FCRL3 is an immunomodulatory receptor that restrains the activation of human memory T lymphocytes

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Table of Contents

Abstract

T lymphocytes are central mediators of adaptive immunity, orchestrating responses that ensure the targeted elimination of pathogens. $CD4^+$ T lymphocytes primarily function through the secretion of cytokines, which coordinate the activity of other immune cells, whereas $CD8^+$ T lymphocytes directly eliminate infected or malignant cells. The initiation of an effective immune response requires accurate antigen recognition, followed by tightly regulated gene expression programs that drive a robust yet controlled immune activation. Such regulation ensures the efficient clearance of infected cells while enabling restoration of immune homeostasis once the pathogen has been eliminated. Failure of these regulatory mechanisms can result in sustained or excessive T cell activity, leading to pathological outcomes such as chronic inflammation and autoimmunity. The family of Fc receptor–like (FCRL) proteins exhibits immunomodulatory functions. The presence of immunoreceptor tyrosine-based activation motifs (ITAMs) and inhibitory motifs (ITIMs) within their cytoplasmic domains suggests that FCRL molecules play a role in coordinating immune responses, contributing to the maintenance of the delicate balance required to initiate or terminate effector functions while limiting collateral damage to host tissues.

In this thesis, I investigated the role of FCRL3, one of the few FCRL family members expressed by T lymphocytes. A functional variant of FCRL3, defined by a single nucleotide polymorphism (SNP) in the *FCRL3* promoter region, has been associated to several autoimmune diseases; however, FCRL3 function and mechanistic contribution to conventional T lymphocytes biology remained poorly defined. To address this, we performed an extensive functional characterization of the subsets of T lymphocytes expressing FCRL3. Our results across conventional T lymphocytes revealed that FCRL3 marks highly differentiated T cells with reduced proliferative and activation capacity but enhanced cytotoxic potential. Furthermore, using CRISPR-Cas9–mediated gene editing and ectopic expression approaches, we identified a direct role for FCRL3 in the negative regulation of T cell activation.

Collectively, these findings advance our understanding of how FCRL3 expression influences T cell effector function, providing novel insights into its potential contribution to immune regulation and autoimmunity.

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L'unica gioia al mondo è cominciare.
È bello vivere perché vivere è cominciare, sempre, ad ogni istante.

Cesare Pavese, *Il mestiere di vivere.*

List of Abbreviations

ACD	Asymmetric Cell Division
AP-1	Activator protein 1
APC	Antigen Presenting Cell
BCR	B Cell Receptor
CDR	Complementary Determining Region
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CTLs	Cytotoxic T Lymphocytes
DAG	Diacylglycerol
DAMP	Damage-Associated Molecular Pattern
DC	Dendritic Cell
DGKα	Diacylglycerol kinase α
DN	Double Negative
DP	Double Positive
ER	Endoplasmic Reticulum
ERK1/2	MAPK extracellular signal-regulated kinase-1 and 2
FCRLs	Fc-Receptor Like proteins
GADS	Grb2-related adaptor downstream of Shc
GCs	Glucocorticoids
Grb2	Growth factor receptor-bound protein 2
HLA	Human Leukocyte Antigen
IP_3	Inositol 1,4,5-trisphosphate
IR	Inhibitory Receptor
irAEs	Immune-Related Adverse Events
ITAM	Immunoreceptor Tyrosine-Based Activation Motif
ITIM	Immunoreceptor Tyrosine-Based Inhibition Motif
ITSM	Immunoreceptor Tyrosine-Based Switch Motifs
LAG-3	Lymphocyte-activation gene 3
LAT	Linker for Activation of T cells

Lck	Lymphocyte-specific protein tyrosine kinase
MALT	Mucosa-Associated Lymphoid Tissue
MAPKs	Ras-Mitogen-Activated Protein Kinases
MHC	Major Histocompatibility Complex
NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear Factor κB
NGS	Next Generation Sequencing
NK	Natural Killer Cells
NSCLC	Non-Small-Cell Lung Cancer
PAMP	Pathogen-Associated Molecular Pattern
PD-1	Programmed Death 1
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PLCγ1	Phospholipase C gamma1
pMHC	Peptide-MHC
PRR	Pattern-Recognition Receptor
pTregs	Peripheral Regulatory T Cells
RA	Rheumatoid Arthritis
RASGRP1	RAS guanyl nucleotide-releasing protein
S1PR1	Sphingosine-1-Phosphate Receptor 1
SH2	Src homology 2
SIgA	Secretory IgAs
SLE	Systemic Lupus Erythematosus
SLP-76	SH2-domain-containing leukocyte protein of 76 kDa
SNP	Small Nucleotide Polymorphism
SOS	Son of Sevenless
SP	Single Positive
T_{CM}	Central Memory Cells
TCR	T Cell Receptor
T_{EM}	Effector Memory Cells

T_{EMRA}	Terminally Differentiated CD45RA^{+} Effector-Memory Cells
TF	Transcription Factor
T_{FH}	T Follicular Helper cells
T_{H}	Helper T cells
TIGIT	T-cell immunoreceptor with Ig and ITIM domains
TME	Tumour Microenvironment
TNF	Tumor Necrosis Factor
TNFRSF	TNF receptor superfamily
TRAFs	TNF receptor-associated factors
Tregs	Regulatory T Cells
T_{RM}	Tissue-Resident Memory T cells
tTregs	Thymic Regulatory T Cells
UBASH3A	Ubiquitin Associated And SH3 Domain Containing A
UBASH3B	Ubiquitin Associated And SH3 Domain Containing B
ZAP-70	Zeta-chain-associated protein kinase 70

1. Foundations of T Cell Biology

1.1 The immune system

The immune system is a complex network of specialized cells, tissues, and molecules that protects the host from pathogenic microorganisms and malignant cells. Pathogens capable of causing disease in humans include viruses, bacteria, fungi, and eukaryotic parasites, which differ substantially in size, structure, and pathogenic mechanisms. To counter this diversity, the immune system comprises two main, interconnected branches: the innate and adaptive immune responses. In 1908, the Nobel Prize was awarded to Elie Metchnikoff and Paul Ehrlich, marking a milestone in the birth of immunology. Metchnikoff's work on phagocytosis established him as the father of innate immunity, while Ehrlich's research on antibodies laid the foundation for our understanding of humoral adaptive immunity (Kaufmann 2008). Today we recognize that innate and adaptive immune responses work together as complementary systems to detect, contain, and eliminate infectious agents and malignant cells, preserving tissue integrity and homeostasis.

