$^+$ γδ T-ALL cells in the blood (**C**), bone marrow (**D**) or spleen (**E**) of mice sacrificed after 18 days of treatment. Each dot represents an animal; T-test *p* values are indicated.

Chapter IV

Discussion and conclusions

IV. DISCUSSION and CONCLUSIONS

The key roles played by γδ T cells in immunity to infection and tumors critically depend on their survival, activation and differentiation into effectors capable of secreting cytokines and killing infected or transformed cells. These processes are controlled, at the molecular level, by surface receptors that capture key extracellular cues and convey downstream intracellular signals that regulate both lymphocyte physiology and pathology. In this PhD thesis we evaluated the contribution of cell receptor signaling pathways both in human γδ T cell differentiation and activation. We further assessed the role of those signaling pathways in γδ T cell malignancy, as they may constitute putative therapeutic targets.

In the first section of this chapter we described the physiology and cytokine signatures of human γδ thymocytes as well as the molecular requirements to differentiate these cells into cytotoxic against tumors. In the second section of this chapter we described the signalling pathways required for human γδ thymocyte survival. In the end, we disclosed a hallmark protein involved in γδ T-ALL tumorigenesis that ultimately could be used as a valuable therapeutic target in patients suffering form TCRγδ^{+} T cells malignancies.

Human γδ thymocytes are functionally immature and differentiate into cytotoxic type 1 effector T cells upon IL-2/IL-15 signaling

T lymphocytes develop from bone marrow precursors only after migrating to the thymus where these cells undergo several lineage decisions during a process of differentiation. In the thymic microenvironment a number of functionally distinct types of T cells are generated including γδ T cells, NKT cells, “CD8αα” innate-like cells, regulatory T cells, CD8^{+} cytotoxic T cells, and CD4^{+} T “helper” cells[10]. While some critical processes involved in differentiation of these cells have been

elucidated, the signalling pathways and molecular events involved in functional differentiation following γδ T cell commitment remain to be fully understood.

In mouse models, γδ T cells can complete their functional differentiation in the thymus[48–50,150] and it is currently accepted that TCR signaling shapes the mature γδ thymocyte pool. The functional differentiation into IFN-γ- or IL-17-producing γδ T cells is tightly controlled both by thymic microenvironment factors and intracellular signalling pathways[49,147]. These include the ligands of TCRγδ expressed by thymic epithelial cells (TECs), downstream TCR signalling strength[39,45,150] and cytokines such as TGF-β[363] or IL-7[364].

In contrast, the data presented here show that freshly isolated human γδ thymocytes (obtained from young children subjected to cardiac surgery) are functionally immature, unable to produce IFN-γ, TNF-α or IL-17. Moreover, we show that these cells do not present activation markers nor cytotoxic functions against tumor cells, opposite to what it is observed in circulating γδ PBLs. Given that circulating γδ PBLs from both adult[104,328] and fetal blood[365] display type 1 effector properties and express memory markers[366], human γδ T cells must thus complete their differentiation in the periphery, as reported for αβ T cells[21,22].

In healthy individuals, γδ PBLs are strongly biased towards IFN-γ production (type 1 effectors), and in our study we failed to identify any significant production of type 2 or type 17 cytokines. Particularly IL-17, which is constitutively expressed by a subset of murine γδ T cells[48,330], is rarely expressed (<1%) in human γδ T cells from blood of healthy donors[104,217,330]. By contrast, IL-17-producing γδ T cells appear to accumulate in high numbers in clinical cases of severe bacterial or viral infections[23] and autoimmune disorders[367–371]. More recently, IL-17-producing γδ T cells were reported to be the major player in protumoral inflammation in colorectal cancer where these γδ T cells acquired the ability to secrete IL-8, tumour necrosis factor (TNF) and granulocyte–macrophage colony-stimulating factor (GM-CSF), responsible for recruiting immunosuppressive MDSCs into the malignant microenvironment[89]. The mechanism whereby human γδ T cells

functionally differentiate into IL-17 producers remains largely unknown. Previous studies in mouse models have demonstrated that IL-17A expression is mainly controlled by the ubiquitously expressed transcription factor retinoic acid-related orphan receptor (ROR)γ and its immune cell-specific isoform RORγt, with additional contributions from cytokines (including, IL-6, IL-21, IL-23 and TGF-β) and transcription factors such as STAT3, IFN-regulatory factor-4 (IRF4), Runx transcription factor 1 and basic leucine zipper transcription factor ATF-like (BAFT)[372–375]. Our laboratory recently showed that mouse γδ T cells are strongly dependent on RORγt but surprisingly do not require accessory transcription factors like BATF for IL-17 production[376]. Interestingly, in humans, Rauen *et al*. reported an RORγt isoform, denoted as RORγt-Δ(5-8), which suppresses IL-17A production through direct transcriptional repression at both the *IL17A* and the *IL21* promoter and counteracting RORγt activity on *IL17A* gene transcription[377]. Along these lines, IL-17 expression seems to be silenced in human γδ T cells and to be induced by TCR stimulation in combination with polarizing cytokines, IL-1β, IL-6, TGF-β, and IL-23[48,216].

In our study, the naïve and immature phenotype of human γδ thymocytes provided an ideal system to investigate the molecular cues required for acquisition of the type 1 effector properties that ultimately characterize γδ PBLs. Our work demonstrated that IL-2 or IL-15 signals are sufficient to drive the differentiation of human γδ T cells into IFN-γ/ TNF-α producers endowed with potent cytotoxicity against tumor targets.

The redundant functions of IL-2 and IL-15 can be explained by the structure of their respective receptors, which share not only the γc chain but also their second signaling subunit, the IL-2Rβ. A third subunit, IL-2Rα or IL-15Rα, is cytokine specific and stabilizes binding but apparently lacks signaling activity. Structural comparisons of IL-2-IL2-Rα and IL-15-IL15-Rα interactions have emphasized their

similarities[24,25,378], and it has been recently demonstrated that they induce similar downstream signaling and the same type of transcriptional effects[378,379].

On the other hand, IL-7 clearly failed to trigger differentiation of cytotoxic type 1 γδ T cells. This is in stark contrast with the major role described for IL-7 in the functional differentiation of human NKT cells[380] and IL-17-producing γδ T cells[364]. These lines of evidence establish an interesting cytokine dichotomy for human γδ T cells: whereas IL-7 promotes type 17 effector functions, IL-2 and IL-15 are the main drivers of the type 1 program. Of note, since IL-15 stimulation induces γδ T cells to produce IL-2, this cytokine could also be the autocrine signal and direct mediator of the type 1 program downstream of IL-15 signals.

Unexpectedly, TCR activation was not required for the cytotoxic type 1 differentiation of human γδ thymocytes. Recent work from Hayday and colleagues on murine γδ T cells suggests that innate-like γδ T cell subsets may lack TCR responsiveness in the periphery following strong TCR signaling during thymic development[153]. In the same line of thought, this data supports the model of different signal strengths results into γδ T cell differentiation towards type 1 or type 17 effectors[41,45].

Due to the technical limitations in manipulating human γδ thymocytes, we cannot assess if human γδ thymocytes have already received TCR signals *in vivo*. Were this to be the case, IL-2 and IL-15 would act as terminal differentiation factors in cells that had been previously selected via their TCR. This is also reminiscent of the two-step model proposed by Hsieh and Farrar for murine Foxp3$^+$ regulatory T cell (Treg) development[381,382]. Importantly, whereas for Treg this two-step process is completed in the thymus, human γδ T cell functional differentiation seemingly involves a second step that takes place in the periphery. Physiologically, this likely depends on the provision of IL-2 by activated T cells (either γδ T cells themselves or their αβ counterparts); or IL-15 by myeloid and epithelial cells. Concerning a potential autocrine IL-2 loop based on γδ T cells, we have previously shown that IL-2 production requires TCR activation in the presence of CD28 costimulation[383].

Therefore, CD80/ CD86 expression by APCs, including dendritic cells but also Vγ9Vδ2 T cells, are likely additional players in the functional differentiation of γδ T cells. For therapeutic purposes in cancer immunotherapy, our results strongly suggest that the provision of exogenous IL-2 or IL-15 may be critical, not only for γδ PBL activation and expansion, but also for extensive differentiation of anti-tumor effectors from recent thymic emigrants and circulating naïve γδ T cells.

Downstream of IL-2/ IL-15 receptors, our data provide the important insight that γδ T cell cytotoxic type 1 differentiation can be manipulated by drugs specifically targeting the MAPK/ ERK pathway[339]. In this context, dual-specificity phosphatases (DUSPs) seem particularly promising targets for modulating MAPK-dependent immune processes[339]. Moreover, our recent and unpublished data shows the existence of an additional regulatory layer for functional differentiation of γδ T cells mediated by microRNAs and controlled by IL-2 that orchestrates both ERK signaling and IFN-γ production.

Altogether, our results detach human γδ T cells from previous paradigms of T cell differentiation: their differentiation program is not completed in the thymus, unlike murine γδ T cells[48–50]; and does not require TCR plus coreceptor activation in the periphery, in contrast with naïve $CD4^+$ T cells[334]. Instead, IL-2/ IL-15 signals are sufficient for functional differentiation of human γδ T cells, which clearly aligns them with NK cells[384] and some naïve $CD8^+$ T cell populations[385]. Thus, the three main cytotoxic type 1 lymphocyte subsets share a common, IL-2/ IL-15-dependent, differentiation program with key implications in cancer immunotherapy.

Protein Kinase CK2 controls the survival of normal thymic and leukemic γδ T cells via promotion of AKT signaling

In order to further elucidate the process of normal/ healthy γδ T cell differentiation and activation as well as γδ T cell tumorigenesis it is essential to understand how multiple extracellular signals are integrated within the cell and how we can instructively manipulate them towards improvement of in

immunotherapy; or, alternatively, block γδ T cell survival and proliferation in the cases of leukemic γδ T cells.

The protein kinase CK2 is a ubiquitous serine/threonine, acidophilic protein kinase implicated in several functions including cell growth and proliferation. Since CK2 is a potent suppressor of apoptosis and supports cell survival, its upregulation is a hallmark acquired by many cancer cells.

However, the CK2 functions, in healthy human cells, remain unclear. In mouse models, CK2 is known to be a key factor in embryonic development and CK2α knockout are lethal by embryonic day 11 (E11) and have apparent gross morphological cardiac and neural tube defects[386–389]. The mouse embryos knockout for CK2β die at E6.5; for unknown reasons[390] and the knockout embryos for CK2α' result in infertile male mice, with defective spermatozoa morphogenesis[391,392]. CK2 transcripts and proteins are differentially expressed in animal models, however CK2 subunits are expressed at higher levels in both neuroepithelial and epithelial cells, connective tissue, skeletal muscle, cartilage, brain and testis compared to other cell types, evidences that support the diverse functions and distribution of this kinase[392].

Recent studies in mice have shown that epithelial cells and peripheral T cells depend on CK2 for their survival and function. CK2 is required for the survival of intestinal epithelial cells in inflammatory colitis[393], for $CD4^+$ T cell activation and differentiation into Th2 or Th17 cells[352] and for the suppressive function of $CD4^+$ $Foxp3^+$ regulatory T cells against allergy-promoting Th2 cells[353].

Despite the broad functions in non-transformed and transformed cells, CK2 is not classified as an oncogene and the mechanism of its regulation remained poorly described[354]. CK2 presents constitutive kinase activity and until now, no actual CK2 gain-of-function mutations have ever been found nor associated with cancer[306]. The increased CK2 activity is typically associated with cell transformation in several hematological and solid tumors[307] but none

physiological role has yet been attributed to CK2 in the human thymus or on healthy human T cells[285,354].

In this thesis we identified a major role for CK2 that is restricted to the γδ T cell lineage of human thymocytes. Our data shows an ~2-fold higher CK2 activity in γδ thymocytes (that are mainly of the Vδ1$^+$ subset) and a strikingly increased sensitivity to CK2 inhibition compared to their αβ T cell counterparts, either CD4$^+$ or CD8$^+$ T cells. The reasons that may justify the increased CK2 activity at steady state in γδ T cells remains to be elucidated. Similarly to what was previously described in different cell types[307,394], we did not find any relationship to the gene expression or protein levels of any CK2 subunit that may justify the increased enzymatic activity. On the other hand, we may speculate that the different basal levels of CK2 activity in γδ versus αβ thymocytes are due to stronger TCR signals received during their development. It is well established that strong TCR signaling favors γδ over αβ T-cell lineage commitment[17,36,37,39], and further impacts subsequent γδ thymocyte development[45]. In this line of reasoning, the high CK2 activity in agonist-selected γδ thymocytes could be an important pro-survival mechanism to counteract the activation-induced cell death underlying thymocyte negative selection.

Very few studies demonstrated the manipulation of CK2 activity by extracellular factors. Raman and colleagues identified that CD5 receptor directly binds to CK2, promotes CK2 downstream signaling and IFN-γ and IL-17 production in CD4$^+$ T cells in mouse models[304,352,395]. The same authors showed that mice that express a CD5 protein containing a microdeletion with selective inability to interact with CK2 (CD5ΔCK2BD); and that CD5$^{-/-}$ mice presented diminished levels of IFN-γ$^+$ IL-17$^+$ double producers T cells in the central nervous system and were resistant or less severely affected to EAE, respectively[396]. More recently, Boussiotis and colleagues identified PD-1 as a new receptor with the ability to modulate CK2 activity [186]. The authors showed that TCR plus CD28 stimulation increased CK2 protein levels and kinase activity, whereas PD-1 suppressed CK2 and resulted in

impaired phosphorylation of PTEN and inhibition of AKT signaling pathway in human $CD4^+$ PBLs, while not affecting cell survival. In contrast, we described increased (~2.5 fold) CK2 activity in γδ thymocytes following TCR stimulation resulting in increased AKT-dependent cell survival. Similarly to what we previously described in peripheral Vγ9Vδ2 T cells, CD27 represents a key costimulatory factor that, together with TCR/CD3, promotes AKT signaling, cell survival and proliferation of γδ thymocytes. We also observed increased levels of CD27 at the cell surface following TCR activation that may serve as a positive feedback mechanism to potentiate TCR/CD27 downstream signaling pathways. These evidences are in agreement with what our group recently showed for Vδ1 T cells from the peripheral blood: CD27 was upregulated and remained stable on the cell surface during Vδ1 T cell expansion and activation *in vitro* and *in vivo*[88].

Various costimulatory receptor domains have been tested in chimeric antigen receptor (CAR) in primary human T cells that give us insightful information about their costimulatory functions. These constructs includes CD28, 41BB, ICOS, OX40 or CD27 associated in tandem with TCR CD3ζ intracellular domain (ICD)[397]. Interestingly, CD27 boosted human CAR T cell survival and resistance to antigen-induced apoptosis, increasing TH1 cytokine secretion (IFN-γ, TNF-α and IL-2) and cytotoxicity against antigen-expressing cancer cells *in vitro* and *in vivo*, compared with CAR T cells with CD3ζ alone[397,398]. Rosenberg *et al.* identified that in adoptive T cell therapy, the $CD27^+$ tumor antigen-specific T cells persist long-term in patients responding to therapy and are remarkably stable in contrast to $CD27^-$ or $CD28^+$ CAR T cells[399]. Collectively, together with TCR, CD27 is a costimulatory receptor involved in CK2-AKT-mediated survival.

AKT phosphorylates the serine/threonine kinase glycogen synthase kinase-3β (GSK3β) at N-terminal regulatory serine residue (S9) inhibiting its activity and promotes downstream D-type cyclins expression (data not showed) that supports cell cycle progression[356,400–403]. Following TCR/CD27 activation we verified an

increased AKT/ mammalian target of rapamycin (mTOR) axis, measured by S6 and 4EBP1 phosphorylation. It is well described that several cellular functions of AKT are mediated by mTOR, which is considered the master controller of protein synthesis and cell proliferation[404]. Activated AKT can phosphorylate and inactivate tuberous sclerosis complex 2 (TSC2), which negatively regulates mTOR[405]. mTOR interacts with either raptor or rictor to form mTOR complex I (mTORC1) or mTOR complex 2 (mTORC2), respectively[406]. While mTORC1 activates 40S ribosomal protein S6 kinase (S6K) and subsequently phosphorylates S6, mTORC2 can phosphorylate AKT, at Ser473 residue, accounting for a feedback loop mechanism[404–407]. In order to further evaluate the involvement of CK2 in TCR/CD27 downstream signaling pathway, we specifically blocked CK2 activity using the clinical grade inhibitor, CX-4945 and we identified an impairment of both basal and TCR/CD27-induced CK2-AKT signaling pathway and associated γδ T cell survival and proliferation.

Protein phosphorylation is integrated in a complex system in all cells and has long been recognized as an important post-translational modification regulating cellular processes. Emphasizing the importance of this modification is the existence of 518 distinct protein kinases in the genome, and the estimate that one third of cellular proteins are phosphorylated, often at several distinct sites. Proper regulation of phosphorylation events is crucial to the proper function of cellular signaling pathways, and loss of regulation in these pathways underlies many human diseases, including cancer. Consequently, the enzymes that regulate protein phosphorylation in cells, namely protein kinases and phosphatases, have emerged as promising therapeutic targets[293].