Innate immunity is a defense mechanism made up of multiple layers: physical barriers, immune cells and soluble proteins that act together to prevent or restrict pathogen invasion. It provides a rapid, non-specific response. Epithelial barriers, including the skin and mucosal surfaces, are the first line of defense against invading microbes; following, cells of the innate immune system carry out different effector functions. Among these there are monocytes, dendritic cells, macrophages, mast cells, neutrophils, eosinophils, basophils, and natural killer cells. Innate immunity cells express pattern-recognition receptors (PRRs), which recognize non-specific microbial molecules that are evolutionarily conserved, called pathogen-associated molecular patterns (PAMPs), or damage-associated molecular patterns (DAMPs), which are expressed by host cells damaged or stressed. Some PRRs are located in the cytoplasm and detect intracellular microbial components. Activation of PRRs after recognition of PAMPs or DAMPs triggers a range of effector responses, including phagocytosis, inflammation, cytokine production, amplification of the immune response by recruitment of other immune cells (Akira, Uematsu et al. 2006). Lastly, the soluble component of innate immunity is the complement system, a network of plasma proteins that strengthens host defense by coating pathogens through a process known as opsonization, promoting their clearance (Ricklin, Hajishengallis et al. 2010).

Adaptive immunity is mediated by lymphocytes, which mount antigen-specific responses and generate immunological memory. Its defining feature is the extensive diversity of antigen receptors generated by somatic gene rearrangement, a mechanism elucidated by Susumu Tonegawa (Hozumi and Tonegawa 1976), that was awarded with the Nobel Prize in 1987. This process enables the human immune repertoire to recognize an estimated $10^{6\text{-}8}$ distinct antigens. Each lymphocyte expresses a single, unique receptor; upon

antigen recognition, the cell proliferates, producing a population of clones with identical specificity. The two major lymphocyte types are B and T cells. B cells express the B cell receptor (BCR), which can bind native antigens directly, and exists also in a secreted form as antibodies, or immunoglobulins (Igs). There are different classes of Igs (IgA, IgD, IgE, IgG or IgM), they are classified according to their constant regions, which determine the downstream responses they trigger. Upon antigen recognition, B cells mediate humoral immunity through antibody secretion. Among the different possible effector functions of secreted antibodies, they can neutralize and coat pathogens, making them easily recognized by the complement system or by phagocytes of innate immunity (LeBien and Tedder 2008). T cells express the T cell receptor (TCR), which recognizes peptide antigens only when they are presented by major histocompatibility complex (MHC) molecules on antigen-presenting cells (APC). The peptides bound to MHC can derive from either intracellular or extracellular sources. There are two main subsets of T cells: $CD4^+$ T lymphocytes and $CD8^+$ T lymphocytes. $CD4^+$ T cells recognize exclusively antigens presented in the context of MHC class II molecules, which are expressed by APCs of the immune system, such as dendritic cells, macrophages and B lymphocytes. In contrast, $CD8^+$ T cells recognize peptides presented by MHC class I molecules, expressed by all nucleated cells. This concept, whereby a T cell recognizes antigens only when they are presented by MHC molecules, is known as MHC restriction (Zinkernagel and Doherty 1997, Greenspan 2020). Functionally, T cells mediate cell-mediated immunity: $CD4^+$ T lymphocytes primarily activate other immune cells and regulate immune responses, whereas $CD8^+$ T lymphocytes directly kill infected or malignant cells (Zinkernagel and Doherty 1974, Mosmann and Coffman 1989).

Although this thesis focuses on T lymphocytes, it is important to emphasize that both B and T cells are essential for productive adaptive immune responses. Also, innate and adaptive immunity are interconnected; effective responses typically require the coordinated activity between the two systems. Cytokines and chemokines mediate this integration, functioning as key signalling molecules that regulate immune cell activation, differentiation, and migration. Cytokines can be pro-inflammatory or anti-inflammatory, while chemokines specifically direct immune cell trafficking to sites of infection or injury (Banyer, Hamilton et al. 2000).

1.2 TCR and Antigen Recognition

T lymphocytes detect the presence of pathogens when APCs express on their surface MHC molecules loaded with peptide fragments coming from pathogens. The TCR is able to recognize and bind these molecules, making it the most distinctive trait of T cells. This chapter will be focused on how this interaction occurs.

The T Cell Receptor and TCR repertoire

In the 1980s, groundbreaking research elucidated the clonality of the TCR, revealing that the genes encoding the TCR undergo somatic rearrangement to generate a unique receptor for each T cell, enabling vast diversity in antigen recognition. In the next decade, major advances were made in defining the molecular and protein structure of the TCR, which was critical for understanding T cell specificity and immune function (Reinherz 2014, Shi, Strasser et al. 2024).

The TCR is a heterodimer that is made of two different transmembrane polypeptide chains, the α and β chains, linked together by a disulfide bond. Therefore, conventional lymphocytes are named αβ T cells. This is in contrast with a rarer subpopulation of γδ T cells, whose TCR consists of a γ and δ chain. This subpopulation of T cells will be further discussed below. The amino-terminal variable region of the α and β chains (Vα and Vβ) forms the antigen-binding domain of the TCR, and indeed the genes which encode the antigen-binding domain during lymphocyte development in the thymus undergo a random somatic recombination process. In the variable region, three hypervariable loops per chain are found, they are named Complementary Determining Regions (CDRs) because they form the portion of the protein that is complementary to the antigen that they bind and to the MHC that is presenting the antigen (**Figure 1**). The CDR sequences have the highest variability in both α and β chains and determine the ability of a T cell to recognize an antigen peptide presented by the MHC molecule. The carboxy-terminal region of α and β chains (Cα and Cβ) instead is constant between different TCRs and it is important for the interactions with the CD3 molecules, that are closely associated with the TCR and play a crucial role in signal transduction. The αβ-TCR heterodimer together with six additional signalling CD3 subunits form the TCR Complex. Specifically, the CD3 molecules are CD3γ, CD3δ, two copies of CD3ε and two copies of CD3ζ. The CD3 portion is not involved in antigen recognition but it is essential for signal transduction (Garcia, Teyton et al. 1999, Rudolph, Stanfield et al. 2006). The binding between the TCR and MHC molecules, with their respective subunits highlighted, is represented in **Figure 1**.

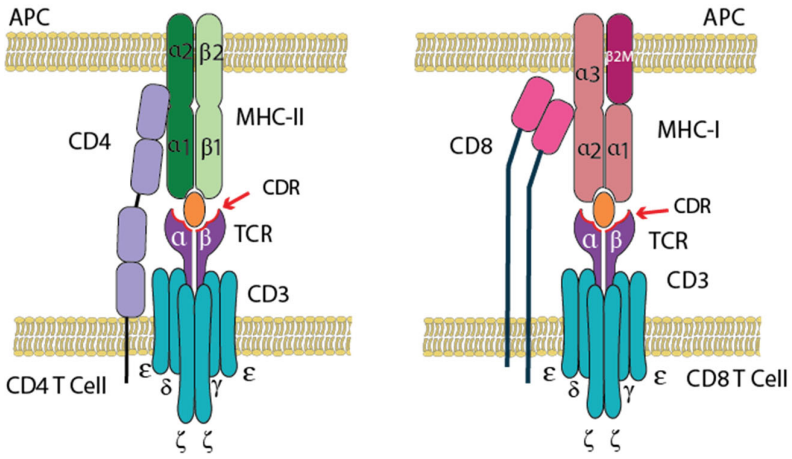

Figure 1. Representation of the T cell receptor (TCR) bound to peptide–MHC class I (pMHC-I, right) and peptide–MHC class II (pMHC-II, left). The TCR α and β chains are shown in purple, while the CD3 complex is shown in blue, with individual subunits (CD3γ, CD3δ, CD3ε, and CD3ζ) indicated. The figure shows the distinct roles of CD4 and CD8 in stabilizing TCR–pMHC interactions: CD4 engages MHC class II molecules, whereas CD8 engages MHC class I molecules.

TCR repertoire. Upon recognition of the MHC-peptide complex and activation, T cells proliferate and produce a progeny of cells that expresses the same TCR of the parental cell. The T cells which share the same TCR derived from the same parental cell are referred to as clones, and a set of clones is defined a clonotype. The total number of distinct TCRs expressed by the estimated $10^{12\text{-}13}$ T lymphocytes circulating in the human body was first quantified in 1999 using TCR gene amplification and Sanger sequencing. It was shown by Arstila, Casrouge and colleagues that the peripheral blood TCR repertoire consists of at least 10^6 different TCR-β chains. Another layer of diversity is introduced by the heterodimerization of TCR chains. On average, one β-chain pairs with at least 25 different α-chains, this yields a total αβ TCR diversity greater than the β-chain diversity alone. The α-chain repertoire itself is less variable, with approximately 0.5×10^6 distinct α-chains detected. When the analysis was restricted to the memory T lymphocytes, cells that expanded after initial antigen recognition, the αβ TCR diversity was estimated to be $2x10^5$. This indicates that naïve T cells, which have not yet encountered an antigen, are responsible for the highly diverse repertoire, while memory T cells account for only 1% of the total repertoire diversity (Arstila, Casrouge et al. 1999). A detailed description of naïve and memory T lymphocytes will be provided in Chapter 1.4. The advent of high-throughput next-generation sequencing (NGS) has revolutionized the characterization of the TCR repertoire, markedly reducing sequencing costs and establishing TCR sequencing as a widely adopted approach to study T cell populations. A commonly employed strategy involves multiplex PCR-based assays performed directly on genomic DNA, using pools of forward and reverse primers designed to maximize coverage and ensure comprehensive representation of gene segments that together encode the antigen-binding region of a TCR (De Simone, Rossetti et al. 2018). NGS techniques confirmed that the diversity of TCR-β chains ranges from approximately 1×10^6 to 1×10^8 unique

sequences (Robins, Campregher et al. 2009, Warren, Freeman et al. 2011, Zarnitsyna, Evavold et al. 2013, Li, Hiroi et al. 2015). However, a critical consideration is that the TCR repertoire data reported in these studies are subject to important technical limitations. TCR diversity is vast, a single blood sample cannot capture the full repertoire; high-throughput sequencing techniques are subject to biases and sequencing errors, and the sequencing depth is insufficient to cover all the clonotypes present in the repertoire. As a result, these estimates may distort the true αβ-TCR diversity (Laydon, Bangham et al. 2015, Sun, Nguyen et al. 2022).

Increasing age is associated with a reduction in αβ-TCR repertoire richness (Britanova, Shugay et al. 2016, Yoshida, Cologne et al. 2017). Of particular interest, Sun, Nguyen, and colleagues performed a longitudinal analysis of age-associated changes in the αβ TCR repertoire across naïve and memory $CD4^+$ and $CD8^+$ T cell subsets in healthy adults over a span of 70 years. They confirmed an overall reduction in αβ-TCR repertoire richness, most pronounced in naïve $CD8^+$ T cells, along with increased clonal expansion, particularly within the memory $CD8^+$ T cell compartment (Sun, Nguyen et al. 2022). Age-related changes in the TCR repertoire are influenced by multiple factors, including thymus involution and reduced thymic output, an individual's history of infections (Khan, Shariff et al. 2002, Wertheimer, Bennett et al. 2014), certain diseases, such as cancer, which can result in an abnormally narrow TCR repertoire (Rudqvist, Pilones et al. 2018, Porciello, Franzese et al. 2022) and genetic elements, including allelic variants of Human Leukocyte Antigen (HLA) genes, coding for MHC molecules, which will be described in the following paragraph. Overall, the TCR repertoire of each adult is unique and serves as a fingerprint of healthy immunity: an abnormal TCR repertoire may indicate underlying pathologies challenging the immune system. A schematic representation of the TCR repertoire shaped by multiple factors is shown in **Figure 2**.

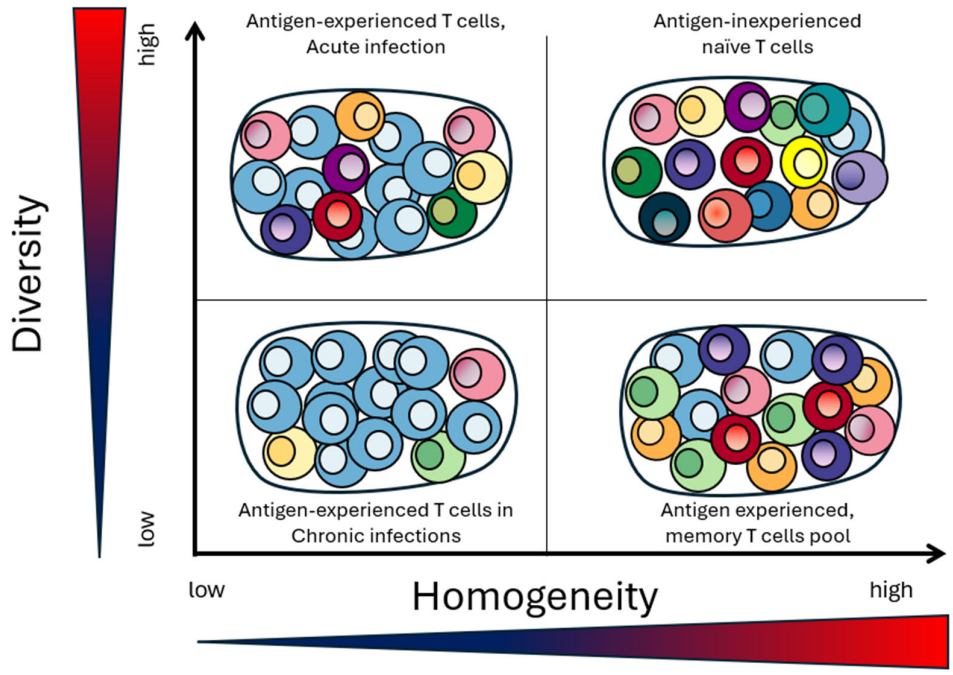

Figure 2 The TCR repertoire is shaped by multiple factors. Naïve T cells, prior to antigen encounter and clonal expansion, contribute to a highly diverse and relatively homogeneous repertoire (top, right). In contrast, memory T cells account for only ~1% of total repertoire diversity, with composition influenced by an individual's infection history, age, and genetic background (bottom, right). Alterations in repertoire composition may reflect underlying immunological challenges. During acute infections, antigen-specific responder clones expand and dominate the repertoire (top, left), whereas chronic infections can drive persistent skewing, resulting in an abnormally restricted repertoire (bottom, left).