In this thesis, we describe that protein kinase CK2 functions are increased in healthy γδ thymocytes, compared to αβ T cell counterparts. Increased CK2 activity is usually an acquired feature of malignant cells. Consistent with our prior

observations that TCRαβ$^+$ primary T-ALL cells display higher levels of CK2 expression and activity as compared to the immunophenotypically equivalent normal αβ thymocytes[283], we now suggest that CK2 can be a putative therapeutic target in TCRγδ$^+$ malignant cells.

Interestingly, we demonstrate that the differential CK2 activity between the γδ and αβ T cell lineages extends from healthy thymocytes to transformed T-ALL cells. Thus, primary γδ T-ALL cells from human patients displayed >2-fold higher CK2 activity compared to αβ T-ALL counterparts. Since we previously showed that endogenous CK2 activity correlates with increased susceptibility to apoptosis upon CK2 inhibition[283,321,350], this differential activity likely explains the higher sensitivity of γδ T-ALL cells observed in this study. Of note, all γδ T-ALL samples analyzed, as well as the PEER cell line, are of the Vδ1$^+$ subset, similarly to the majority of γδ T cells in the thymus; this is therefore consistent with γδ T-ALL initiating in the thymus.

Albeit rare, γδ T-ALL accounts for up to 10% of T-ALL cases, which is significantly higher than the proportion (1%) of γδ thymocytes in the healthy thymus. This raises the possibility that γδ thymocytes have increased potential for malignant transformation[343,344,408]. A possible contributor to this phenomenon could be CD27 costimulation, since it increases CK2 activity (synergistically with TCR stimulation) in γδ T-ALL cells expressing high levels of CD27.

The ability of CK2 to impact on AKT signaling was previously reported in T-ALL[283,321,350]. Here we showed, for the first time, that a CK2-AKT link exists in γδ thymocytes and γδ T-ALL cells. More importantly, our studies demonstrated that AKT is essential for CK2-mediated effects: i) chemical AKT inhibition (with MK-2206) mimicked CK2 inhibition (with CX-4945); ii) the latter extinguished AKT signaling (AKT phosphorylation and downstream effects); and iii) ectopic expression of a constitutively active form of AKT partially rescued the apoptosis due to CK2 inhibition. These results suggest that, although AKT activation is not

sufficient to fully mimic CK2 activity, it is absolutely required for CK2-mediated effects in γδ T cells. Activated AKT promotes cell survival through direct phosphorylation of anti-apoptotic molecules, or indirectly through the transcriptional activation of anti-apoptotic genes and increased metabolic capacity as previously discussed[409,410]. In addition to DiMaria and colleagues' data shows that the maximal AKT activation requires phosphorylation at Ser129 by CK2 both *in vitro* and *in vivo*[411,412], we described that the inhibition of CK2 activity in γδ thymocytes or γδ T-ALL cells abrogated the AKT/ GSK3β and AKT/ mTOR signaling pathway and had a major impact on cell survival and proliferation independently of mitogen-activated protein kinases/ extracellular signal-regulated kinases (MAPK/ ERK) (data not shown). Interestingly, the strong dependence on AKT may be specific for human γδ T-cells, since AKT-deficient mice were reported to have a normal γδ T-cell pool in the periphery[413]. The phosphatase PTEN is inhibited by phosphorylation and its protein levels are stabilized, in agreement to the previous reports[354,414,415], following TCR/CD27 stimulation. However we saw a similar effect in cell survival in both $PTEN^{+}$ or $PTEN^{null}$ γδ T-ALL samples following CX-4945 treatment both *in vitro* and *in vivo* (data not shown).

Moreover, our data shows a dose-dependent effect of CX-4945 *in vitro* and *in vivo*, both in tumor primary site and in blood, bone marrow and spleen metastasis. Overall, our observation of the high sensitivity γδ T-ALL cells to CK2 inhibition (with CX-4945) *in vitro* and *in vivo* clearly supports its use as a putative therapy for γδ T-ALL. Importantly, CX-4945 is safety approved in humans and it is currently in phase II clinical trials in patients with multiple myeloma or advanced solid cancers[288].

It appears that elevated levels of CK2 activity alone are not indicative of deregulation since it is intrinsically present in certain cell types as we describe here in healthy γδ thymocytes compared to αβ thymocytes. However, stable levels of CK2 appear to be critical to cell homeostasis. Indeed, we show that γδ T-ALL

present elevated CK2 kinase activity and that deregulation of CK2 is likely to be related to the severity of disease and can even be used as a prognostic indicator since it is implicated in tumor growth and severity[416,417]. The diagnosis and treatment of T-ALL remains a challenge and further characterization of subtypes of T-ALL, such as TCRγδ$^+$ T-ALL, could reframe our ability to further characterize this disease and categorize these patients and respective treatments. Since CK2 affects several signaling pathways in γδ T-ALL, CX-4945 treatment may be a valuable therapeutic approach that could replace the current therapies that target downstream pathways, including inhibitors of PI3K[418], AKT[419,420], mTOR[421], dual PI3K-mTOR inhibitors[422], and BCL2 inhibitors[261,423,424].

Finally, our study has implications, not only for hematology, but also for cancer immunotherapy, since γδ T cells are known to play important roles in anti-tumor responses[72]. The success of their clinical application, particularly in adoptive cell therapy, will strongly depend on the capacity to survive *ex vivo* TCR-mediated activation and *in vivo* establishment and expansion. Thus, we believe that a better knowledge of the molecular determinants of γδ T-cell survival, as described in this thesis, for CK2, will be critical to optimize their performance in cancer immunotherapy.

Chapter V

Future perspectives

V. FUTURE PERSPECTIVES

The laboratories involved in this thesis, the Silva-Santos and Barata groups, have performed various studies with hematological tumors that provided seminal data on (i) altered mechanisms acquired by malignant T cells during tumor initiation, progression and response to treatment; and (ii) the potential of using γδ T lymphocytes to target aggressive leukemias.

Mechanistically, the Barata lab dissected the major signaling pathways responsible for leukemia development. IL-7 mediates abnormal PI3K/AKT/mTOR signaling pathway as result of mutations, post-translation modifications, cell-intrinsic aberrations or microenvironmental factors that support irregular cell survival, proliferation and tumor development and progression. The characterization of the signaling pathways involved in T-ALL allows us to identify new molecular targets to be used as a personalized therapeutic approach such as PI3K-, γ-secretase-, AKT-, mTOR- or CK2-inhibitors that could be combined to the standard therapies. Thus we strongly believe that the improved characterization of the malignant cells including their TCR, CK2, PI3K, AKT and mTOR analysis, will help us to define more effective treatments against T-ALL.

On the other hand, the Silva-Santos group established a major role for activating NKRs in tumor cell recognition by human γδ T cells. This was observed for both Vγ9Vδ2^{+} and Vδ1^{+} T-cell subsets, in which NKG2D and NCRs (NKp30 and NKp44) mediated leukemia cell recognition. We hope to build on this knowledge to devise more effective cancer immunotherapies, which will overcome the limitations of previous γδ T cell-based clinical trials; that is the underlying principle of the novel Vδ1^{+} T cell (DOT) technology developed in our lab. DOT cells employ NKp30 and NKp44, besides the signature TCR, to recognize tumor cells (**Figure 15**). These NCRs are a primate acquisition, as they are not expressed in mice, and even selectively among non-human primates (macaques lack NKp44, for instance). The

two NCRs are located in distinct human chromosomes and share no homology. They are therefore two complementary molecular tools to induce cytotoxicity in lymphocytes, which DOT cells employ effectively to target tumor cells. Of note, a very attractive property of Vδ1⁺ T cells (and γδ T cells as a whole) aiming at allogeneic cellular therapies is their lack of MHC-restriction, which dictates their non-involvement in graft versus host disease.

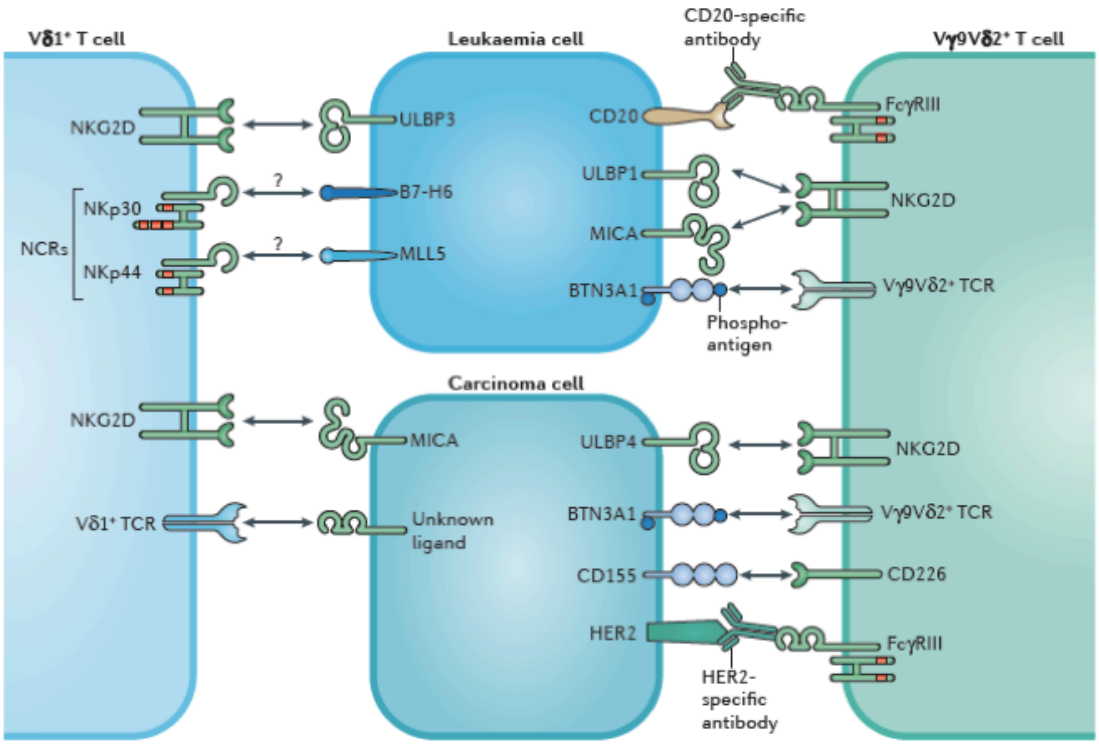

Figure 15: TCR and NKR receptor-ligand interactions mediating tumor cell recognition by human γδ T cells.

While the ligands recognized by Natural Cytotoxicity Receptors (NCR) in this context have not been formally characterized, other systems suggest that B7 homologue 6 (B7-H6) could be the putative ligand of NKp30 and Mixed Lineage Leukemia-5 protein (MLL5) that of NKp44. Tumour specificity for the signaling through the FcγRIII (CD16) is dictated by anti-tumour mabs used in combination with the Vγ9Vδ2 T cell therapy; mAb 1: e.g. Rituximab with CD20 as TAA 1, mAb2: e.g. Trastuzumab with HER2 as TAA 2. BTN3A1: butyrophilin-3A1, an isoform of CD277; MICA: MHC class I polypeptide-related sequence A; ULBPs: UL Binding Proteins; DNAM-1: DNAX accessory molecule-1 (CD226) (From Silva-Santos et al. *Nat Rev Immunol* 2015, 15: 683-91).

A critical issue to investigate in the future is the relevant ligands expressed on the tumor cell surface to engage the TCR and the NCRs (on DOT cells). Our current conceptualization postulates that they will be stress/ transformation-induced molecules, absent (or very low) in healthy cells but strongly upregulated in their malignant counterparts. That is the case for B7-H6, the known ligand for NKp30. B7-H6 transcripts have not been detected in most normal adult tissues, consistent with the absence of the protein on circulating cells, isolated from healthy individuals. In contrast, B7-H6 surface expression is observed in a restricted panel of tumor cell lines from various origins including lymphoma, leukemia, melanoma, and carcinoma as well as on primary tumor blood cells. Following on from this, it will be important to establish how much tumors downregulate their expression throughout progression, as potential immune evasion mechanisms. This is particularly relevant since NK cells constitutively express NCRs, which may confer immune surveillance but subsequently provoke immunoediting. Notwithstanding, we believe that the non-redundant contributions of the TCR and NCRs will reduce the possibilities of complete evasion of DOT-cell surveillance, which will be the key to guarantee durable responses and the prevention of disease relapses.

Building on these considerations, our current working model includes two stages of T-cell activation/ differentiation and tumor cell recognition. First, γδ T cells are potently activated by TCRγδ and costimulatory ligands in the presence of IL-2 or IL-15. This endows them with potent cytolytic (and cytokine-secreting) function, but requires a subsequent phase of target identification, namely for discrimination between tumor and healthy cells. We propose this is mainly determined by activating NKRs that bind stress-inducible proteins that selectively accumulate on the surface of tumor cells. The NKRs can either be constitutively expressed (like NKG2D) or induced upon activation (like NCRs). We believe the integration of γδ T cell activation with tumor cell recognition, as we demonstrated for DOT cells, will

be the key for success of γδ T cell-based protocols in the clinic. In addition, we are highly encouraged by recent data, published by Gentles and colleagues, ranking γδ T cells as the number 1 most favorable immune prognostic population in tumor biopsies derived from 25 types of cancer[79].

As we continue our quest to manipulate γδ T cells for cancer immunotherapy, various major questions remain unanswered. For instance, it will be very important to decipher the full repertoire of tumor antigens involved in γδ T cell recognition, including the ligands of TCRs or NKRs, and to find additional determinants of tumor cell killing. γδ T cells express a very diverse panel of inhibitory and activating receptors that directly impact on their activation state and function. However, we still lack a dynamic picture of the receptors elicited along tumor-induced γδ T cell activation, and a deep understanding of the interplay between the numerous signaling cascades induced upon sequential or concomitant receptor engagement.

We also believe that γδ T cell-based therapies can be further improved following specific and personalized *ex vivo* protocols for γδ T cell expansion and activation. In this context, the combination of therapeutic drugs that may improve γδ T cell survival, proliferation, homing and cytotoxicity *in vivo* remain to be elucidated but can be highly valuable for future immunotherapy approaches.

Chapter VI

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VI. REFERENCES

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Chapter VII

Additional files

VII. ADDITIONAL FILES

Peer-reviewed articles associated to this thesis:

Sérgio T Ribeiro; Julie C Ribot; Bruno Silva-Santos. Five Layers of Receptor Signaling in γδ T-Cell Differentiation and Activation. *Frontiers in Immunology*. 6: 1-9. 2015 doi: 10.3389/fimmu.2015.00015

Julie C Ribot; Sérgio T Ribeiro; Daniel V Correia; Ana E Sousa; Bruno Silva-Santos; Human gamma-delta Thymocytes are functionally immature and differentiate into cytotoxic type 1 effector T cells upon IL-2/IL-15 signaling. *Journal of Immunology*. 192(5):2237-43. 2014 doi: 10.4049/jimmunol.1303119

Sérgio T Ribeiro; Melania Tesio; Julie C Ribot; Elizabeth Macintyre; João T Barata; Bruno Silva-Santos. Casein Kinase 2 controls the survival of normal thymic and leukemic γδ T-cells via promotion of AKT signaling. *Leukemia*. 1-8. 2016 doi: 10.1038/leu.2016.363.

frontiers in IMMUNOLOGY

REVIEW ARTICLE
published: 26 January 2015
doi: 10.3389/fimmu.2015.00015

Five layers of receptor signaling in γδ T-cell differentiation and activation

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The contributions of γδ T-cells to immunity to infection or tumors critically depend on their activation and differentiation into effectors capable of secreting cytokines and killing infected or transformed cells. These processes are molecularly controlled by surface receptors that capture key extracellular cues and convey downstream intracellular signals that regulate γδ T-cell physiology. The understanding of how environmental signals are integrated by γδ T-cells is critical for their manipulation in clinical settings. Here, we discuss how different classes of surface receptors impact on human and murine γδ T-cell differentiation, activation, and expansion. In particular, we review the role of five receptor types: the T-cell receptor (TCR), costimulatory receptors, cytokine receptors, NK receptors, and inhibitory receptors. Some of the key players are the costimulatory receptors CD27 and CD28, which differentially impact on pro-inflammatory subsets of γδ T-cells; the cytokine receptors IL-2R, IL-7R, and IL-15R, which drive functional differentiation and expansion of γδ T-cells; the NK receptor NKG2D and its contribution to γδ T-cell cytotoxicity; and the inhibitory receptors PD-1 and BTLA that control γδ T-cell homeostasis. We discuss these and other receptors in the context of a five-step model of receptor signaling in γδ T-cell differentiation and activation, and discuss its implications for the manipulation of γδ T-cells in immunotherapy.

Keywords: γδ T-cells, T-cell receptor, T-cell costimulation, cytokines, natural killer receptors

INTRODUCTION

γδ cells endow the T-cell compartment with a rapid, innate-like reaction to insults, which places them in the afferent phase of the immune response. Namely, γδ T-cells are responsible for "lymphoid stress surveillance," i.e., sensing and responding immediately to infections or non-microbial stress without the need of clonal expansion or *de novo* differentiation, in synchrony with prototypic innate immune responses (1). Critically, this implicates γδ T-cells in inflammation (2), autoimmunity (3), infectious diseases (4, 5), and tumor surveillance (6–8).