The Major Histocompatibility Complex

MHC molecules are specialized glycoproteins that bind peptide fragments derived from pathogens and expose them on the surface of cells, so that they will be recognized by T lymphocytes. As described more in detail below, MHC molecules are polygenic and polymorphic: these characteristics make them able to bind a large variety of antigens so that specific clones of T cells are activated. In humans, the cluster of MHC genes is called Human Leukocyte Antigen (HLA), and it contains more than 200 genes. In this locus there are genes that code for MHC class I and II molecules, that differ in terms of structure and in their expression pattern in the human body (Neefjes, Jongsma et al. 2011, Rock, Reits et al. 2016).

The three main genes that are coding for MHC Class I molecules are HLA-A, HLA-B and HLA-C; each one of these encodes for the full α-chain of their respective MHC molecule. An essential protein for the stability of the MHC Class I complex is β2-microglobulin, which is encoded by a gene not located in HLA locus. MHC Class I molecules are expressed by all nucleated cells and are bound by the surface-protein CD8, expressed by $CD8^+$ lymphocytes (Bjorkman, Saper et al. 1987, Trowsdale and Knight 2013). The interaction pMHC Class I:CD8 is represented in **Figure 1, on the right.**

The human MHC Class II locus contains HLA-DR, HLA-DP, HLA-DQ and HLA-DM, which encode the α and β subunits of their respective MHC II molecule. HLA Class II region includes also other genes that are involved in the processing and presentation of antigen to the immune system, such as the LMP/TAP cluster. MHC Class II molecules are expressed only by immune cells and are bound by the surface protein CD4, expressed by $CD4^+$ lymphocytes (Stern, Brown et al. 1994, Trowsdale and Knight 2013). The binding pMHC Class II:CD4 is represented in **Figure 1, on the left.**

As mentioned above, MHC molecules are polygenic: every person has 3 genes encoding MHC I molecules and 4 genes encoding MHC II molecules. These molecules are all co-dominantly expressed, meaning that both maternal and paternal genes are expressed at the same time. In this way, every individual can express 6 different MHC I molecules and 8 different MHC II molecules simultaneously. The human MHC loci encodes for the most polymorphic genes in humans, with many HLA genes bearing hundreds to thousands of alleles that diversify peptide-binding specificities. The MHC I and MHC II molecules that they produce bind short peptides derived from pathogens or self-neoplastic cells, and display them to T cells (Choo 2007). The IPD-IMGT/HLA Database, launched in 1998 by the Anthony Nolan HLA Informatics Group, currently lists 28`983 class I alleles and 13`210 class II alleles; HLA-B alone exceeds 10`000 alleles, underscoring its extreme diversity (Trowsdale and Knight 2013, Robinson, Barker et al. 2020). Every individual expresses two alleles per gene locus, but at the population level this polymorphism will determine a huge diversity. Because HLA genes are co-dominantly expressed, individuals typically express both parental alleles at each locus, while population-level polymorphism generates vast inter-individual diversity in antigen presentation. From an evolutionary perspective, the high polymorphism of MHC within the population ensures that, in the event of a completely new pathogen emerging, at least some individuals in a population are capable of mounting an effective immune response, surviving otherwise lethal pathogens.

MHC molecules bind peptides generated by proteolysis, not whole proteins, lipids, or polysaccharides. The αβ-TCR recognizes and bind both the MHC molecule and the peptide presented, therefore αβ T cells effectively encounter and respond only to peptide antigens. Instead, the co-receptors CD4 and CD8 bind to invariant portions of MHC II and MHC I, respectively (Pishesha, Harmand et al. 2022, Margulies, Jiang et al. 2023). 1-5% of T cells circulating in peripheral blood belong to the minor γδ-T cell lineage. Unlike αβ T cells, γδ T cells are not MHC-restricted and can recognize a wide range of non-peptide antigens, such as metabolites produced by pathogens and cancer cells. Similarly to αβ-TCR, γδ-TCR is associated to CD3 molecules that are crucial for signal transduction (Hu, Hu et al. 2023).

The TCR signalling pathways and T cell activation

Proximal TCR signalling

Following peptide-MHC (pMHC) complex engagement to the TCR, the information that a specific antigen has been recognized is transduced into the intracellular compartment through conformational changes. Ligand affinity and concentration provide signals of varying intensity: if the pMHC signal is high-affinity and long-lasting, allosterically regulated changes are induced into the αβ chains of the TCR and are propagated to CD3 molecules through mechanical force and biophysical alterations. CD3 chains contain a total of 10 ITAMs distributed in different CD3 molecules: after conformational changes are induced, CD3 molecules expose their ITAMs on the intracellular side (Acuto, Di Bartolo et al. 2008, Lanz, Masi et al. 2021, Shah, Al-Haidari et al. 2021). ITAM motifs mediate transduction of activation signals and are defined as (E/D)-X-XY-X-X-(L/I)-$X_{6–8}$-Y-X-X-(L/I). The tyrosine residues of these motifs are phosphorylated by Src family kinases, such as Lck, and upon phosphorylation they can recruit other tyrosine kinases, such as Syk and ZAP-70, initiating signalling cascades that culminate in cellular activation (Reth 1989, Cambier 1995, Turner, Schweighoffer et al. 2000, Fodor, Jakus et al. 2006, Wang, Kadlecek et al. 2010).

Upon engagement of peptide-MHC by the TCR, the intracellular tails of the co-receptors (CD4 or CD8) deliver Lck, a tyrosine kinase of the Src-family, to the CD3 chains, triggering phosphorylation of the exposed ITAMs and the onset of proximal signalling. This is the earliest phosphorylation event known upon TCR engagement. The extent of ITAM phosphorylation depends on the strength and duration of the TCR-peptide-MHC interaction and on the availability of the active isoform of Lck, which is tightly regulated. A fraction of constitutively active Lck is always present in lymphocytes, but the equilibrium between the active and inactive isoform is controlled via the action of kinases and phosphatases, establishing a dynamic equilibrium between the different forms of Lck. Primarily, the membrane protein CD45 inactivates Lck by dephosphorylating tyrosine residues within the protein. Conversely, the kinase Csk phosphorylates Lck, promoting its kinase activity. This regulatory network tunes Lck availability and enables initiation of TCR signaling upon antigen engagement (Salmond, Filby et al. 2009, Fernández-Aguilar, Vico-Barranco et al. 2023).