Many of the studies elucidating the physiological roles of γδ T-cells have been performed in murine models, where a major breakthrough has been the identification of pro-inflammatory subsets naturally producing either IFNγ or IL-17 (9–11). Moreover, these studies have been greatly facilitated by the identification of cell surface markers that segregate the two functional γδ T-cell subsets: CD27, CD122, and NK1.1 mark IFNγ-producing γδ cells, whereas their IL-17-expressing counterparts display a $CD27^-$ $CCR6^+$ phenotype (9–11). Moreover, the two subsets show distinct Vγ chain usage in their TCR repertoires, with a bias toward Vγ1 among IFNγ-producing γδ cells, and an enrichment in Vγ4 and Vγ6 in IL-17-producing γδ cells (12).

In humans, γδ T-cells are primarily identified by their Vδ chain usage, with $Vδ1^+$ cells predominating in the thymus and in peripheral tissues, while $Vδ2^+$ cells (mostly co-expressing a Vγ9 chain) constitute the majority of blood-circulating γδ T-cells. Both human γδ T-cell subsets are highly prone to secrete IFNγ, but IL-17 can be induced in highly inflammatory conditions triggered by infections (13) or tumors (14, 15).

In both murine and human γδ T-cells, functional responses are initiated upon recognition of antigens that are likely induced by stress signals and sensed by either T-cell or natural killer receptors. Some γδ T-cell populations are also particularly responsive to cytokines or innate toll-like receptor (TLR) agonists (16, 17). Following proliferation and effector responses, the return to homeostasis is controlled by inhibitory receptors. Here, we discuss the various layers of contributions of T (TCR and costimulatory/inhibitory receptors), NK, and cytokine receptors to the activation and differentiation of effector γδ T-cell populations in mice and humans.

SIGNAL 1: T-CELL RECEPTOR

The γδTCR complex is composed by the γδTCR itself and various CD3 chains following the stoichiometry: $TCRγδCD3ε_2γδζ_2$ in humans and $TCRγδCD3ε_2γ_2ζ_2$ in mice (18). The assembly of a γδTCR complex in thymic progenitors has immediate consequences for γδ T-cell development. The "strong" signals stemming from the γδTCR (when compared to the "weaker" pre-TCR signaling) drive γδ/αβ common precursors into the γδ lineage (19, 20). These "stronger" γδTCR signals associate with increased phosphorylation of ERK1/2, abundant calcium release and induction of early growth response (Egr) transcription factors (21, 22).

The TCR complex does not present intrinsic kinase activity but the intracellular signaling is initiated after phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) in the

CD3 cytoplasmic domains by the Src-family kinases (SFKs) Lck and Fyn (23). The recruitment of these SFKs to the TCR complex in γδ T-cells remains obscure since these cells do not express the CD4 or CD8 co-receptors, which have been shown, in αβ T-cells, to be responsible for recruiting SFKs upon αβTCR ligation (23). Nonetheless, the importance of SFKs in γδ T-cells is underscored by the substantial phosphorylation of ERK upon inhibition of Csk, a potent inhibitor of SFKs (24).

SFK-mediated phosphorylation of the ITAMs on CD3 chains allows the recruitment, phospholylation, and activation of Zap70 that facilitates phosphorylation of the scaffolding proteins SLP-76 and LAT. This lead to the formation of a supramolecular signalosome that recruits the phospholipase PLCγ1, resulting in propagation of downstream signaling events (22). Here again, γδ T-cell signaling is different from αβ T-cells, since mutations on the binding site of PLCγ1 on LAT resulted in a severe block in murine αβ thymocyte development while γδ T-cell numbers were only modestly reduced in the thymus, intestine, and liver, and remained normal in the skin. Unexpectedly, a population of γδ T-cells in the secondary lymphoid organs in these mice underwent uncontrolled expansion and caused autoimmune pathology, suggesting distinct functions for LAT/PLCγ1-mediated signaling in subpopulations of γδ T-cells (21, 25).

In humans, the major γδ T-cell subset in the peripheral blood, Vγ9Vδ2 T-cells, are uniquely and specifically reactive to self- and foreign non-peptidic phosphorylated intermediates of isoprenoid synthesis – "phosphoantigens" or "phosphoagonists" (P-Ags) (26–28). These P-Ags were shown to trigger bona fide Vγ9Vδ2 TCR signaling in various studies. Cipriani and colleagues showed that the activation of Vγ9Vδ2 T-cells with the P-Ag isopentenyl pyrophosphate (IPP), induced rapid and persistent PKC-dependent phosphorylation of ERK1/2, p38 MAPK, and JNK, resulting in NF-κB and AP-1 activation as well as the release of MIP-1α, MIP-1β, IFN-γ, and TNF-α (29). Moreover, P-Ag stimulation and CD3-crosslinking produced identical phosphorylation of the signaling proteins Zap70, PI3K, LAT, ERK1/2, and p38 MAPK (30, 31); and induced highly sustained calcium signaling in Vγ9Vδ2 T-cells (32). Importantly, activation by P-Ags is the basis of current cancer immunotherapy strategies involving Vγ9Vδ2 T-cells (33).

Recent work has produced some puzzling results on the role of the γδTCR in the development of effector subsets of murine γδ T-cells (34–36), namely, $CD27^+$ $CD122^+$ γδ T-cells producing IFN-γ or $CD27^-$ $CCR6^+$ γδ T-cells making IL-17 (9, 10). First, Chien and co-workers showed that T10/T22-specific γδ T-cells required thymic expression of their TCR ligand to differentiate into IFN-γ producers, in contrast with "ligand naïve" IL-17 producers (9). Consistent with this, TCR-dependent thymic selection was also shown to set the functional potential of dendritic epidermal T-cells (DETC) progenitors away from IL-17 production (37). Furthermore, peripheral IL-17-producing $CD27^-$ $CCR6^+$ γδ T-cells were shown to expand and produce IL-17 independently of TCR activation (38). However, a subsequent study by Chien and collaborators demonstrated that a subset of phycoerythrin (PE)-specific γδ T-cells produced IL-17 specifically upon TCR ligation (39). Moreover, a recent study by Hayday and colleagues suggested that an impairment in Zap70 signaling (in SKG mice) mostly affected the development of $IL\text{-}17^+$ rather than $IFN\text{-}\gamma^+$ γδ T-cells (40). The authors further proposed that "innate-like" γδ T-cell populations, including IL-17 producers and some subsets of IFN-γ producers, receive strong TCR signals during thymic development to become hyporesponsive to TCR stimulation in the periphery (40). Future research should aim to resolve the apparent contradictions of the available data, namely, by clarifying the requirement on TCR ligand engagement, as well as the developmental effects of manipulating distinct γδTCR signaling pathways and their downstream (transcriptional and post-transcriptional) mechanisms on γδ T-cell subsets.

SIGNAL 2: COSTIMULATORY RECEPTORS

A series of T-cell costimulatory receptors are known to induce qualitative and quantitative changes that lower activation thresholds, prevent "anergy" and enhance T-cell functions. Typical costimulatory receptors are type I transmembrane proteins that can be divided into two groups, based on their structural characteristics: immunoglobulin (Ig) or tumor necrosis factor receptor (TNFR) superfamilies. Ig superfamily members have a variable Ig-like extracellular domain and a short cytoplasmic tail, whereas TNFR family members present extracellular domains rich in six cysteine repeats (which form disulfide bridges) and a more complex cytoplasmic tail [reviewed in Ref. (41)]. These two main types of costimulatory receptors display different modes of intracellular signaling: whereas the CD28 family members associate directly with protein kinases (like PI3K or ITK), TNFR superfamily co-receptors require the adaptor proteins TRAF (TNFR-associated factor), namely TRAF2 and TRAF5, to link to downstream signaling mediators (**Table 1**). Here, based on their specific roles in γδ T-cells, we shall discuss CD28 (of the Ig superfamily) and the TNFR superfamily members, CD27, CD30, and CD137 (4-1BB).

The best studied costimulatory receptor, CD28, has historically yielded paradoxical results on γδ T-cells (46). We have recently readdressed this issue for both human and mouse γδ T-cells. We described that CD28 is constitutively expressed on lymphoid γδ T-cells and promotes survival and proliferation via IL-2 production. CD28 receptor agonists enhanced γδ T-cell expansion, which was conversely inhibited by blocking antibodies against its B7 ligands (42). Importantly, CD28-deficient mice displayed lower (relative to controls) numbers of total or activated γδ T-cells upon *Plasmodium berghei* infection, and failed to expand both their $IFN\text{-}\gamma^+$ and $IL\text{-}17^+$ subsets (42). In contrast, Hayes and colleagues reported that both functional γδ T-cell subsets differentiated and expanded normally in a *Listeria* model (80). It would be interesting to determine how variable is the dependence on CD28 costimulation for γδ T-cell responses to distinct infectious agents.

In naïve mice, while CD28 is not required for the development of either $IFN\text{-}\gamma^+$ or $IL\text{-}17^+$ γδ T-cell subsets (80), the TNFR superfamily member CD27 is selectively implicated in the generation of $IFN\text{-}\gamma^+$ γδ T-cells (10). In fact, we showed that CD27 expression segregates $IFN\text{-}\gamma^+$ ($CD27^+$) and $IL\text{-}17^+$ ($CD27^-$) γδ T-cells. Most interestingly, these phenotypes are established in the thymus, and since embryonic stages. Based on the results from our (10) and Chien's (9) teams, the development of IFN-γ-producing γδ T-cells seemingly requires strong TCR signaling and CD27 costimulation in the thymus.

Table 1 | Co-receptors of γδ T-cells – extracellular ligands and intracellular signaling pathways.

Receptor	Ligands	Intracellular signaling initiators/adaptors	Downstream signaling pathway	Target molecules	Reference
CD28	B7.1 (CD80) B7.2 (CD86)	PI3K ITK Grb2	PI3K/AKT Grb2/MEK/ERK	IL-2, NF-κB, AP-1, Bcl-x_L, NFAT	(42–45)
CD27	CD70	TRAF2 TRAF5 Siva	IKK/NF-κB JNK	NF-kB, Ca^{2+}, *cyclinD2*, *Bcl2a1*, Bcl-x_L	(46–49)
CD30	CD30L	TRAF2 TRAF5	TRAF/IKK/IkB Ca^{2+}	NF-κB, IL-4, IFNγ, IL-8, CC chemokines	(46, 50, 51)
4-1BB (CD137)	CD137L	TRAF2		NF-κB, IFNγ	(52–54)
IL-2R IL-15R	IL-2 IL-15	Jak1 Jak3	PI3K/AKT Jak/STAT4/STAT5 MEK/ERK STAT1	IFNγ, TNF-α, T-bet, eomesodermin	(55–58)
IL-7R	IL-7	Jak1 Jak3	STAT3	IL-17, SOCS3	(59)
IL-21R	IL-21	Jak1 Jak3	STAT3	CXCL13, CXCR5	(60)
NKG2D	MIC(A–B) ULBP (1–6) H60 MULT1 RAE1	DAP10	PI3K/AKT Grb2/VAV1/SOS1 PKCθ/Ca^{2+}	NF-κB, RelB, Bcl-x_L, Bcl-2	(32, 46, 61–63)
NKp30	B7-H6 BAT3	CD3ζ	cAMP/PKA	CC chemokines: CCL3, CCL4, CCL5	(64–67)
NKp44	NKp44L	DAP12	Zap70/Syk		(64, 68–70)
DNAM-1 (CD226)	Nectin-like-5 Nectin-2	PKC LFA-1 Fyn	SLP-76/VAV1/ERK		(71, 72)
PD-1	PD-L1 (B7-H1) PD-L2 (B7-DC)	SHP-1 SHP-2	CK2/PTEN/PI3K/AKT MEK/ERK	GSK-3, Bcl-x_L Smad3, Cdc25A, IFNγ, IL-2	(73–76)
BTLA	HVEM	SHP-1 SHP-2	Zap70/ERK	IL-17, TNF, IL-2	(77–79)

Beyond its role in thymic differentiation, CD27 is critical for the expansion of peripheral IFN-γ-producing γδ T-cells upon infection with herpes viruses or malaria parasites in mice (81). We showed that, in the context of TCR stimulation and upon ligation to CD70, CD27 signaling activates the non-canonical NF-κB pathway and enhances the expression of anti-apoptotic and cell cycle-related genes, thus promoting murine γδ T-cell survival and proliferation (81).

We have also addressed the impact of CD27 costimulation on the activation of human γδ T-cells. Administration of soluble recombinant CD70 enhanced, whereas anti-CD27 (or anti-CD70) antibodies reduced, Vγ9Vδ2 T-cell expansion *in vitro* (82). Moreover, CD27 signals induced calcium fluxes and upregulated the expression of *Cyclin D2* and the anti-apoptotic gene *Bcl2a1*. Given the typical IFN-γ secretion and cytotoxicity of activated Vγ9Vδ2 T-cells (30), our work suggests that the modulation of CD70–CD27 signals may be beneficial in the context of γδ T-cell-based cancer immunotherapy.

Upon activation, human γδ T-cells can also express another TNFR superfamily member, CD30 (83). CD30 signaling, which potentiated calcium fluxes induced by TCR activation, also enhanced pro-inflammatory cytokine production (50). Recently, Yoshikai and colleagues compared γδ T-cell homeostasis and response to *Listeria monocytogenes* in CD30-sufficient versus

deficient mice. They demonstrated a selective depletion of IL-17-producing Vγ6$^+$ T-cells in mucosal tissues in the steady-state and upon infection (84). This associated with reduced bacterial clearance, which could be rescued, alongside the IL-17$^+$ Vγ6$^+$ T-cell pool, by agonistic anti-CD30 antibody administration. In contrast, Lee et al. reported that agonistic anti-CD137 (4-1BB) antibodies promoted the expansion of IFN-γ$^+$ Vγ1$^+$ T-cells, which protected (in an IFN-γ-dependent manner) also from *Listeria* infection (52). This study also showed that 4-1BB was expressed and functional on activated human γδ T-cells, and its ligation upon cell transfer protected NOD/SCID mice against *Listeria* infection.

Interestingly, activated Vγ9Vδ2 T-cells also express high levels of 4-1BBL (CD137L) (85), which besides acting as a ligand for 4-1BB on T and NK-cells, may also participate in Vγ9Vδ2 T-cell activation due to its known reverse signaling ability (86). This may, in fact, also apply to CD70 (CD27-ligand), which is highly induced upon phosphoantigen-mediated stimulation of Vγ9Vδ2 T-cells (82, 87). These possibilities deserve further investigation.

SIGNAL 3: CYTOKINE RECEPTORS

Interleukins are key determinants of T-cell survival, proliferation, and differentiation. IL-7, IL-15, and IL-2 are essential for lymphocyte development and homeostasis; upon inflammation, other cytokines, namely, IL-1β, IL-12, IL-18, IL-21, and IL-23, take a central role in determining T-cell functions. Here, we review the main contributions of homeostatic and inflammatory cytokines specifically to γδ T-cell physiology.

IL-7 and IL-15 are seemingly the key determinants of murine γδ T-cell development (88–90) and homeostasis (91). A recent study that depleted IL-7 specifically from (Foxn1$^+$) thymic epithelial cells showed that γδ T-cells were significantly reduced in the adult thymus and in the gut, whereas they were completely absent in the fetal thymus and epidermis (89). In the dermis, it was also IL-7, but not IL-15, that supported the development and survival of the resident γδ T-cell population (92). Conversely, in the gut, IL-15 seems to play the primordial role in sustaining the local intraepithelial γδ T-cell compartment (93).

Unexpectedly, IL-7 was recently reported to promote the selective expansion of murine IL-17-producing γδ T-cells (59). STAT3-dependent IL-7 signals allowed CD27$^-$ γδ T-cells to resist activation-induced cell death (AICD) and undergo proliferative responses to TCR agonists. Such an IL-7/IL-17 axis was also reported to be required for the γδ T-cell response to viral hepatitis infection *in vivo* (94). Moreover, IL-7 also seems to support the expansion of human IL-17-producing γδ T-cells (59).

We recently assessed the functional differentiation of human γδ thymocytes, which are >80% of the Vδ1 subtype. We observed that IL-15 and IL-2, but not IL-7, induced the cytotoxic type 1 (IFN-γ-producing) program in functionally immature γδ thymocytes (55). This was consistent with previous data on peripheral γδ T-cells isolated from cancer patients (95). However, additional reports on peripheral Vγ9Vδ2 T-cell cultures showed that IL-15 or IL-2 stimulation, despite efficient ERK and AKT activation, were not sufficient to induce effector responses; these required phosphoantigen-dependent TCR activation and downstream calcium mobilization (56, 96). Unexpectedly, in our cultures of γδ (mostly Vδ1) thymocytes, TCR stimulation was not required for neither ERK activation nor T-bet and eomesodermin induction and the acquisition of effector functions (55).

IL-2 and IL-15 play key roles in the peripheral expansion of Vγ9Vδ2 T-cells in response to microbial phosphoantigens or synthetic drugs like bisphosphonates (56, 97). This notwithstanding, it is important to note, toward the therapeutic application of Vγ9Vδ2 T-cells, that optimal effector responses seemingly require the combination of these cytokines with TCR agonists. Thus, recent work from Chen and colleagues demonstrated that the differentiation of cytotoxic type 1 Vγ9Vδ2 T-cells capable of controlling *Mycobacterium tuberculosis* infection in macaques required a phosphoantigen/IL-2 combination (98).

Effector γδ T-cell differentiation is also greatly impacted by inflammatory cytokines, particularly IL-12 and IL-18 that typically promote IFN-γ production; and IL-1β and IL-23 that mostly drive IL-17 production.

High expression of IL-12Rβ expression on activated murine γδ T-cells guarantees a dominance of type 1 (IFN-γ$^+$) over type 2 (IL-4$^+$) effector fates (99). Type 1 differentiation is also predominant in human γδ T-cells, and can be further enhanced by IL-18 (100, 101) or IL-21 (102). The induction of a type 17 program in human γδ T-cells requires persistent stimulation with IL-23 for neonatal Vγ9Vδ2 T-cells (15); and IL-23 and IL-1β in the presence of TGF-β for adult Vγ9Vδ2 T-cells (13, 103). In mice, IL-1β and IL-23 are also the main drivers of abundant IL-17 production by peripheral γδ T-cells (3, 5, 81, 104–106), although recent data surprisingly suggest that IL-18 can replace IL-1β in combining with IL-23 to induce IL-17 expression (107). In contrast, IL-1β upstream of IL-1R seems essential for GM-CSF production by γδ T-cells (108).

Finally, IL-21 was recently suggested to endow human Vγ9Vδ2 T-cells with B-cell helper activity associated with a T follicular helper cell-like phenotype (60, 109), which may impact on the generation of high affinity antibodies against microbial infections.

SIGNAL 4: NATURAL KILLER RECEPTORS

An important key characteristic that allows the recognition of transformed cells by γδ T-cells is the expression of a wide set of germline-encoded receptors that were initially described in NK-cells and hence are collectively known as NK receptors (NKRs), including natural cytotoxicity receptors (NCRs).

The C-type lectin-like NK receptor group 2 member D (NKG2D) is the best studied NKR in γδ T-cells. NKG2D binds extracellularly to multiple ligands of the MIC(A–B) and ULBP (1–6) families in humans; and to H60, MULT1, and various RAE1 molecules in mice (110). NKG2D ligands are induced upon cellular stress, for example, downstream of the DNA-damage response pathway in tumor cells (111, 112). The biological significance of this recognition system is underlined by the increased susceptibility of NKG2D-deficient mice to tumor development (113).

Intracellularly, NKG2D binds to DNAX-activating protein of 10 kDa (DAP10), which carries an YXNM motif that after tyrosine phosphorylation recruits PI3K or a Grb2–Vav1–SOS1 signaling complex (**Table 1**). This motif is similar to that in CD28, and thus, NKG2D/DAP10 may provide T-cells with costimulatory signals that synergize with the ITAM-based TCR/CD3 complex (61). However, unlike αβ T-cells but similarly to NK-cells, γδ T-cells can

express both DAP10 and DAP12 (62). The latter contains an ITAM motif, which after tyrosine phosphorylation recruits and activates Syk and ZAP70. Interestingly, only murine but not human NKG2D is able to associate with DAP12 (in addition to DAP10).

The controversy on a primary stimulatory versus costimulatory role of NKG2D in γδ T-cells has been discussed elsewhere (46, 114). Briefly, the costimulatory function of NKG2D in human Vγ9Vδ2 T-cells was supported by additive effects on TCR-mediated activation: an upregulation of cytokine production upon MICA-NKG2D interactions (115); and an increase in intracellular calcium mobilization and cytotoxic activity (32). However, other lines of evidence have suggested that NKG2D signals can activate γδ T-cells in the absence of TCR engagement: NKG2D ligation can upregulate CD69 expression in Vγ9Vδ2 T-cells to similar extent as TCR stimulation (116); NKG2D but not TCR blockade can inhibit Vγ9Vδ2 T-cell cytotoxicity against various hematological tumors (117); and murine DETC can target tumors upon recognition of NKG2D ligands (6, 118).

Another NKR implicated in tumor cell recognition by Vγ9Vδ2 T-cells is DNAX accessory molecule-1 (DNAM-1). DNAM-1 is an Ig-like family glycoprotein composed of a cytoplasmic domain containing three putative sites of phosphorylation by intracellular kinases. The phosphorylation of the Ser329 by protein kinase C (PKC) was shown to be critical for the association between DNAM-1 and LFA-1, which recruits the Fyn Src kinase to phosphorylate the Tyr322 of DNAM-1, thus initiating downstream signaling leading to SLP-76 and Vav1 phosphorylation (**Table 1**) (119). Antibody-mediated DNAM-1 blockade impaired Vγ9Vδ2 T-cell cytotoxicity and IFN-γ production against hepatocellular carcinoma lines expressing Nectin-like-5 (71).

Recently, we characterized a $V\delta1^+$ T-cell population capable of targeting hematological tumors resistant to fully activated Vγ9Vδ2 T-cells (120). Unexpectedly, the enhanced killer function resulted from induced NCR expression, namely NKp30 and NKp44, which had been previously regarded as NK-specific markers. Although neither $V\delta1^+$ nor $V\delta2^+$ cells express NCRs constitutively, these can be upregulated selectively in $V\delta1^+$ cells by PI3K/AKT-dependent signals provided by γc cytokines (IL-2 or IL-15) and TCR stimulation. Once expressed on the cell surface, NKp30 and NKp44 can signal via CD3ζ and DAP12, respectively (64). We further showed that NKp30 and NKp44 are both functional in NCR^+ $V\delta1^+$ T-cells and synergize with NKG2D to target lymphocytic leukemia cells (120).

In sum, NKRs seem critical for tumor recognition and deployment of the cytotoxic program that is endowed by TCR/γc cytokine-dependent differentiation, thus defining distinct mechanisms to be integrated in γδ T-cell-mediated cancer immunotherapy.

SIGNAL 5: INHIBITORY RECEPTORS

Beyond efficient activation and deployment of effector functions, it is necessary to negatively regulate the T-cell response in order to return to the homeostatic baseline. Inhibitory receptors like PD-1 or CTLA-4 are known to be critical for this contracting phase of the T-cell response and have become major clinical targets in cancer immunotherapy. Although γδ T-cells rarely express CTLA-4, they can upregulate PD-1 upon activation, while they constitutively express BTLA, and thus these two receptors may be the key to control γδ T-cell responses.

Programed death-1 (PD-1) is absent or low expressed on circulating Vγ9Vδ2 T-cells but is rapidly induced upon activation (121). The cytoplasmic tail of PD-1 contains conserved immunoreceptor tyrosine-based inhibitory motif (ITIM) and switch motif (ITSM), both of which are phosphorylated to recruit negative regulators that block Lck activity downstream of the TCR complex (122). Moreover, PD-1 ligation can augment the activity of the protein phosphatase and tensin homolog (PTEN), a cellular phosphatase that inhibits PI3K/AKT signaling and thus leads to impaired survival, proliferation, and IL-2 release (123). The expression of the ligand PD-L1 on tumor cells inhibited Vγ9Vδ2 T-cell cytotoxicity and IFN-γ production (121). However, zoledronate-induced accumulation of P-Ags in tumor cells and consequent Vγ9Vδ2 TCR activation seemed to overcome the inhibitory effect of PD-1/PD-L1 interactions. More research is required to understand the full extent to what PD-1 may control γδ T-cell functions and homeostasis.

B- and T-lymphocyte attenuator (BTLA) is another inhibitory receptor, member of the CD28 family and structurally related to PD-1 and CTLA-4. Binding to its ligand, herpesvirus entry mediator (HVEM), induces phosphorylation of the ITIM domain and association with SH2 domain-containing protein tyrosine phosphatase 1 (SHP-1) and SHP-2, which leads to attenuation of cellular activation and growth (124). Recent data showed that BTLA engagement with HVEM reduced P-Ag/TCR-mediated signaling and inhibited Vγ9Vδ2 T-cell proliferation, including in response to lymphoma cells (77). Conversely, BTLA-HVEM blockade using monoclonal antibodies enhanced Vγ9Vδ2 TCR signaling and may thus have therapeutic potential for the positive manipulation of γδ T-cells.

A detailed study on BTLA function in murine γδ T-cells has revealed a selective involvement in the homeostasis of the IL-17-producing $CD27^-$ γδ T-cell subset (78). Although these cells constitutively express low levels of BTLA, it is upregulated by IL-7 stimulation and thereby limits γδ T-cell numbers. Consequently, BTLA-deficient mice accumulated $IL\text{-}17^+$ $CD27^-$ γδ T-cells and were more susceptible (than wild-type controls) to dermatitis, which could be reversed by agonist BTLA antibodies. Thus, BTLA may be an important target for controlling pathogenic γδ T-cells in inflammatory and autoimmune diseases.

CONCLUDING REMARKS

A multitude of surface receptors has been shown to participate in γδ T-cell differentiation and activation. However, some crucial aspects remain to be elucidated, such as the identity of most γδTCR ligands. Most importantly, we must improve the transfer of past and current basic research into future protocols for γδ T-cell-based immunotherapy. In this context, some key questions are: how to balance γδTCR activation with "exhaustion" due to chronic stimulation? What can be achieved by manipulating the NK-like activation mode of γδ T-cells? Which costimulatory receptors should be modulated, and at what stages, to boost the desired γδ T-cell responses? Which combinations of cytokines enable the best effector γδ T-cells for each therapeutic application? Which receptors are most useful to tune down or switch off pathogenic effector

γδ T-cells? The answers to these questions must be obtained in appropriate *in vivo* pre-clinical models and hopefully next in the clinic.

For now, we would like to propose that the five types of receptor signals reviewed here define five distinct layers of regulation of γδ T-cell differentiation, activation, and function. The γδTCR is critical for the initial stages of differentiation and for proliferative responses; both processes further require cytokine signals that promote cell survival, proliferation, and terminal effector function. Costimulatory and inhibitory receptors control the extent of γδ T-cell expansion, with interesting biases toward specific effector subsets. Finally, NK receptors play a decisive role in tumor cell targeting by γδ T-cells. Thus, we believe that the recognition of "stressed self" can be mediated by the γδTCR but also chiefly by NK receptors like NKG2D. As such, the characterization of both type of ligands on tumors may be critical to design protocols, select and monitor patients, and increase the chances of efficacious γδ T-cell-based cancer immunotherapies.

ACKNOWLEDGMENTS

We thank Daniel Correia for helpful discussions on these topics. Our work is supported by Fundação para a Ciência e Tecnologia (SFRH/BD/84123/2012 to Sérgio T. Ribeiro; SFRH/BPD/78135/2011 and EXPL/IMI-IMU/0170/2013 to Julie C. Ribot) and European Research Council (StG_260352 to Bruno Silva-Santos).

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Conflict of Interest Statement: Bruno Silva-Santos is a co-founder and share holder of LymphAct S.A. The other co-authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Received: 21 October 2014; accepted: 08 January 2015; published online: 26 January 2015.
*Citation: Ribeiro ST, Ribot JC and Silva-Santos B (2015) Five layers of receptor signaling in γδ T-cell differentiation and activation. Front. Immunol. **6**:15. doi: 10.3389/fimmu.2015.00015*
This article was submitted to T Cell Biology, a section of the journal Frontiers in Immunology.

Published January 31, 2014, doi:10.4049/jimmunol.1303119

The Journal of Immunology

Human γδ Thymocytes Are Functionally Immature and Differentiate into Cytotoxic Type 1 Effector T Cells upon IL-2/IL-15 Signaling

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Cytotoxicity and IFN-γ production by human γδ T cells underlie their potent antitumor functions. However, it remains unclear where and how human γδ T cells acquire these key effector properties. Given the recent disclosure of a major contribution of the thymus to murine γδ T cell functional differentiation, in this study we have analyzed a series of human pediatric thymuses. We found that ex vivo–isolated γδ thymocytes produced negligible IFN-γ and lacked cytolytic activity against leukemia cells. However, these properties were selectively acquired upon stimulation with IL-2 or IL-15, but not IL-4 or IL-7. Unexpectedly, TCR activation was dispensable for these stages of functional differentiation. The effects of IL-2/IL-15 depended on MAPK/ERK signaling and induced de novo expression of the transcription factors T-bet and eomesodermin, as well as the cytolytic enzyme perforin, required for the cytotoxic type 1 program. These findings have implications for the manipulation of γδ T cells in cancer immunotherapy. ***The Journal of Immunology*****, 2014, 192: 000–000.**

All jawed and jawless vertebrates have evolved three lineages of lymphocytes based on somatic gene diversification of Ag receptors (1). Within the lymphocyte trilogy, γδ T cells clearly remain the most poorly understood lineage, both in terms of Ag recognition and differentiation into effector cell subsets (2). This notwithstanding, γδ T cells are already being targeted in cancer immunotherapy (3, 4) based on multiple promising preclinical studies in both mice (5–8) and humans (3, 9–11).

Human γδ PBLs are endowed with potent cytotoxicity against hematological (3, 9, 10) and epithelial (11, 12) malignant cells. Moreover, γδ PBLs are highly polarized toward IFN-γ production since early life, as preterm babies harbor significant proportions of IFN-γ^+ γδ (but not αβ) T cells in the blood (13), and CMV infection in utero promotes the differentiation of IFN-γ^+ and perforin$^+$ γδ T cells (14). γδ T cells thus constitute the first functional population of circulating T cells (13).

Given an individual's history of infections, circulating γδ PBLs can display very heterogeneous phenotypes ranging from naive to effector/memory and terminally differentiated effector cells (15). It is therefore difficult to inquire where and how human γδ T cells acquire their effector functions. In healthy individuals, these are tightly linked to IFN-γ production, as alternative functional states, such as IL-17 or IL-22 secretion, are very rare (16–18).

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Received for publication November 19, 2013. Accepted for publication January 3, 2014.

This work was supported by Fundação para a Ciência e Tecnologia Grant EXPL/BIM-ONC/0490/2012 and by the Young Investigator Program of the European Molecular Biology Organization.

J.C.R. participated in the execution of all experiments, designed the study, and wrote the paper; S.T.R. performed the experiment in Fig. 4A; D.V.C. performed the experiment in Fig. 2G; A.E.S. contributed to the design of the study; and B.S.-S. designed and supervised the study and wrote the paper.

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The online version of this article contains supplemental material.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1303119

Interestingly, we (19) and others (20, 21) have shown that murine γδ T cells acquire their effector properties during thymic development, in a process regulated by TCRγδ (and coreceptor) signaling (22). For example, IFN-γ–producing γδ T cells require TCR and CD27 signals for differentiation in the mouse thymus (19–21). This raises the question whether human γδ thymocytes can also complete their functional differentiation before being exported to the periphery. Although thymic commitment to the γδ T cell lineage is controlled by Notch signaling (23, 24), much less is known about the subsequent steps of functional differentiation of human γδ T cells (16, 25, 26). This will likely have major implications for their manipulation in cancer immunotherapy.

Building on these considerations, in this study we have used pediatric thymic tissue to address the molecular mechanisms of human γδ T cell differentiation toward antitumor lymphocytes. Our results reveal an NK-like mode of differentiation that is dependent on IL-2/IL-15 signals but surprisingly not on TCR activation. Interestingly, this process must take place in the periphery, because, unlike their murine counterparts, human γδ thymocytes are devoid of cytotoxic type 1 effector functions. Finally, our data disclose an MAPK/ERK-mediated differentiation pathway that may constitute an important target for future modulation of γδ T cell activity in the clinic.

Materials and Methods

Ethics

Thymic specimens were routinely obtained during pediatric corrective cardiac surgery, after parent's written informed consent. The study was approved by the Ethics Board of the Faculdade de Medicina da Universidade de Lisboa.

Lymphocyte preparations

Thymic samples (from newborn to 9-y-old children) were processed by tissue dispersion and Histopaque-1077 (Sigma-Aldrich) density gradient. Peripheral blood was collected from anonymous healthy volunteers, diluted with 1 volume of PBS (Invitrogen Life Technologies), and separated on a Histopaque-1077 density gradient. γδ T cells were isolated (to >95% purity) by magnetic cell sorting by positive selection (Miltenyi Biotec). Alternatively, CD1a$^+$ γδ T cells or CD3$^-$CD4$^-$CD8$^-$TCRγδ$^-$ thymic progenitors were electronically sorted on a FACSAria cell sorter (BD Biosciences).

Cell culture

Isolated γδ T cells were cultured at 10^6 cells/ml at 37°C, 5% CO_2 in round-bottom 96-well plates with RPMI 1640 and 2 mM L-glutamine (Invitrogen Life Technologies) supplemented with 10% FBS (Invitrogen Life Technologies), 1 mM sodium pyruvate (Invitrogen Life Technologies), and 50 mg/ml penicillin and streptomycin (Invitrogen Life Technologies). Indicated cytokines were added when mentioned (all from PreproTech, 10 ng/ml). To study the effects of chemical inhibitors of signal transduction, the MEK inhibitor UO126, the PI3K inhibitor LY294002, and the STAT5 inhibitor Ssi (all from Calbiochem) were added at 20 μM after 4 d in culture. $CD3^-CD4^-CD8^-TCR\gamma\delta^-$ thymic progenitors were seeded at 2×10^5 cells/well into 48-well tissue culture plates (BD Biosciences) containing a subconfluent monolayer of OP9-DL1 cells. Cocultures were performed in culture medium consisting of DMEM (Invitrogen Life Technologies) supplemented with 20% FCS, 100 IU/ml streptomycin, penicillin, and L-glutamine (Invitrogen Life Technologies) in the presence of 10 ng/ml IL-7 (PreproTech). Where mentioned, IL-2 (10 ng/ml; PreproTech) was added. Every 4–5 d, cells were harvested by forceful pipetting and transferred to a fresh confluent monolayer of OP9-DL1 cells.