When doubly phosphorylated by Lck, CD3 ITAMs recruit the cytoplasmic tyrosine kinase ZAP-70 by binding to its Src homology 2 (SH2) domains. SH2 domains are protein modules of approximately 100 amino acids whose function is to bind tyrosine-phosphorylated sequences, linking receptor activation to downstream signalling, and are frequently found in signalling cascades. Normally, multiple copies of ZAP-70 are recruited at different subunits of CD3. Lck phosphorylates ZAP-70, catalytically activating it, and

further ZAP-70 auto-phosphorylation completes its own activation (**Figure 3**) (Waksman, Kumaran et al. 2004, Wang, Kadlecek et al. 2010, Fernández-Aguilar, Vico-Barranco et al. 2023).

ZAP-70 is the pivotal kinase that propagates signals downstream of TCR engagement. Two related negative regulators, the de-phosphatases UBASH3A and UBASH3B (also known as STS-2/TULA and STS-1/TULA-2), limit ZAP-70 activity. Both proteins contain a ubiquitin-associated (UBA) domain, which binds ubiquitin (Hofmann and Bucher 1996), and a Src-homology 3 (SH3) domain that recognizes proline-rich motifs common in signalling proteins (Kurochkina and Guha 2013). Ubiquitination of ZAP-70 promotes its engagement by UBASH3A/B; these phosphatases then dephosphorylate ZAP-70 and act partly redundantly to dampen proximal TCR signalling (Carpino, Turner et al. 2004, Feshchenko, Smirnova et al. 2004, San Luis, Sondgeroth et al. 2011, Yang, Chen et al. 2015, Tsygankov 2024). ZAP-70 ubiquitination is controlled by the ubiquitin-ligase NRDP1, which promotes recruitment of the phosphatases UBASH3A/B to dephosphorylate ZAP-70 (**Figure 3**) (Yang, Chen et al. 2015); removal of this ubiquitin mark by the deubiquitinating enzyme OTUD7B counteracts the brake and prolongs TCR signalling (Hu, Wang et al. 2016).

The central role of ZAP-70 in propagating TCR signals is underscored by human loss-of-function mutations that cause severe combined immunodeficiency (SCID), with absent peripheral $CD8^+$ T cells and numerically preserved but signalling-impaired $CD4^+$ T cells (Elder, Lin et al. 1994, Wang, Kadlecek et al. 2010). In mice, complete ZAP-70 deficiency results in a near-complete absence of mature peripheral T cells (Negishi, Motoyama et al. 1995).

Sustained activation of ZAP-70 molecules drives the next signalling module. Activated ZAP-70 phosphorylates the adaptor proteins LAT and SLP-76. Adaptors are non-catalytic molecules but rich in modular binding domains that form multiprotein signalosomes, integrating and amplifying receptor-proximal signals. LAT is a transmembrane adaptor with a long cytoplasmic tail containing nine conserved tyrosines that, upon phosphorylation, serve as docking sites for cytosolic proteins such as the adaptors Grb2 and GADS, and PLCγ1. Proline-rich motifs in SLP-76 engage the PLCγ1 SH3 domain, promoting assembly of the LAT–GADS–SLP-76–PLCγ1 complex (**Figure 3**) (Bubeck Wardenburg, Fu et al. 1996, Zhang, Sloan-Lancaster et al. 1998, Fernández-Aguilar, Vico-Barranco et al. 2023). PLCγ1 is a central signalling molecule in T cells: it is the connecting link between proximal and distal signalling pathways. Phosphorylation on its multiple tyrosine residues is required for its full activation. The Tec-family kinase ITK is recruited to the LAT–GADS–SLP76–PLCγ1 complex via SLP-76; Lck-mediated phosphorylation activates ITK, which then phosphorylates its substrate PLCγ1 (Nishibe, Wahl et al. 1990, Shah, Al-Haidari et al. 2021).

Activated PLCγ1 hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP_2), a phosphoinositide enriched at the plasma membrane, producing diacylglycerol (DAG), a membrane-retained lipid, and inositol 1,4,5-

trisphosphate (IP_3), a soluble second messenger that binds IP_3 receptors on the endoplasmic reticulum to trigger Ca^{2+} release. PLCγ1 activation is a pivotal step in T-cell signalling, as these products start three distinct signalling branches that culminate in the activation of transcription factors NFAT, NF-κB and AP-1 (Hwang, Byeon et al. 2020, Shah, Al-Haidari et al. 2021). These pathways will be explained below in the distal signalling paragraph. The essential role of PLCγ1 in T cell development and activation is highlighted by mice in which PLCγ1 deletion is confined to the T cell lineage: these animals show defective thymic maturation, reduced proliferation and cytokine production, profound peripheral T cell lymphopenia, and spontaneous autoimmune disease (Fu, Chen et al. 2010). PLCγ1 is also one of the frequently altered genes found in human cancers (Mandal, Bandyopadhyay et al. 2021, Sakihama and Karube 2022).

VAV is another protein recruited at the LAT–GADS–SLP-76 complex. It is an adaptor protein which acts as a guanine nucleotide exchange factor (GEF), a protein that activates small GTPases by catalysing the exchange of GDP for GTP, switching them to the active state. VAV downstream signalling pathways lead to actin polymerization and cytoskeletal rearrangements (Fischer, Kong et al. 1998, Shah, Al-Haidari et al. 2021).

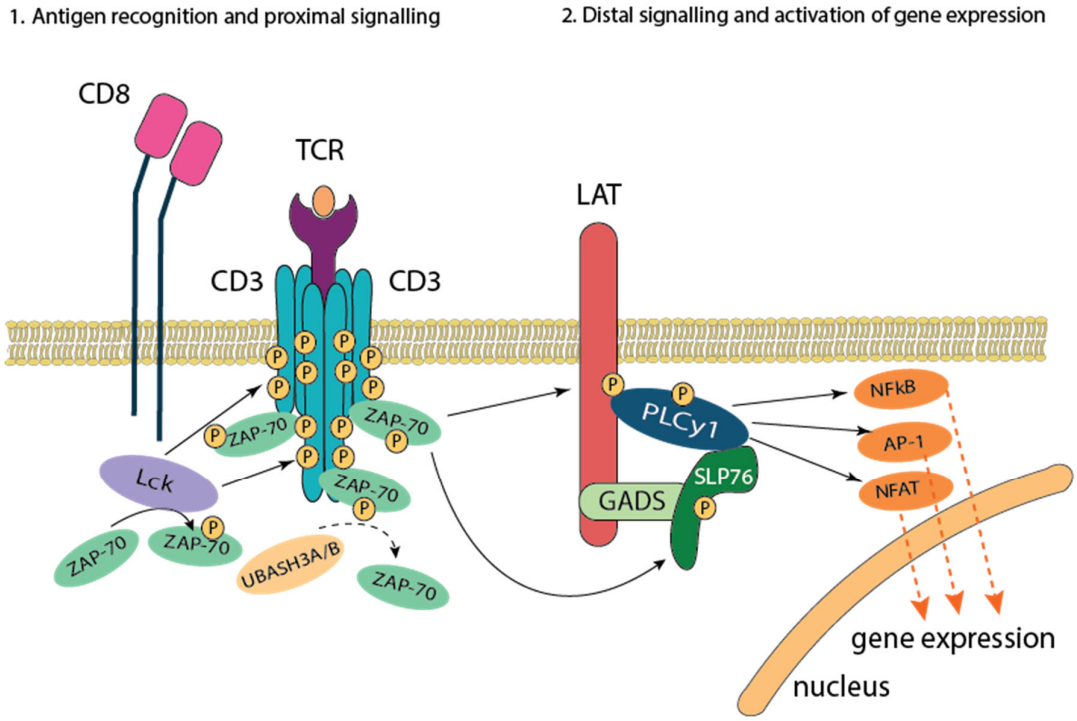

Figure 3. T-cell receptor proximal and distal signalling modules. 1) The initial events following antigen recognition involve co-receptors (CD4 or CD8) delivering Lck to the CD3 chains, leading to phosphorylation of exposed ITAMs and initiation of proximal signalling. Doubly phosphorylated ITAMs recruit the cytoplasmic kinase ZAP-70, whose activity is negatively regulated by the phosphatases UBASH3A and UBASH3B. Sustained ZAP-70 phosphorylation drives module 2), in which activated ZAP-70 phosphorylates the adaptor proteins LAT and SLP-76. These adaptors function as scaffolds for the recruitment of PLCγ1, a central signalling molecule that links proximal events to distal pathways. The downstream cascades culminate in the activation of three key transcription factors, NFAT, AP-1, and NF-κB, ultimately driving gene transcription and T cell activation.

Another key early step is clustering of the TCRs at the plasma membrane. Clustering locally concentrates receptors, adaptors, and kinases, raising the effective molarity of enzymes and substrates; this enhances encounter rates, stabilizes ITAM phosphorylation, and promotes sustained signal propagation (Acuto 2024). The complexes that assemble couple the TCR-proximal signalling to distal pathways, converting antigen recognition into transcriptional programs that drive T-cell activation and differentiation. TCR-proximal signalling to distal pathways are represented in **Figure 3**.

Distal TCR signalling

Ca^{2+}–calcineurin–NFAT pathway. PLCγ1 is the main molecule connecting the TCR proximal to distal signalling cascades. It produces IP_3, which triggers the activation of a Ca^{2+} -dependent calcineurin nuclear factor of activated T cells (NFAT) pathway. When IP_3 binds calcium channel receptors on the endoplasmic reticulum (ER), Ca^{2+} stored in the ER is released in the cytoplasm (Kania, Roest et al. 2017). Increased intracellular Ca^{2+} activates a protein phosphatase, calcineurin, that dephosphorylates NFAT, thereby causing its nuclear translocation. In resting cells, NFAT is kept phosphorylated, and inactive, in the cytosol; after calcineurin-dependent activation, NFAT activates a wide array of genes. If NFAT forms a complex with another transcription factor, AP-1, they induce the expression of various genes crucial for T cell activation and survival, including cytokines genes, such as interleukin 2 (IL-2), and other effector molecules (Macian 2005). If AP-1 is absent, NFAT alone activates various genes, like several ubiquitin ligases and diacylglycerol kinase α (DGKα), that are responsible for T cell anergy, a state of T cell unresponsiveness. Thus, two opposite T cell functions, activation and anergy, are controlled by NFAT (Macián, García-Cózar et al. 2002, Shah, Al-Haidari et al. 2021). To emphasize the importance of this pathway in the physiology of T lymphocytes, calcineurin inhibitors (CIs) are clinically employed to suppress T cell function in autoimmune diseases, such as severe complications of systemic lupus erythematosus (SLE) (Ponticelli, Reggiani et al. 2021, Chen, Song et al. 2022).

RASGRP1–RAS–ERK1/2–AP1 pathway. PLCγ1 generates DAG, which induces the activation of another key molecule, a RAS guanyl nucleotide-releasing protein (RASGRP1), and recruits it to the plasma membrane. RASGRP1 and Son of Sevenless (SOS) are two known guanine nucleotide exchange factors (GEFs) responsible for Ras activation in T cells. Ras, a small G protein, in the activated state binds to GTP and initiates the Ras-mitogen-activated protein kinases (MAPK) cascade. MAPKs phosphorylate and activate other kinases, called MAPK extracellular signal-regulated kinase-1 and 2 (ERK1/2), which in turn control T cell development, differentiation, and TCR-induced signal strength (Kolch 2005). ERK1/2 promote activation of the dimeric complex AP-1 (Fos/Jun), a transcription-factor complex that cooperates with NFAT to drive immune-response gene expression (Shah, Al-Haidari et al. 2021).

PKCθ–IκB–NFκB pathway. DAG and Ca^{2+} regulate activation of the serine/threonine kinase PKC-θ, which initiates a series of steps that results in the activation of nuclear factor κB (NFκB). NFκB is a transcription factor that plays important roles in regulating genes involved in inflammatory and immune responses, cell growth, survival, and differentiation. In resting cells, NFκB is held in the cytoplasm in its inactive state by its inhibitor, IκB; PKC-θ starts a signalling that leads to the degradation of IκB, making NFκB able to enter the nucleus and activate is targets (Matsumoto, Yamada et al. 2002, Blanchett, Boal-Carvalho et al. 2021, Shah, Al-Haidari et al. 2021). Glucocorticoids (GCs) are among the most potent anti-inflammatory and immunosuppressive agents. A principal mechanism is the GR-dependent induction of IκB, which restrains NFκB activity and thereby limits transcription of pro-inflammatory cytokines (Auphan, DiDonato et al. 1995). In parallel, GCs repress the transcriptional programs of NFκB and AP-1, further dampening inflammatory gene expression (Hudson, Vera et al. 2018). GC-based therapies are widely used to control inflammation and allergic disease and to suppress T cell–mediated immune responses in solid organ transplantation, underlying the importance of NFκB-controlled pathways for T cell function (Rhen and Cidlowski 2005, Dashti-Khavidaki, Saidi et al. 2021).