In vitro tumor-killing assays

The MOLT-4 leukemia cell line was stained with CellTrace Far Red 7-hydroxy-9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one–succinimidyl ester (1 μM; Molecular Probes/Invitrogen) and each batch of 3×10^4 tumor cells was incubated with 3×10^5 γδ T cells in RPMI 1640 for 3 h in a round-bottom plate with 96 wells. Cells were stained with annexin V–FITC (BD Biosciences) and analyzed by FACS.

Flow cytometry (FACS) analysis

Surface and intracellular stainings were performed as previously described (19). Cells were labeled with the following fluorescent mAbs: anti–TCRγδ-FITC (SA6.E9) from Invitrogen; anti–Vδ1-FITC (TS8.2) from Thermo Scientific; anti–Vδ2-PE (B6), anti–CD3-PerCP-Cy5.5 (UCHT1), anti–CD69-PE-Cy7 (FN50), anti–TNF-α-PE-Cy7 (MAb11), anti–CD107a-Pacific Blue (H4A3), and anti–CD45RA-allophycocyanin (HI100) from BioLegend; and anti–CD8α-PE (HIT8a), anti–IL-2-PE (MQ1-17H12), anti–CD27-PE-Cy7 (LG.7F9), anti–IFN-γ-allophycocyanin (4S.B3), anti–CD4-eFluor 450 (RPA-T4), and anti–CD1a-eFluor 450 (HI49) from eBioscience. Cell proliferation was measured by following a standard CFSE staining protocol (19) (CellTrace CFSE cell proliferation kit from Invitrogen; final concentration, 0.5 mM), whereas apoptosis was assessed by annexin V–FITC (BD Pharmingen) staining. Cells were analyzed on a FACSFortessa (BD Biosciences) and using FlowJo software (Tree Star).

Immunoblotting

Cells were lysed in lysis buffer (50 mM Tris [pH 7.6], 150 mM EDTA, 1% Nonidet P-40 in PBS) enriched with a protease inhibitor mixture (Roche) and a phosphatase inhibitor mixture (Roche). The total proteins were quantified using a Bradford assay (Bio-Rad), following the manufacturer's instructions. For following analysis, 30 μg total protein was denatured in Laemmli buffer (Bio-Rad), boiled for 5 min, and loaded in a 10% SDS-PAGE. After electrophoretic separation, the proteins were transferred to nitrocellulose blotting paper (Amersham Biosciences). The membranes were blocked with 5% BSA and 0.5% Tween 20 (Sigma-Aldrich) in PBS and probed with the following primary Abs: p-ERK1/2 (Thr^{202}/Try^{204}), ERK1, p-AKT (Ser^{473}) (Cell Signaling Technology), p-STAT5 ($Tyr^{694/699}$) (Merck/Millipore), STAT5a (Santa Cruz Biotechnology), and connexin (Sicgen). These were detected by the appropriate HRP-conjugated secondary Abs and developed by chemiluminescence.

Real-time quantitative PCR

Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Concentration and purity were determined by spectrophotometry, and integrity was confirmed using an Agilent 2100 bioanalyzer with an RNA 6000 Nano assay (Agilent Technologies). Total RNA was reverse-transcribed into cDNA using random hexamers and SuperScript II first-strand synthesis reagents (Invitrogen). Real-time quantitative PCR was performed on an ABI Prism 7500 Fast sequence detection system using SYBR Green detection system (both from Applied Biosystems). For each transcript, quantification was done using the calibration curve method. The following primers were used: *B2m*, forward, 5′-CTATCCAGCGTACTCCAAAGATTC-3′, reverse, 5′-CTTGCTGAAAGACAAGTCTGAATG-3′; *Tbx21*, forward, 5′-CACCTGTTGTGGTCCAAGTTT-3′, reverse, 5′-AACATCCTGTAGTGGCTGGTG-3′; *Pfn*, forward, 5′-GCAATGTGCATGTGTCTGTG-3′, reverse, 5′-GGGAGTGTGTACCACATGGA-3′.

Statistical analysis

Differences between populations were assessed using the Student *t* test and are indicated in the figures when significant.

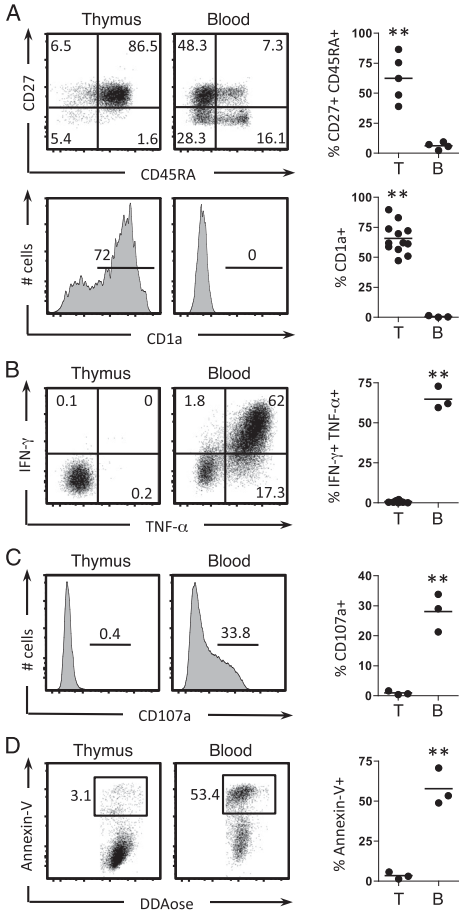

FIGURE 1. Human γδ thymocytes are devoid of IFN-γ production and cytotoxic functions. $TCR\gamma\delta^+CD3^+$ cells were isolated from pediatric thymic biopsies (Thymus/T) or from the peripheral blood of healthy donors (Blood/B) and analyzed ex vivo by flow cytometry. Dead cells were excluded from the analysis using LIVE/DEAD Fixable Dead Cell Stain Kits (Molecular Probes). (**A**) Surface expression of CD27, CD45RA, and CD1a. (**B** and **C**) Intracellular staining for IFN-γ and TNF-α (B) or the degranulation marker CD107a (C) following 4 h of stimulation with PMA and ionomycin. (**D**) Cytotoxic activity against MOLT-4 leukemia cells. Percentage of apoptotic annexin V^+ within 7-hydroxy-9*H*-(1,3-dichloro-9,9-dimethylacridin-2-one–succinimidyl ester ($DDAO\text{-}SE^+$) (prelabeled) tumor cells after 3 h of coincubation with γδ T cells at a 10:1 E:T ratio. Results in this figure are representative of 3–12 independent experiments; each dot represents an individual sample. $^{**}p < 0.005$.

Results

Human γδ thymocytes are devoid of cytotoxicity and IFN-γ production

Inspired by the recent identification of fully differentiated effector γδ T cell subsets in the murine thymus (19–21, 26), we started this study by analyzing the surface phenotype and functional potential of γδ T cells isolated from human pediatric thymic samples. Based on the differentiation markers CD1a (27), CD27, and CD45RA (15), the vast majority of γδ thymocytes showed an immature and naive phenotype, which contrasted with the dominant effector/memory phenotype (15) of γδ PBLs (Fig. 1A). Also unlike these, γδ thymocytes produced negligible proinflammatory cytokines, particularly IFN-γ and TNF-α (Fig. 1B). Moreover, γδ thymocytes lacked cytolytic activity (Fig. 1C), namely against leukemia target cells, which were promptly killed by their PBL counterparts (Fig. 1D). These data clearly demonstrate that, unlike murine γδ thymocytes (19–21, 26), human γδ T cells do not complete their functional differentiation in the thymus, and they thus lack the cytotoxic type 1 characteristics of γδ PBLs.

IL-2 and IL-15 signals drive human γδ cytotoxic type 1 cell differentiation

The functional immaturity of human γδ thymocytes made them an ideal system to investigate the molecular cues required for acquisition of antitumor effector properties. Focusing first on IFN-γ

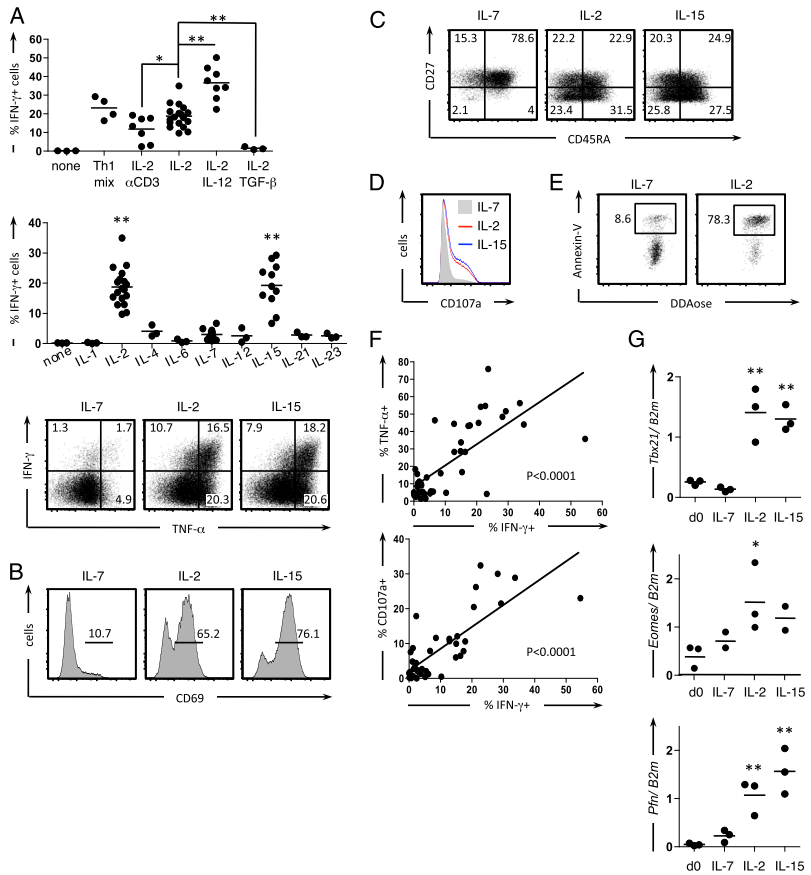

FIGURE 2. IL-2 and IL-15 signals differentiate γδ thymocytes into cytotoxic type 1 effector T cells. MACS-purified γδ thymocytes were cultured for 7 d in the presence of 10 ng/ml of the indicated cytokines; anti-CD3 mAb (1 μg/ml) was added when noted, and as part of the Th1 mix also contained anti-CD28 mAb (5 μg/ml), IL-2, and IL-12. (**A**) Intracellular staining for IFN-γ following 4 h of stimulation with PMA and ionomycin. (**B**) Surface expression of the activation marker CD69. (**C**) Surface staining for CD27 and CD45RA. (**D**) Intracellular staining for CD107a. (**E**) Cytotoxic activity against MOLT-4 leukemia cells (as in Fig. 1D). (**F**) Correlations between fractions of cells expressing IFN-γ, TNF-α, or CD107a. Each dot represents a specific culture condition from the experiments in Fig. (A) and (D). (**G**) Real-time PCR data for the expression of T-bet (*Tbx21*, *upper panel*), eomesodermin (*Eomes*, *middle panel*), and perforin (*Pfn*, *lower panel*), normalized to housekeeping β_2-microglobulin (*B2m*), on γδ thymocytes either freshly isolated or cultured for 7 d with the indicated cytokines. Results in this figure are representative of 3–18 independent experiments; each dot represents an individual sample. $*p < 0.05$, $**p < 0.005$.

production, we considered that naive CD4$^+$ T cells typically require TCR/CD3 and CD28 ligation in the presence of IL-2 and IL-12 for differentiation along the "T helper 1" pathway (28). Although such a "Th1 mix" was indeed capable of generating IFN-γ$^+$ γδ T cells, we surprisingly found that IL-2 alone was also sufficient (Fig. 2A, *upper panel*). This effect was potentiated by IL-12, whereas TGF-β abrogated the process. Unexpectedly, the addition of TCR stimulation via anti-CD3 mAb did not enhance, but rather reduced, the IL-2–mediated differentiation of human IFN-γ$^+$ γδ T cells (Fig. 2A, *upper panel*).

We next tested a large panel of individual cytokines and observed that besides IL-2, only IL-15 (but notably not IL-4 or IL-7) was able to induce IFN-γ production in γδ thymocyte cultures (Fig. 2A, *middle panel*). Both IL-2 and IL-15 treatments also promoted TNF-α expression (Fig. 2A, *lower panel*), upregulated the activation marker CD69 (Fig. 2B), and drove thymocytes along the effector/memory differentiation pathway, toward a CD45RA$^+$ CD27$^-$ T effector memory stage (Fig. 2C).

Concerning γδ T cell cytotoxicity, IL-2 and IL-15 (but not IL-7) induced the expression of the degranulation marker CD107a on γδ thymocytes (Fig. 2D) and endowed them with potent killing capacity against leukemia target cells (Fig. 2E). Of note, exogenous IL-2 and IL-15 also enhanced the effector functions of γδ PBLs, especially their degranulation/cytotoxic potential (Supplemental Fig. 1).

The acquisition of IFN-γ/TNF-α production and cytolytic capacity by γδ thymocytes were positively correlated, suggesting a common pathway of cytotoxic type 1 differentiation (Fig. 2F). Moreover, IL-2/IL-15 signals induced de novo expression of the type 1 master transcription factors T-bet and eomesodermin, as well as the cytolytic molecule perforin (Fig. 2G). These data firmly demonstrate that IL-2 and IL-15 are key functional differentiation factors for human γδ T cells. Importantly, they also show that IL-2 and IL-15 signals are sufficient, in the absence of TCR activation, to generate fully functional γδ T cells from immature thymocytes.

Vδ1 and Vδ2 T cell subsets follow similar rules of functional differentiation

Given that human γδ T cells comprise two major subsets, Vδ1$^+$ cells (5–30% of γδ PBLs but more abundant in tissues) and Vδ2$^+$ cells (60–95% of γδ PBLs), both strongly biased toward cytotoxic type 1 functions (3, 29), we next assessed whether they followed similar rules of differentiation. Consistent with the literature (25), the thymic γδ repertoire was largely biased for Vδ1$^+$ thymocytes,

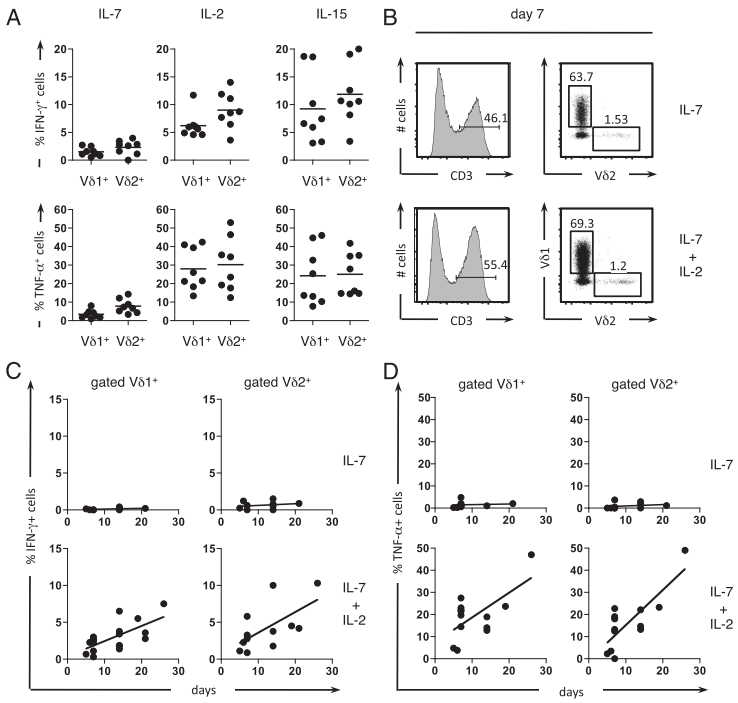

FIGURE 3. Vδ1 and Vδ2 subsets of γδ T cells follow similar rules of functional differentiation. (**A**) CD1a$^+$ γδ thymocytes were FACS sorted and cultured for 7 d with the indicated cytokines (at 10 ng/ml). Cells were restimulated for 4 h with PMA and ionomycin and stained for TCRVδ1, TCRVδ2, and intracellular IFN-γ and TNF-α. Histograms depict percentages of IFN-γ$^+$ cells (*upper panels*) or TNF-α$^+$ cells (*lower panels*) within pregated Vδ1$^+$ or Vδ2$^+$ cells. (**B** and **D**) CD3$^-$CD4$^-$CD8$^-$TCRγδ$^-$ thymic progenitors were FACS sorted and cultured on OP9-DL1 monolayers in the presence of IL-7 with or without IL-2. At indicated time points, cells were harvested and stained for surface CD3, TCRVδ1, TCRVδ2 (B), and intracellular IFN-γ (**C**) and TNF-α (D). Graphs represent percentages of IFN-γ $^+$ cells (C) or TNF-α$^+$ cells (D) within pregated Vδ1$^+$ or Vδ2$^+$ cells. Dead cells were excluded from the analysis using LIVE/DEAD Fixable Dead Cell Stain Kits. Data are representative of six to eight independent experiments with similar results; each dot represents an individual sample.

with an average Vδ1/Vδ2 ratio of 25, which was maintained in vitro upon IL-7, IL-2, or IL-15 treatment (Supplemental Fig. 2A). In all thymic samples analyzed, Vδ1$^+$ T cells behaved as expected: they were functionally immature ex vivo and differentiated into type 1 effectors in response to IL-2 or IL-15 stimuli (Supplemental Fig. 2B). However, the results obtained by gating on the Vδ2$^+$ population were affected by an important intersample variation (Supplemental Fig. 2B). We considered that this could be due to blood contamination or recirculation (back to the thymus) of mature Vγ9Vδ2 cells, which are much more abundant in the blood than in the thymus (<3% of total γδ thymocytes). To overcome these problems, we purified CD1a$^+$ γδ T cells, which are exclusive to the thymus (Fig. 1A, *lower panel*) and cultured them for 7 d with IL-7, IL-2, or IL-15. We found that both Vδ1$^+$ and Vδ2$^+$ cells similarly acquired type 1 effector properties in response to IL-2 and IL-15, but not IL-7 (Fig. 3A).