Co-stimulatory receptors

Although TCR engagement is in principle sufficient to initiate signal transduction, in order to reach full T cell activation, with proliferation, enhanced survival, cell differentiation and metabolic reprogramming, secondary signals provided by co-stimulatory receptors are also required. In addition to presenting the antigen via MHC molecules, APCs express on their surface co-stimulatory molecules, that bind to co-stimulatory receptors expressed by T cells. These additional inputs amplify TCR signalling and/or trigger non-redundant pathways. The most important co-stimulatory molecules are members of the B7/CD28 family and of the TNF receptor superfamily (TNFRSF). CD28 is the principal and most extensively studied co-stimulatory receptor on T cells. Its ligands, B7-1 (CD80) and B7-2 (CD86), are displayed on dendritic cells with distinct kinetics: CD86 is constitutive and rapidly enhanced upon activation, whereas CD80 is induced later during inflammatory responses. Engagement of CD28 triggers in T cells Lck-dependent phosphorylation of Lck cytoplasmic tyrosine motifs, recruiting several kinases and adaptor proteins. Together these interactions lead to the activation of NFAT, AP-1 and NFκB. This drives the activation of robust IL-2 production, survival signalling, and metabolic reprogramming (Greenwald, Freeman et al. 2005, Esensten, Helou et al. 2016, Burke, Chaudhri et al. 2024). Another important co-stimulatory receptor of the same family as CD28 is ICOS (Amatore, Gorvel et al. 2020). Co-stimulatory receptors of the TNFRSF family include CD27, Ox40 (or CD134) and 4-1BB (or CD137). When TNFRSF members bind to their respective ligand, they recruit TNF receptor-associated factors (TRAFs), that link TNFRSFs to

NFκB and MAPKs activation. TNFRSFs ligands are generally upregulated in APCs after Toll-like receptor and/or interferon-signalling (Watts, Yeung et al. 2025).

As a counterbalance to the positively acting elements downstream of the TCR, T cells possess a variety of mechanisms to limit the duration and the intensity of signals generated by receptor activation (Koretzky and Myung 2001, Veillette, Latour et al. 2002). The B7/CD28 family includes Cytotoxic T Lymphocyte Antigen 4 (CTLA-4), Programmed Death 1 (PD-1) and TIGIT, which have opposing roles of CD28 and ICOS: they inhibit TCR signalling, with important implications for immunomodulatory therapies that will be described in Chapters 2.2 and 2.3 (Esensten, Helou et al. 2016). The interplay of co-stimulation and inhibition refines T cell fate and function, ensuring protective immunity while limiting inflammation-driven injury.

1.3 T cell development in thymus

T cell differentiation begins early in fetal life and continues, at reduced levels, into adulthood. T cells derive from hematopoietic stem cells that originate in the bone marrow. Later they migrate to the thymus to continue their maturation and differentiation, which is why these organs are referred to as primary lymphoid organs.

To originate mature T cells, the principal processes occurring in the thymus are:

1. The differentiation of T cell lineages, including $CD4^+$ T cells, $CD8^+$ T cells and regulatory T cells (Tregs).
2. The maturation of the TCR and the establishment of the TCR repertoire.

During differentiation, T cells precursors are called thymocytes. At the beginning of their development, thymocytes lack signature surface markers that define mature T cells, including the CD3 complex and the co-receptor molecules CD4 and CD8, that define the two major subsets of αβ T lymphocytes. For this reason, at this stage they are called double negative (DN) thymocytes. They will commit either to the minority γδ T cell lineage (10% of all T cells), and follow an independent fate, or to the major αβ T cells lineage. The enzymes Recombination-Activating Gene 1 and 2 (RAG-1/2) mediate rearrangements in the *TRB* locus, which encodes the β-chain of the TCR, and T cells will express a TCR formed by a recombined β-chain associated with an invariant α-chain. The expression of this pre-TCR complex induces constitutive signalling, leading to the expression of both co-receptor molecules CD4/CD8, starting the double positive (DP) phase in the thymic cortex. DP thymocytes undergo recombination of the *TRA* locus, encoding the α-chain, and once expressing a functional αβ-TCR DP thymocytes will undergo the positive selection process. Cortical thymic epithelial cells (cTECs) display a distinctive set of self-peptides on their MHC, testing TCR functionality and MHC restriction: recognition of pMHC I or II delivers survival signals and biases lineage choice (**Figure 4**) (Kadouri, Nevo et al. 2020). MHC class I directs differentiation into the $CD8^+$ lineage, whereas recognition of MHC class II drives commitment to the $CD4^+$ lineage, initiating the single-positive (SP) phase (Klein, Kyewski et al. 2014, Baldwin and Robey 2024). During the SP phase also other lineages can arise, most notably Tregs, whose phenotype will be discussed in Chapter 1.5.

Positively selected T cells migrate into the thymic medulla, where negative selection occurs. The affinity model of thymocyte selection centers on the strength of the interaction of the TCR with self-peptide–MHC complexes as a crucial determinant of cell fate. Medullary thymic epithelial cells (mTECs) express many self-antigens that are normally found only in specific tissues of the body, and they interact SP thymocytes (Kadouri, Nevo et al. 2020). Weak interactions are required to generate survival signals that protect thymocytes from death by neglect. Strong interactions cause negative selection by apoptosis. A surprisingly

broad range of affinities seems to be permissive for Tregs-lineage differentiation, which play a critical role in maintaining immune tolerance. Only 2-4% of the original pool of thymocytes survives positive and negative selection: in this way a balance is reached in which TCR diversity is generated while highly self-reactive clones are eliminated (Klein, Kyewski et al. 2014, Baldwin and Robey 2024). Positive and negative selection processes are illustrated in **Figure 4**.

To exit the thymus, mature T lymphocytes upregulate sphingosine-1-phosphate receptor 1 (S1PR1). S1PR1 ligand, sphingosine-1-phosphate, is maintained at high concentration in the blood by red blood cells and endothelial cells, and chemotactically draws $S1PR1^{+}$ T lymphocytes into the peripheral circulation (Cyster 2005).