As an alternative developmental strategy, we differentiated γδ T cells from sorted CD3$^-$TCRγδ$^-$CD4$^-$CD8$^-$ thymic precursors cultured on OP9-DL1 monolayers, as previously described (24). In the presence of IL-7 alone, Vδ1 and Vδ2 T cells developed normally (Fig. 3B) to a Vδ1/Vδ2 ratio similar to that observed ex vivo (Supplemental Fig. 2A). We therefore think this is an elegant model to characterize γδ T cell differentiation from very early developmental stages. Most importantly, the further addition of IL-2 was necessary to generate IFN-γ– (Fig. 3C) and TNF-α– (Fig. 3D) producing γδ T cells, and this occurred similarly for Vδ1 and Vδ2 T cell subsets (Fig. 3C, 3D). These data demonstrate that IL-2 (or IL-15) signals drive the functional differentiation of both major subsets of human γδ T cells.

IL-2/IL-15 signals induce γδ type 1 cell differentiation via the MAPK/ERK pathway

To gain further mechanistic insight into the type 1 differentiation pathway of human γδ T cells, we probed the three major signaling pathways downstream of common γ-chain cytokine receptors and observed that IL-2 stimulation hyperphosphorylated ERK1/2 (MAPK pathway), STAT5 (JAK/STAT pathway), and AKT (PI3K pathway) (Fig. 4A). To determine which of these signaling pathways was critical for functional differentiation of γδ T cells, we analyzed the effect of specific chemical inhibitors on γδ thymocyte cultures. When added at the start of the cultures, all of these drugs interfered with γδ T cell proliferation and prevented their functional differentiation (Supplemental Fig. 3A). Of note, although proliferation was necessary for IFN-γ induction, this specifically required IL-2 or IL-15 signals, as IL-7 failed to do so even after five cell divisions (Fig. 4B). To dissociate proliferation from differentiation, we added the inhibitors at day 4 of culture, when cells had already undergone five rounds of division (Fig. 4B). Whereas blocking STAT5 or PI3K/AKT had no detectable effect, the MAPK/ERK inhibitor UO126 (30, 31) completely abrogated the differentiation of IFN-γ$^+$ or TNF-α$^+$ γδ T cells (under IL-2 or IL-15 treatment) (Fig. 4B, Supplemental Fig. 3B).

Interestingly, IL-2 or IL-15 signals also enhanced IL-2 production by γδ T cells, thus providing an autocrine mechanism to sustain their functional differentiation (Supplemental Fig. 3B). The production of IL-2 (as well as TNF-α) by γδ T cells was also completely abolished by the addition of UO126, but not LY294002 or Ssi (Supplemental Fig. 3B). These data demonstrate that γδ T cell cytotoxic type 1 differentiation can be manipulated by drugs specifically targeting the MAPK/ERK pathway (32).

Collectively, our results identify MAPK-mediated IL-2/IL-15 signaling as the major functional differentiation pathway of human γδ T cells toward antitumor (cytotoxic type 1) effector lymphocytes.

Discussion

Seminal studies in murine models have shown that γδ T cells can complete their functional differentiation in the thymus (19–21, 26). In contrast, the data presented in the present study show that human γδ thymocytes (obtained from young children subjected to cardiac surgery) are functionally immature. Given that circulating γδ PBLs display type 1 effector properties (13, 18) and express memory markers (15), human γδ T cells must thus complete their differentiation in the periphery, as reported for αβ T cells (33, 34).

In healthy individuals, γδ PBLs are strongly biased toward IFN-γ production (type 1 effectors), and throughout our study we failed to identify any significant production of type 2 or type 17

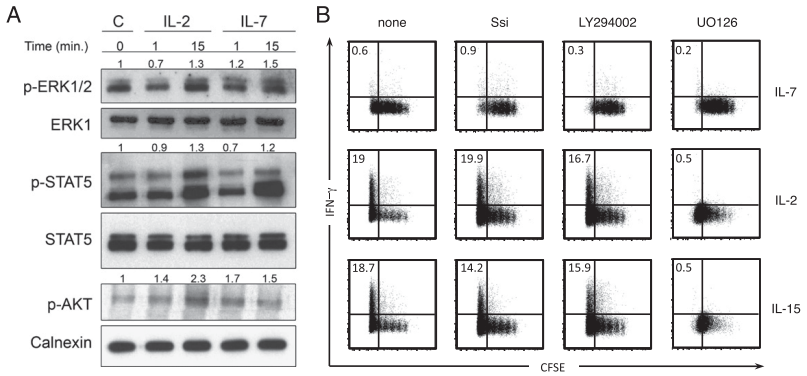

FIGURE 4. The MAPK/ERK signaling pathway is required for IL-2–mediated type 1 differentiation of human γδ T cells. MACS-purified γδ thymocytes were cultured in the presence of 10 ng/ml IL-2, IL-7, or IL-15. (**A**) Western blot analysis of p-ERK1/2 (Thr^{202}/Try^{204}), ERK1, p-AKT (Ser^{473}), p-STAT5 (Tyr$^{694/699}$), and STAT5a in cell lysates obtained at the indicated timepoints. C, control medium without cytokines. The numbers above the blots correspond to desitometric analysis of the corresponding bands. (**B**) Effect of chemical inhibitors on the production of IFN-γ in γδ thymocyte cultures. At day 4 (after five cell divisions, based on CFSE dilution), specific inhibitors of STAT5 (Ssi), PI3K (LY294002), or MEK phosphorylation (UO126) were added (20 μM each), and at day 7 cells were restimulated for 4 h with PMA and ionomycin and stained intracellularly for IFN-γ. Dead cells were excluded from the analysis using LIVE/DEAD Fixable Dead Cell Stain Kits. Data are representative of three to five independent experiments with similar results.

cytokines. Namely, IL-17, which is constitutively expressed by a subset of murine γδ T cells (19, 26), is rarely expressed in human γδ T cells from healthy donors (16–18). In contrast, IL-17–producing γδ T cells seem to accumulate to high numbers in clinical cases of bacterial meningitis, and their rules of differentiation have been previously dissected (17, 35).

In our study, the naive and immature phenotype of human γδ thymocytes provided an ideal system to investigate the molecular cues required for acquisition of type 1 effector properties. Our work demonstrated that IL-2 or IL-15 signals are sufficient to drive the differentiation of human γδ T cells into IFN-γ/TNF-α producers also endowed with potent cytotoxicity against tumor targets.

The redundant functions of IL-2 and IL-15 can be explained by the structure of their respective receptors, which share not only the common γ-chain but also their second signaling subunit, IL-2Rβ. A third subunit, IL-2Rα or IL-15Rα, is cytokine specific and stabilizes binding but apparently lacks signaling activity. Structural comparisons of IL-2/IL2-Rα and IL-15/IL15-Rα interactions have emphasized their similarities (36, 37), and it has been recently demonstrated that they induce similar downstream transcriptional effects (38).

On the other hand, IL-7 clearly failed to trigger differentiation of cytotoxic type 1 γδ T cells. This is in stark contrast with the major role described for IL-7 in the functional differentiation of human NKT cells (39) and IL-17–producing γδ T cells (40). These lines of evidence establish an interesting cytokine dichotomy for human γδ T cells: whereas IL-7 promotes type 17 effector functions, IL-2 and IL-15 are the main drivers of the type 1 program. Of note, because IL-15 stimulation induces γδ T cells to produce IL-2 (Supplemental Fig. 3B), this cytokine could also be the direct (autocrine) mediator of the type 1 program downstream of IL-15 signals.

Unexpectedly, TCR activation was not required for the functional differentiation of human γδ thymocytes. Recent work from Hayday and colleagues (41) on murine γδ T cells suggests that innate-like γδ T cell subsets may lack TCR responsiveness in the periphery following strong TCR signaling during thymic development. Naturally, we cannot assess whether human γδ thymocytes (the starting point of our in vitro experiments) have already received TCR signals in vivo. Were this to be the case, IL-2 and IL-15 would act as terminal differentiation factors in cells that had been previously selected via their TCR. This is also reminiscent of the two-step model proposed by Lio and Hsieh (42) and Farrar and colleagues (43) for murine $Foxp3^+$ regulatory T cell development. Importantly, whereas for regulatory T cells this two-step process is completed in the thymus, human γδ T cell functional differentiation seemingly involves a second step that takes place in the periphery. Physiologically, this likely depends on the provision of IL-2 by activated T cells (either γδ T cells themselves or their αβ counterparts) or IL-15 by myeloid and epithelial cells. Concerning a potential autocrine IL-2 loop based on γδ T cells, we have previously shown that IL-2 production requires TCR activation in the presence of CD28 costimulation (44). Therefore, CD80/CD86, as expressed by APCs, including dendritic cells but also Vγ9Vδ2 T cells, are likely additional players in the functional differentiation of γδ T cells. For therapeutic purposes (in cancer immunotherapy), our results strongly suggest that the provision of exogenous IL-2 or IL-15 may be critical, not only for γδ PBL activation and expansion, but also particularly for extensive differentiation of antitumor effectors from recent thymic emigrants and circulating naive γδ T cells.

Downstream of IL-2/IL-15 receptors, our data provide the important insight that γδ T cell cytotoxic type 1 differentiation can be manipulated by drugs specifically targeting the MAPK/ERK pathway (32). In this context, dual-specificity phosphatases seem particularly promising targets for modulating MAPK-dependent immune processes (32).

In conclusion, our results detach human γδ T cells from paradigms of T cell differentiation: their differentiation program is not completed in the thymus, unlike murine γδ T cells (19, 20, 26), and it does not require TCR/co-receptor activation in the periphery, in contrast with naive $CD4^+$ T cells (28). Instead, IL-2/IL-15 signals are sufficient for functional differentiation of human γδ T cells, which clearly aligns them with NK cells (45) and some naive $CD8^+$ T cell populations (46). Thus, the three main cytotoxic type 1 lymphocyte subsets share a common, IL-2/IL-15–dependent differentiation program with key implications in cancer immunotherapy.

Acknowledgments

We thank Miguel Abecasis and Rui Anjos at Hospital of Santa Cruz for provision of the pediatric thymic samples; João Barata, Vanda Póvoa, and Daniel Ribeiro for reagents and expert advice; Helena Nunes Cabaço, Iris Caramalho, Catarina Mota, Vânia Silva, and Francisca Matos for technical assistance; and Marc Bonneville, Emmanuel Scotet, António Lopes, Daniel Pennington, and Henrique Veiga-Fernandes for helpful discussions.

Disclosures

The authors have no financial conflicts of interest.

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OPEN

Leukemia (2016), 1–8

www.nature.com/leu

ORIGINAL ARTICLE

Casein kinase 2 controls the survival of normal thymic and leukemic γδ T cells via promotion of AKT signaling

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The thymus is the major site for normal and leukemic T-cell development. The dissection of the molecular determinants of T-cell survival and differentiation is paramount for the manipulation of healthy or transformed T cells in cancer (immuno)therapy. Casein kinase 2 (CK2) is a serine/threonine protein kinase whose anti-apoptotic functions have been described in various hematological and solid tumors. Here we disclose an unanticipated role of CK2 in healthy human thymocytes that is selective to the γδ T-cell lineage. γδ thymocytes display higher (and T-cell receptor inducible) CK2 activity than their αβ counterparts, and are strikingly sensitive to death upon CK2 inhibition. Mechanistically, we show that CK2 regulates the pro-survival AKT signaling pathway in γδ thymocytes and, importantly, also in γδ T-cell acute lymphoblastic leukemia (T-ALL) cells. When compared with healthy thymocytes or leukemic αβ T cells, γδ T-ALL cells show upregulated CK2 activity, potentiated by CD27 costimulation, and enhanced apoptosis upon CK2 blockade using the chemical inhibitor CX-4945. Critically, this results in inhibition of tumor growth in a xenograft model of human γδ T-ALL. These data identify CK2 as a novel survival determinant of both healthy and leukemic γδ T cells, and may thus greatly impact their therapeutic manipulation.

Leukemia advance online publication, 16 December 2016; doi:10.1038/leu.2016.363

INTRODUCTION

T cells develop in the thymus. The dissection of the cell-intrinsic and -extrinsic signals that regulate thymocyte survival, proliferation and differentiation is critical to understand their potential for transformation and to devise new therapies for T-cell acute lymphoblastic leukemia (T-ALL).

T-cell commitment is coupled to somatic T-cell receptor (TCR) rearrangements, generating thymocytes bearing either an αβ or a γδ TCR.[1] The expression of a pre-TCR composed of TCRβ and the invariant pTα chain in αβ thymocyte progenitors results in a massive proliferative burst ('β-selection') that dictates that αβ T cells largely outnumber their γδ counterparts. Likely a consequence, although significant progress has been made in our understanding of human αβ T-cell development, the molecular determinants of γδ thymocytes remain poorly characterized.[1]

Most of what we know about thymic γδ T-cell differentiation comes from studies performed in mice, showing how various receptors (namely, TCRγδ, CD27 and LTβR) and downstream transcription factors (such as Id3, Sox13, TCF1 and Lef1) control various maturation steps, from divergence from the αβ lineage to the acquisition of effector functions such as pro-inflammatory cytokine production.[2–7] In contrast, much less is known about human thymic γδ T-cell differentiation. Notwithstanding, we recently showed that interleukin (IL-2) or IL-15 differentiate human γδ thymocytes into cytotoxic type 1 effector T cells, rendering them highly efficacious against leukemic cells *in vitro* and *in vivo*.[8,9]

γδ thymocytes can themselves transform into leukemic cells, causing a rare ($<$ 10% of all cases) form of T-ALL with distinctive clinical features.[10–12] Given that malignant γδ T cells have been very poorly studied and lack defined molecular targets, we have here addressed the potential role of casein kinase 2 (CK2), a signaling effector molecule previously implicated in chronic lymphocytic leukemia,[13,14] multiple myeloma,[15] B-ALL,[16,17] T-ALL[18,19] and other hematological disorders (reviewed in Piazza *et al.*[20]).

CK2 is a ubiquitous and constitutively activated serine/threonine protein kinase that regulates multiple pathways including phosphatidylinositol 3-kinase/AKT and WNT signaling, nuclear factor-κB transcription and the DNA damage response.[20] CK2 displays pro-survival and anti-apoptotic functions that were described in several cancer cell types. CK2 is frequently over-expressed or hyperactivated in both solid tumors and hematological malignancies, thus making it a promising target for cancer treatment.[20] In contrast, the physiological function of CK2 in nontransformed cells is less established. Recent studies in mice have demonstrated that CK2 activity is necessary for peripheral T-cell activation and function: interference with CK2 signaling impaired $CD4^+$ T-cell activation and differentiation into T helper type 2 (Th2) or Th17 cells,[21], whereas the genetic deletion of CK2 in $CD4^+$ $Foxp3^+$ regulatory T cells abolished their suppressive activity against allergy-promoting Th2 cells.[22] However, no functional role has yet been attributed to CK2 in the human thymus.

In this study we identified a novel role for CK2 in controlling the survival of normal γδ thymocytes and γδ T-ALL cells. We analyzed CK2 activity in γδ versus αβ thymocytes and T-ALL cells, its regulation by cell-extrinsic signals, the downstream signaling mechanisms and the effect of its inhibition *in vitro* and *in vivo* in a xenograft model of γδ T-ALL.

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Received 17 September 2015; revised 12 October 2016; accepted 18 November 2016; accepted article preview online 30 November 2016

MATERIALS AND METHODS

Statement of Ethics

Thymic specimens (from newborn to 15-year-old children) were obtained during pediatric corrective cardiac surgery after parents' written informed consent. The study was approved by the Ethics Board of Faculdade de Medicina da Universidade de Lisboa. Primary T-ALL blasts derived from diagnostic samples (peripheral blood or bone marrow), obtained after informed content and amplified upon xenografting into NSG (NOD.Cg-Prkdc(scid)Il2rg(tm1Wjll)/SzJ) mice.

Isolation, cell culture and viral transduction

Thymic T cells were collected after thymus tissue dispersion and separation by Histopaque-1077 (Sigma-Aldrich, St Louis, MO, USA) density gradient separation. TCRγδ-positive T cells were isolated (to >97% purity) by magnetic positive selection; TCRαβ-positive T cells were isolated (to >96% purity) by magnetic positive selection from the TCRγδ-negative fraction (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were used as fresh or, when indicted, cells were cultured at 37 °C with 5% CO_2 in complete RPMI-1640 as previously described[23] on indicated conditions. For long-term *in vitro* cell culture of thymocytes (7 days), recombinant human IL-2 was added to the medium. The PEER T-ALL (DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and MOLT-4T-ALL (ATCC CRL-1582) were cultured in 90% RPMI-1640+10% fetal bovine serum following the manufacturer's instructions. When indicated, PEER cell line was transduced using a bicistronic retroviral DNA construct, either empty vector (LZRS) expressing only IRES followed by eGFP (LZRS-IRES-eGFP) or vector co-expressing myrPKB/AKT (constitutively activated AKT) and eGFP (LZRS-myrPKB/AKT-IRES-eGFP) as previously described.[24] To increase the percentage of transduced cells for the following experiments, GFP^+ cells were sorted (≈100% purity) using a FACSAria high-speed cell sorter (BD Biosciences, San Jose, CA, USA).

Chemicals and antibodies

Anti-human monoclonal antibodies were used against: CD3 (UCHT1), CD27 (LG.7F9), CD4 (RPA-T4), CD7 (4H9) and panαβTCR (IP26) from eBioscience (San Diego, CA, USA); CD28 (CD28.2), CD8 (SK1), CD45 (HI30), Vδ2 (B6), CD3 (OKT3), CD45RA (HI100), Annexin-V and 7-aminoactinomycin D (7-AAD) from Biolegend (San Diego, CA, USA); panγδTCR (5A6.E9) from Thermo-Fisher (Rockford, IL, USA); Vδ1 (REA173) from Miltenyi Biotech; p-S129-AKT, AKT, p-S9-GSK3β, GSK3β, p-S380-PTEN, PTEN, p-S235/236-S6 and S6 from Cell Signaling (Danvers, MA, USA); Calnexin and GAPDH from Sicgen (Cantanhede, Portugal); 7-AAD from Invitrogen (Carlsbad, CA, USA) and B-cell lymphoma 2 (Bcl-2) from Dako (Glostrup, Denmark). Recombinant human sCD27 ligand and recombinant human IL-2 were purchased from Peprotech (Rocky Hill, NJ, USA); CX-4945 (Silmitasertib) from Adooq Bioscience (Irvine, CA, USA) and Biorbyt (Cambridge, UK); TBB from Sigma-Aldrich; TG-003 and Harmine from Focus Biomolecules (Plymouth Meeting, PA, USA).

Flow cytometry, cell viability, cell cycle and proliferation analysis

Cells were stained for the indicated cell surface markers, and intracellular staining was performed using fixation/permeabilization and permeabilization buffers (both from eBioscience), following the manufacturer's instructions. Cell apoptosis was analyzed by flow cytometry using Annexin-V/7-AAD staining as previously reported.[25] For cell cycle analysis, cells were stained for 1 h at 37 °C with 30 μl of 7-AAD (BD Pharmingen, San Diego, CA, USA) in permeabilization buffer (eBioscience). CFSE-based proliferation assays (CellTrace CFSE kit, Invitrogen, final concentration 0.5 mM) were performed as previously described.[4] Samples were acquired using LSRFortessa or Accuri C6 (both from BD Biosciences). Data were analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

CK2 kinase activity assay

CK2 activity was measured in cell lysates (from equal cell numbers) using the casein kinase-2 assay kit (17-132) from Upstate Biotechnology (Lake Placid, NY, USA), following the manufacturer's instructions. Briefly, total protein lysates were incubated for 10 min at 30 °C in a reaction mixture containing: CK2α-specific peptide, [γ-32 P]ATP and protein kinase A inhibitor cocktail. The radioactivity incorporated into the substrate was determined in P81 phosphocellulose paper-squares by scintillation counting as previously reported.[14] CK2 activity in $\gamma\delta^+$ and $\alpha\beta^+$ xenograft-derived blasts was measured on samples that showed comparable percentages of human engraftment (defined by fluorescence-activated cell sorting analysis based on the expression of CD45 and CD7 antigens).

Western blot analysis

Cell lysates were used for immunobloting as previously described.[14] Briefly, the cells were lysed, at 4 °C, in cold lysis buffer (50 mM Tris (pH 7.6), 150 mM EDTA, 1% Nonidet P-40 in phosphate-buffered saline) enriched with protease and phosphatase inhibitor cocktails (Roche, Burgess Hill, UK). The total proteins were quantified using a Bradford assay (Bio-Rad, Hercules, CA, USA), following the manufacturer's instructions. Equal amounts of total protein was denatured in Laemmli buffer (Bio-Rad), boiled for 5 min at 95 °C and loaded in a 10% SDS–polyacrylamide gel electrophoresis. After electrophoretic separation, the proteins were transferred to nitrocellulose blotting paper (Amersham Biosciences, Little Chalfont, UK). The membranes were blocked with 5% bovine serum albumin and 0.5% Tween-20 (Sigma-Aldrich) in phosphate-buffered saline and probed with the indicated primary antibodies overnight. After rinse, the membranes were probed using appropriate horseradish peroxidase-conjugated secondary antibodies and developed by chemiluminescence using the ChemiDoc XRS + imaging system (Bio-Rad).

In vivo mouse experiments

All experimental procedures were performed in strict accordance with the recommendations of the European Commission (Directive 2010/63/UE), French National Committee (87/848) and Portuguese authorities (Decreto-Lei 113/2013) for the care and use of laboratory animals. TCRαβ- or TCRγδ-positive T-ALL cells obtained from patient diagnostic were injected into 8–12-week-old NSG mice (1×10^6 cells/mouse, tail vein injections). Mice were monitored weekly by flow cytometry for human leukemic load ($hCD7^+$, $hCD45^+$ cells) in peripheral blood. Mice were killed when terminally ill and blast cells from bone marrow were collected. For the *in vivo* experiment of γδ T-ALL treatment, 10–12-week-old NRGS mice were injected subcutaneously in the right flank with 2×10^6 PEER cells resuspended in 100 μl of phosphate-buffered saline. At day 20, all mice presented palpable tumors (100–150 mm^3) and were randomly distributed into two groups ($n \geqslant 3$). The animals were treated with CX-4945 (75 mg/kg), by oral gavage twice daily (b.i.d.) or vehicle control (25 mM sodium bisphosphate buffer) as previously described.[26] Mice were monitored daily and weighed frequently. Tumors were measured every 2 days with a caliper and tumor volume was calculated (volume = (length × width)^2/2). At day 13, after starting the treatment, all mice were killed (an ethical requirement for the control group, when tumor reached 2000 mm^3).

Statistical analysis

Statistical significance of differences between indicated conditions was assessed using Student's *t*-test with Welch's correction and is indicated when significant as $^*P < 0.05$; $^{**}P < 0.01$; $^{**}P < 0.001$. All statistical analyses were performed using GraphPad Prism software (San Diego, CA, USA).

RESULTS

Human γδ thymocytes have enhanced CK2 activity and are highly sensitive to its inhibition

This study initiated with the analysis of CK2 activity in normal thymocyte subsets obtained from pediatric thymic biopsies. We measured CK2 activity using a substrate-specific kinase assay in freshly isolated TCRγδ+ or TCRαβ+ cells, and unexpectedly found twofold higher activity in γδ thymocytes relative to their αβ counterparts (Figure 1a). To address its physiological relevance, we treated thymocytes for 24 h with a highly specific ATP-competitive inhibitor of CK2, CX-4945.[27] Flow cytometry analysis of Annexin-V/7-AAD-stained cells revealed increased apoptosis of γδ compared with $CD4^+$ and $CD8^+$ αβ thymocytes (Figure 1b). To assess the longer-term impact of CX-4945 treatment, thymocytes were cultured in the presence of TCR plus costimulation for 7 days. The γδ thymocytes were highly susceptible to apoptosis upon CK2 inhibition in a dose dependent-manner (Figure 1c). In contrast, as previously reported,[18] $CD4^+$ or $CD8^+$ αβ thymocyte survival was not significantly affected following CK2 inhibition (Figure 1c).

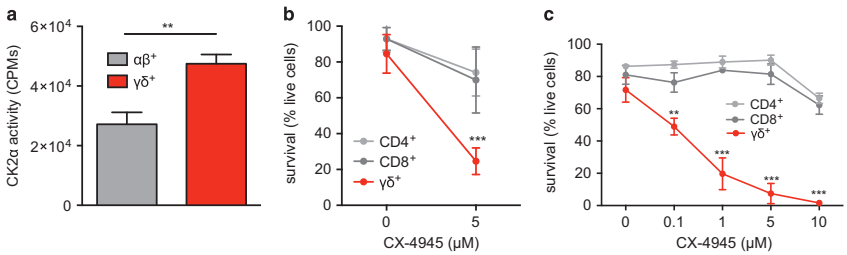

Figure 1. Human γδ thymocytes have enhanced CK2 activity and are highly sensitive to CX-4945. (**a**) *In vitro* CK2α activity (kinase assay) in freshly isolated human thymic γδ and αβ T-cells (2×10^6 cells per assay). CPM, counts per min. (**b**) Survival (% of live cells) of human thymic γδ, $CD4^+$ and $CD8^+$ T cells following 24 h of incubation with 5 μM of the CK2 inhibitor, CX-4945, analyzed by flow cytometry using Annexin-V/7-AAD staining. (**c**) Survival (% of live cells) of human thymic γδ, $CD4^+$ and $CD8^+$ T cells to different concentrations of CX-4945 (or vehicle), analyzed by Annexin-V/7-AAD staining following 7 days in culture with recombinant human IL-2 (rhIL-2) plus CD3+CD27 or CD3+CD28 stimulation of sorted thymic γδ or αβ T cells, respectively. Data in this figure are representative of at least three independent experiments; $^{**}P < 0.01$, $^{***}P < 0.001$ (*T*-test).

These data revealed that healthy γδ thymocytes are exquisitely dependent on their high basal CK2 activity for survival, and thus extremely sensitive to chemical inhibition using CX-4945. To verify that these effects were mediated by inhibition of CK2 rather than other kinases, CLK2 and DYRK1A, recently reported to be sensitive to CX-4945 treatment,[28,29] we also tested another CK2 inhibitor, 4,5,6,7-tetrabromobenzotriazole (TBB); TG-003 that specifically targets CLK2;[28,30] and Harmine that selectively inhibits DYRK1A.[31] Importantly, only the other CK2 inhibitor, TBB, reproduced the effects of CX-4945, whereas TG-003 and Harmine failed to affect γδ thymocyte survival (Supplementary Figure S1). These results strongly suggest that the physiological target of CX-4945 in γδ thymocytes is CK2.

CK2 activity in γδ thymocytes is modulated by TCR stimulation and promotes AKT signaling

We next asked which signals regulated CK2 activity in γδ thymocytes. Very few studies have documented CK2 modulation by physiological stimuli in T cells.[21,32] When we stimulated (for 6 h) isolated thymocyte subsets via the TCR complex using agonist anti-CD3ε antibodies, we observed an approximately threefold enhancement of CK2 activity selectively in γδ thymocytes (Figure 2a). We also tested the impact of costimulation, particularly through CD27 that we have shown to play a major role in γδ T-cell development and expansion.[4,33,34] However, the addition of soluble recombinant CD27-ligand/CD70 (sCD70) had no additive effect on CK2 activity (Figure 2a). Thus, our data suggest that CK2 activity in healthy γδ thymocytes is modulated primarily by TCR signals.

To gain insight into the downstream effects of CK2 signaling and its inhibition in γδ thymocytes, we focused on the AKT signaling pathway that is involved in cell survival and proliferation and known to be regulated by CK2 in both normal and malignant αβ T lymphocytes.[18,35] We observed that TCR/CD27 stimulation inhibited PTEN (phosphatase and tensin homolog), as measured by the increase in its phosphorylated form, and potentiated the AKT signaling pathway in γδ but not αβ thymocytes, as shown by the phosphorylation of AKT and its downstream targets glycogen synthase kinase-3β (GSK3β) and S6 (Figure 2b and Supplementary Table S1). These effects were completely reversed by CX-4945 (Figure 2b). As functional outcomes of CK2 inhibition, we observed decreased γδ thymocyte proliferation (Figure 2c) and survival (Figure 2d). Moreover, in agreement with the implication of AKT signaling downstream of CK2, we found a similarly striking effect on γδ thymocyte survival upon treatment with the specific AKT inhibitor, MK-2206 [ref. 36] (Figure 2d).

CD27-dependent upregulation of CK2 activity and downstream AKT signaling in γδ T-ALL

We next asked how CK2 activity would affect γδ T-ALL. First, we compared CK2 activity in normal γδ and αβ thymocytes versus γδ and αβ T-cell blasts obtained from T-ALL patients (and expanded in NSG mice, with similar engraftment, as detailed in the Materials and methods section). As expected, αβ T-ALL cells displayed higher levels of CK2 activity than αβ thymocytes (Figure 3). Notably, we detected markedly higher CK2 activity in γδ T-ALL cells as compared with healthy thymocytes and αβ T-ALL blasts (Figure 3). Moreover, the γδ T-ALL cell line PEER reproduced the very high CK2 activity observed in γδ T-ALL blasts (Figure 3), making it a good model for further biochemical and functional CK2 tests in γδ T-ALL.

The CK2 inhibitor, CX-4945, suppressed CK2 activity in γδ T-ALL cells in a dose-dependent manner (Figure 4a). As with healthy γδ thymocytes, the effects of CX-4945 were only reproduced by another CK2 inhibitor, TBB, but not by TG-003 or Harmine that selectively target CLK2 and DYRK1A, respectively (Supplementary Figure S1). Thus, in γδ T-ALL cells also, CX-4945 exerts its effects by suppressing CK2 activity. On the other hand, CK2 activity was enhanced upon activation, with CD27 costimulation having a synergistic contribution in γδ T-ALL cells (Figure 4b), in contrast with γδ thymocytes (Figure 2a). Of note, PEER cells are $CD27^+$ $V\delta1^+$ γδ T-ALL cells (Supplementary Figure S2). A CD27-dependent effect was also observed on AKT signaling (Figure 4c), and was completely abrogated upon CX-4945 treatment, also in a dose-dependent manner (Supplementary Figure S3).

Functionally, CK2 inhibition led to γδ T-ALL cell cycle arrest at G2/M phase, cell apoptosis and decreased Bcl-2 protein levels (Figure 4d). To further examine the functional impact of AKT activation downstream from CK2, we tried to rescue this apoptotic phenotype by expressing a myristoylated, constitutively active form of AKT.[37] Cells expressing myristoylated AKT displayed high levels of AKT phosphorylation that were insensitive to CK2 inhibition (Figure 4e). Importantly, AKT hyperactivation partially rescued γδ T-ALL survival under CX-4945 treatment (Figure 4f). On the other hand, CK2 activity was not affected by AKT hyperactivation (Supplementary Figure S4), collectively suggesting that AKT phosphorylation is

downstream rather than upstream of CK2, and that AKT is a key mediator of CK2 functions.

γδ T-ALL cells are highly sensitive to CK2 inhibition *in vitro* and *in vivo*

To further evaluate the functional impact of CK2 inhibition on γδ T-ALL survival, we compared the effect of CX-4945 treatment on primary γδ versus αβ T-ALL cells, as well as representative cell lines, PEER and MOLT-4, respectively.

Upon 48 h of *in vitro* treatment with CX-4945, we observed increased cell apoptosis in primary γδ T-ALL samples compared with αβ T-ALL cells (Figure 5a). We performed a more detailed test with the T-ALL cell lines, at various time points of incubation with CX-4945, and found a profound and dose-dependent effect on PEER (γδ T-ALL) that were significantly more susceptible to apoptosis that MOLT-4 (αβ T-ALL) cells (Figure 5b). These data suggest that γδ T-ALL cells, like healthy γδ thymocytes (Figures 1b and c), are considerably more sensitive to CK2 inhibition than αβ T-ALL cells.

Finally, the increased sensitivity of γδ T-ALL cells to CX-4945 treatment *in vitro* led us to explore its therapeutic potential *in vivo*. For this purpose, we established a xenograft model of human γδ T-ALL by injecting 2×10^6 PEER cells subcutaneously in immune-deficient NRGS (NOD-Rag1^{null} IL2Rgamma^{null}) mice. After the detection of palpable tumor, mice were equally distributed

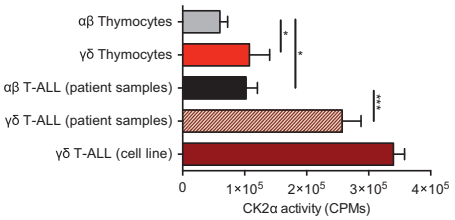

Figure 3. γδ T-ALL cells display higher CK2 activity than αβ counterparts. *In vitro* CK2α activity (kinase assay; 6.6×10^6 cells per assay) in freshly isolated γδ ($n=4$) and αβ ($n=4$) thymocyte samples; γδ ($n=6$) and αβ ($n=14$) T-cell samples obtained from T-ALL patients and expanded in NSG mice (as described in the Materials and methods); and the γδ T-ALL cell line, PEER ($n=4$). *T*-test, $^*P<0.05$, $^{***}P<0.001$.

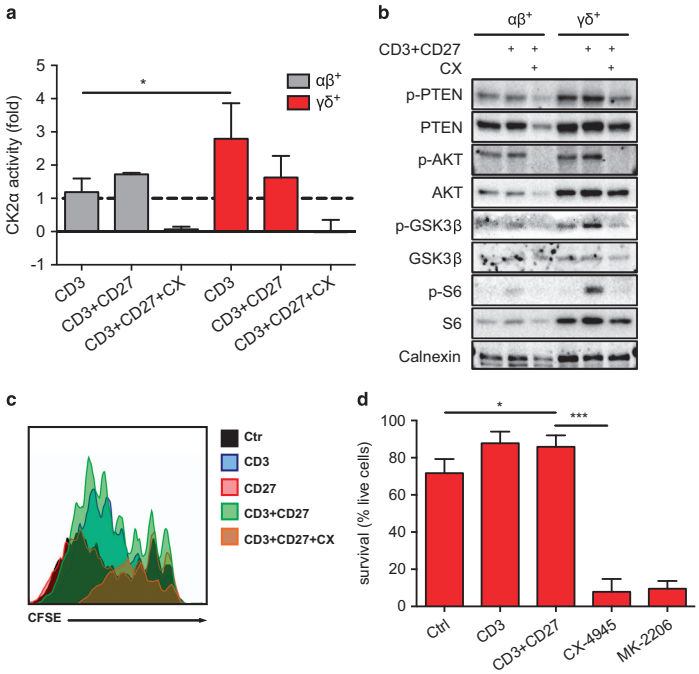

Figure 2. CK2 activity in γδ thymocytes is modulated by TCR stimulation and activates AKT signaling. (**a**) *In vitro* CK2α activity in sorted γδ and αβ thymocytes (2×10^6 cells per sample) after 6 h of stimulation with anti-CD3 antibodies (CD3), plus soluble CD27-ligand (CD3+CD27) or plus 5 μM CX-4945 (CD3+CD27+CX); values were normalized to unstimulated control (dashed line). (**b**) Western blot analysis of (phospho)proteins implicated in AKT signaling, in γδ and αβ thymocytes (1×10^6 cells per sample) stimulated as in (**a**). (**c**) Proliferation (CFSE dilution assay) of γδ thymocytes after 7 days in culture with recombinant human IL-2 (rhIL-2) under the indicated conditions: medium only (Ctrl); anti-CD3 antibody stimulation (CD3); soluble CD27-ligand (CD27); their combination (CD3+CD27); and with 5 μM CX-4945 (CD3+CD27+CX). (**d**) Survival (% of live cells) of γδ thymocytes after 7 days of stimulation (or not, Ctrl for control) with anti-CD3 antibodies (CD3), plus soluble CD27-ligand (CD3+CD27), plus 5 μM of CX-4945 or 10 μM of MK-2206. Data in this figure are representative of at least three independent experiments; $^*P<0.05$, $^{***}P<0.001$ (*T*-test).

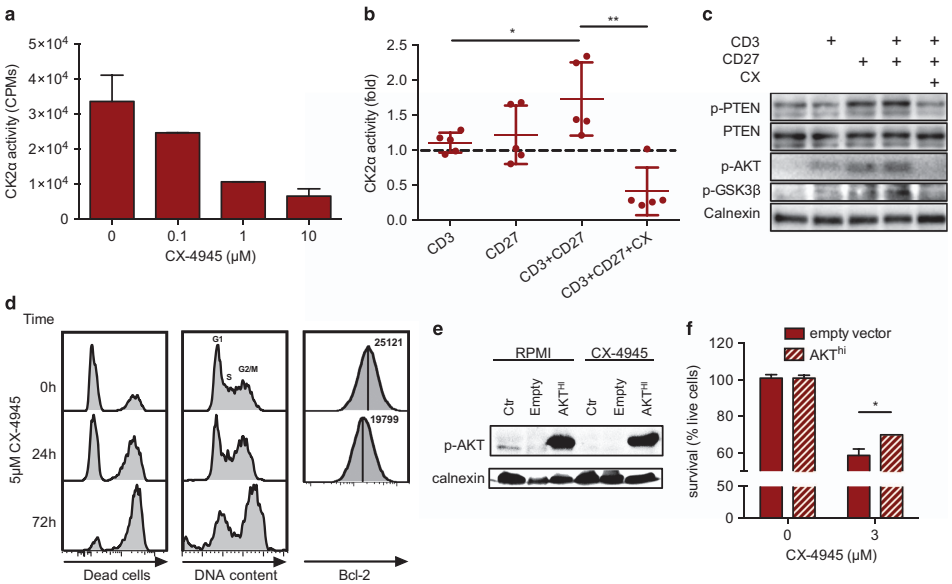

Figure 4. CK2 activity in γδ T-ALL cells is potentiated by CD27 costimulation and promotes AKT signaling. (**a**) CK2α activity in the γδ T-ALL cell line, PEER (2×10^6 cells per condition), after 6 h of treatment with indicated concentrations of CX-4945. (**b**) CK2α activity in lysates from γδ T-ALL (PEER) cells (2×10^6 cells per condition) after 6 h of stimulation under the indicated conditions (*T*-test, $^*P<0.05$; $^{**}P<0.01$). (**c**) Western blot analysis of (phospho)proteins implicated in AKT signaling, in γδ T-ALL (PEER) cells treated for 6 h with anti-CD3 antibodies (CD3), plus soluble CD27-ligand (CD3+CD27) or plus 5 μM CX-4945 (CD3+CD27+CX). Data are representative of five independent experiments. (**d**) Flow cytometry analysis of apoptosis (Annexin-V$^+$; left panel), cell cycle/DNA staining (middle panel) and intracellular Bcl-2 protein staining (right panel; values indicate mean fluorescence intensity (MFI)) of γδ T-ALL (PEER) cells treated with CX-4945 (5 μM) during the indicated times. (**e**, **f**) Western blot analysis of phospho-AKT (and calnexin loading control) (**e**) and cell survival after 48 h (**f**) of PEER cells transduced with a bicistronic retroviral DNA construct: either empty vector (LZRS) expressing only IRES followed by eGFP (LZRS-IRES-eGFP) or vector co-expressing myrPKB/AKT and eGFP (LZRS-myrPKB/AKT-IRES-eGFP) (AKT^{hi}) and treated with 3 μM CX-4945 or vehicle (*T*-test, $^*P<0.05$).

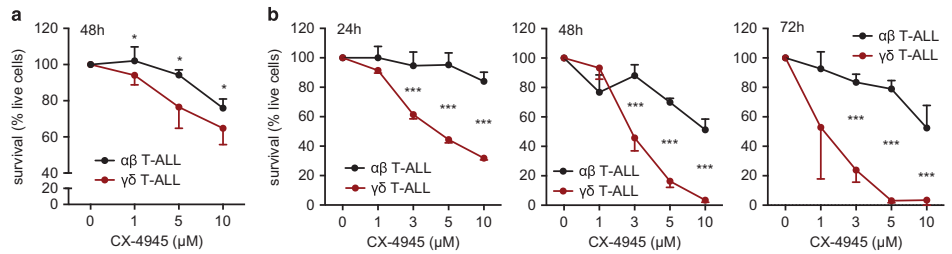

Figure 5. γδ T-ALL cells are more susceptible than αβ T-ALL to apoptosis induced by CX-4945. Flow cytometry analysis of the survival (Annexin-V/7-AAD staining) of (**a**) γδ ($n=5$) and αβ ($n=5$) T-cell blast samples (obtained from T-ALL patients and expanded in NSG mice) or (**b**) γδ (PEER) or αβ (MOLT-4) T-ALL cell lines, cultured for the indicated times with increasing concentrations of CX-4945 (*T*-test, $^*P<0.05$; $^{***}P<0.001$).

according to tumor burden into two groups to receive CX-4945 (orally, twice a day) or vehicle control. We observed a striking impact of CX-4945 treatment on tumor growth (Figure 6a). Upon killing the mice at day 18 (an ethical requirement for the control group), we scored great reductions in the CX-4945-treated group concerning the tumor weight (Figure 6b), as well its dissemination to the blood, bone marrow and spleen (Figures 6c–e). Of note, this therapeutic effect was dose dependent, as it was only observed with 75 mg/kg (Figures 6a–e) but not with 25 mg/kg (data not shown) of CX-4945. These data collectively demonstrate the potential of CK2 inhibition for treatment of γδ T-ALL.

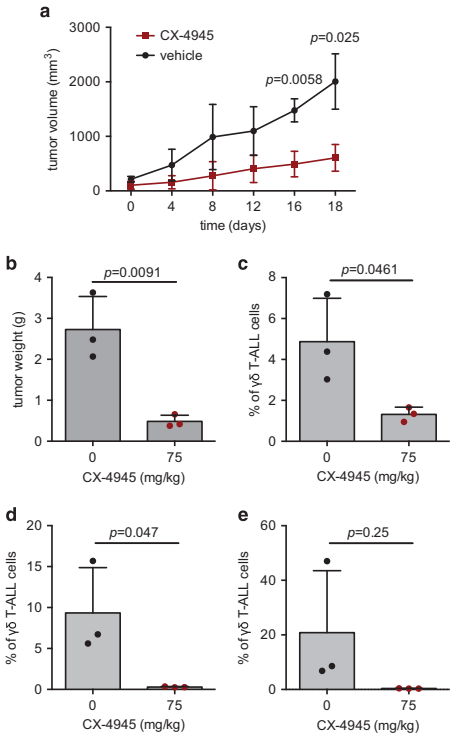

Figure 6. CX-4945 treatment inhibits γδ T-ALL growth *in vivo*. (**a**) Tumor volume following injection of 2×10^6 PEER γδ T-ALL cells subcutaneously into NRGS mice, treated with 75 mg/kg CX-4945 or vehicle (*T*-test, $*P < 0.05$, $**P < 0.01$). Day 0 refers to the start of treatment of mice bearing palpable tumors. (**b–e**) Tumor weight (**b**) or percentage of $CD45^+$ $CD7^+$ γδ T-ALL cells in the blood (**c**), bone marrow (**d**) or spleen (**e**) of mice killed after 18 days of treatment. Each dot represents an animal; *T*-test *P*-values are indicated.

DISCUSSION

Increased CK2 activity is typically associated with cell transformation in several hematological and solid tumors,[38] but few reports have demonstrated its relevance in physiological conditions.[32,39] Recent studies in mice have shown that epithelial cells and peripheral T cells depend on CK2 for their survival and function. CK2 was required for the survival of intestinal epithelial cells in inflammatory colitis,[40] for $CD4^+$ T-cell activation and differentiation into Th2 or Th17 cells[21] and for the suppressive function of $CD4^+$ $Foxp3^+$ regulatory T cells against allergy-promoting Th2 cells.[22] However, no physiological role has yet been attributed to CK2 in the human thymus or on healthy human T cells.

Here we identify a major role for CK2 that is restricted to the γδ lineage of human thymocytes. These display approximately twofold higher CK2 activity and are strikingly more sensitive to CK2 inhibition than their αβ counterparts. As we show that TCR stimulation increases (~2.5-fold) CK2 activity in γδ thymocytes, we may speculate that the different basal levels of CK2 activity in γδ versus αβ thymocytes are because of stronger TCR signals received during their development. It is well established that strong TCR signaling favors γδ over αβ T-cell lineage commitment[1,41–43] and further affects subsequent γδ thymocyte development.[44] In this line of reasoning, the high CK2 activity in agonist-selected γδ thymocytes could be an important pro-survival mechanism to counteract the activation-induced cell death underlying thymocyte negative selection.

We previously showed that αβ primary T-ALL cells display higher levels of CK2 expression and activity than immunophenotypically equivalent normal αβ thymocytes.[18] Most interestingly, we now demonstrate that the differential CK2 activity between the γδ and αβ T-cell lineages extends from healthy thymocytes to transformed T-ALL cells. Thus, primary γδ T-ALL cells displayed more than twofold higher CK2 activity compared with αβ T-ALL counterparts. As we previously showed that endogenous CK2 activity correlates with increased susceptibility to apoptosis upon CK2 inhibition,[14,18,19] this differential activity likely explains the higher sensitivity of γδ T-ALL cells observed in the present study.

Although rare, γδ T-ALL accounts for up to 10% of T-ALL cases, and this is significantly higher than the proportion (1%) of γδ thymocytes in the healthy thymus. This raises the possibility that γδ thymocytes have increased potential for malignant transformation.[10,11,45] A possible contributor to this phenomenon could be CD27 costimulation, as it increases CK2 activity (synergistically with TCR stimulation) in γδ T-ALL cells expressing high levels of CD27. Of note, the importance of CK2 in T-cell biology is also underscored by its modulation by the inhibitory receptor PD-1 that decreases CK2 activity and AKT signaling in $CD4^+$ T cells.[35]

The ability of CK2 to affect AKT signaling was previously reported in T-ALL.[14,18,19] Here we showed for the first time that a CK2-AKT link exists in γδ thymocytes and γδ T-ALL cells. Most important, we considerably extended previous knowledge by demonstrating that AKT is essential for CK2-mediated effects: (1) chemical AKT inhibition (with MK-2206) mimicked CK2 inhibition (with CX-4945); (2) the latter extinguished AKT signaling (AKT phosphorylation and downstream effects); and (3) ectopic expression of a constitutively active form of AKT partially rescued the apoptosis because of CK2 inhibition. These results suggest that, although AKT activation is not sufficient to fully mimic CK2 activity, it is absolutely required for CK2-mediated effects in γδ T cells.

Maximal AKT activation requires phosphorylation at Ser129 (as reported in our western blot analyses) by CK2 both *in vitro* and *in vivo*.[46,47] Activated AKT promotes cell survival through direct phosphorylation of anti-apoptotic molecules, or indirectly through the transcriptional activation of anti-apoptotic genes and increased metabolic capacity.[48,49] AKT inhibits GSK3β activity[50] by direct phosphorylation of an N-terminal regulatory serine residue, allowing glycogen and protein synthesis.[51] Inhibition of CK2 activity in γδ thymocytes or γδ T-ALL cells abrogated the AKT/GSK3β signaling pathway and had a major impact on cell survival and proliferation. Interestingly, the strong dependence on AKT may be specific for human γδ T cells, as AKT-deficient mice were reported to have a normal γδ T-cell pool in the periphery.[52]

Overall, our demonstration of the high sensitivity γδ T-ALL cells to CK2 inhibition with CX-4945 *in vitro* and *in vivo* clearly supports its use for γδ T-ALL treatment. Importantly, CX-4945 is currently in phase II clinical trials in patients with multiple myeloma or advanced solid cancers.[27]

Finally, our study has implications, not only for hematology, but also for cancer immunotherapy, as γδ T cells are known to play important roles in protective (antitumor) responses.[53] In particular, we have recently documented the potent antileukemia properties of $V\delta1^+$ γδ T cells expressing natural cytotoxicity receptors.[9] The success of their clinical application, particularly in adoptive cell therapy, will strongly depend on the capacity to survive *ex vivo*

TCR-mediated activation and *in vivo* establishment and expansion. We thus believe an increased knowledge of the molecular determinants of γδ T-cell survival, as disclosed here for CK2, will be key to optimize their performance in cancer immunotherapy.

CONFLICT OF INTEREST

BSS is co-founder and share holder of Lymphact SA. The other authors declare no conflict of interest.

ACKNOWLEDGEMENTS

We thank Miguel Abecasis and Rui Anjos (Hospital de Santa Cruz) and Ana E Sousa (IMM) for provision of the pediatric thymic samples; Paul Coffer for the myrPKB/AKT construct; and Francisco Caiado, Daniel Correia, Alice Melão, Daniel Ribeiro, Natacha Sousa, Hiroshi Kubo, Helena Nunes Cabaço, Pedro Oliveira and Tiago Amado (all IMM) for technical assistance. This work was supported by project grants from the European Research Council, CoG_646701 (to BSS), Fundação para a Ciência e Tecnologia (FCT), PTDC/DTP-PIC/4931/2014 (to BSS), EXPL/IMI-IMU/0170/2013 (to JCR), PTDC/SAU-ONC/113202/2009 and PTDC/SAU-ONC/122428/2010 (to JTB); and ARC–Association pour la Recherche sur le Cancer (to MT); and by individual fellowships from FCT, SFRH/BD/84123/2012 (to STR) and IF/00013/2014 (to JCR).

AUTHOR CONTRIBUTIONS

STR planned and performed the experiments and wrote the paper; MT planned and performed some experiments; JCR and EM helped to plan and/or perform the experiments; BS-S and JTB supervised the project, planned experiments and wrote the paper.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)