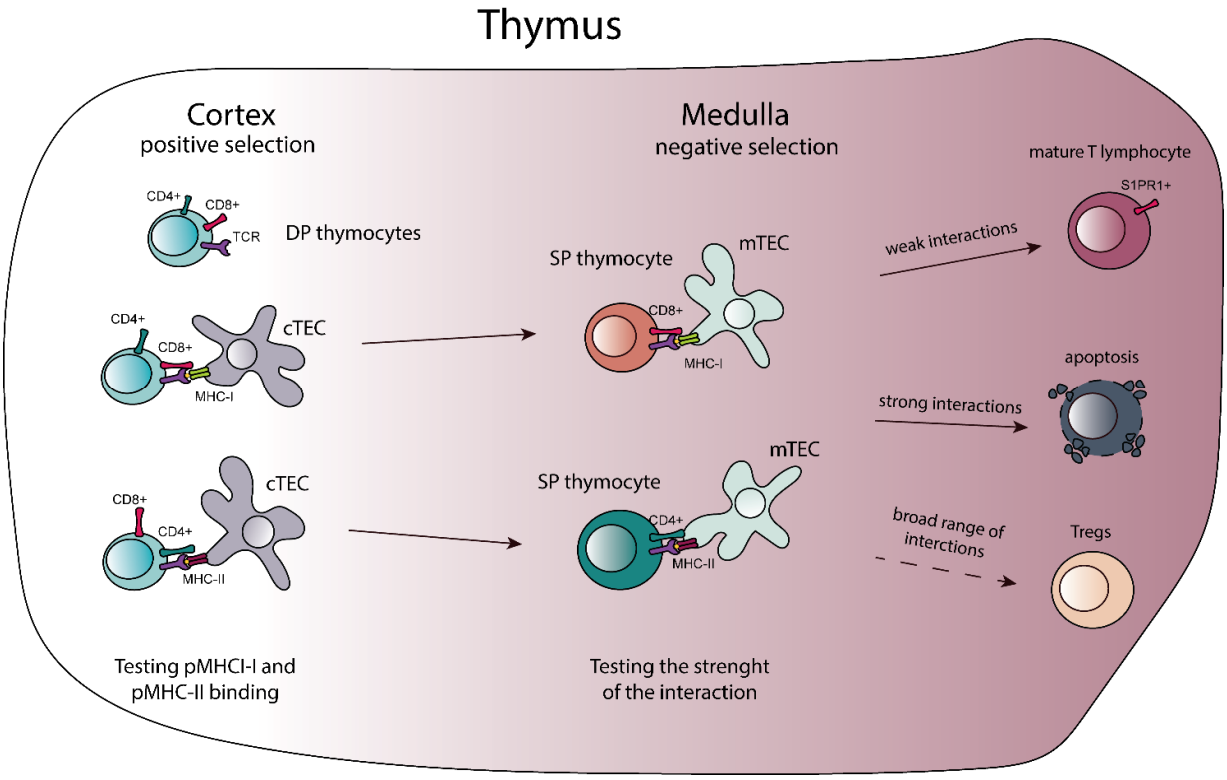

Figure 4. Thymocyte development in the thymus. The left panel illustrates positive selection, where DP thymocytes express the TCR complex along with both CD4 and CD8 co-receptors. DP thymocytes interact with cTECs, which assess TCR functionality and MHC restriction, guiding thymocytes toward either the CD4 or CD8 lineage. The right panel depicts negative selection, in which SP thymocytes interact with mTECs, leading to the elimination of clones highly reactive to self-peptides.

1.4 Naïve T Cell Priming and Immunological Memory

Mature, antigen-inexperienced T lymphocytes that have completed development and exited the thymus but have not yet recognized their specific foreign peptide–MHC are called naïve T lymphocytes. Naïve T cells are in a quiescent state and continuously re-circulate from the bloodstream to secondary lymphoid organs (SLO). SLOs include lymph nodes, the spleen and the Mucosa-Associated Lymphoid Tissue (MALT) of the gut, respiratory tract and urogenital tract. They are specialized structures in which APCs travel to after encountering an antigen in other tissues. As explained in Chapter 1.2, the naïve T cell pool harbors a highly diverse TCR repertoire, therefore antigen-specific naïve T cells are exceedingly rare. Concentrating antigens and APCs in SLOs maximizes encounter rates, ensures proper co-stimulation in a controlled microenvironment, and enables rapid, clonal expansion of the T cells which encountered their specific antigens (Kenneth M. Murphy 2022).

Naïve T cells and APCs express the chemokine receptor CCR7 to migrate to lymphoid organs. Within these organs, stromal cells produce the CCR7 ligands, CCL19 and CCL21, to attract naïve $CCR7^+$ T cells and $CCR7^+$ APCs. Efficient lymph-node entry of naïve lymphocytes also depends on the adhesion molecule CD62L, which recognizes and bind glycoproteins expressed by endothelial cells, such as GlyCAM-1 and MAdCAM-1 (Berg, McEvoy et al. 1993, Butcher and Picker 1996, Hwang, Singer et al. 1996, Sallusto, Lenig et al. 1999). Human naïve T cells express CD45RA on their surface, an isoform of the receptor-type protein tyrosine phosphatase CD45, a key regulator of TCR signalling. Together, CCR7 and CD45RA surface markers can also be used to experimentally identify naïve T cells by Flow-Cytometry (Hermiston, Xu et al. 2003, Al Barashdi, Ali et al. 2021). Naïve T cells patrol SLOs for 12-24 hours before migrating elsewhere. When a naïve T cell recognizes and binds an APC displaying its cognate peptide–MHC complex, the two cells establish long-lasting contacts through the immunological synapse (Douanne and Griffiths 2021). This specialized junction enables highly specific communication and will be described in detail in Chapter 1.5, in the context of T cell effector functions. During this time, sustained TCR signalling activates the T cell and triggers rapid cytoskeletal remodelling and metabolic reprogramming. 24-48 hours after activation, the T cell begins proliferating and undergoes rapid clonal expansion to produce a progeny which can respond to the same antigen. TCR and CD28 signalling activate the mTOR pathway through Akt kinase. Engagement of the TCR and costimulatory receptor CD28 activates the kinases PI3K–Akt, which in turn stimulates mTOR signalling. mTOR coordinates metabolic reprogramming, boosting glycolysis and biosynthetic pathways, to couple growth signals to the bioenergetic demands of activated T cells during clonal expansion. This process—from initial antigen recognition to clonal expansion—is referred to as the priming of naïve T lymphocytes (Malissen and Bongrand 2015, Geltink, Kyle et al. 2018, Kenneth M. Murphy 2022). During clonal expansion, $CD4^+$ and $CD8^+$ naive T cells differentiate into

effector cells, respectively acquiring helper or cytotoxic properties which are essential for orchestrating a productive immune response and executing pathogen clearance. $CD4^+$ and $CD8^+$ effector properties will be discussed in the following chapter (Chapter 1.5, Effector Subsets). A particular important role during the early stage of the immune response is played by the cytokine IL-2. TCR-activated T lymphocytes upregulate the IL-2 receptor α-chain (IL-2Rα or CD25), that together with other 2 subunits, the IL-2 receptor β chain (IL-2Rβ or CD122) and a γ subunit (γc, or CD132), forms the high-affinity IL-2 receptor (IL-2R). Co-expression of all three subunits is required for high-affinity IL-2 binding and robust signalling. Although IL-2R signalling is dispensable for activation onset, it is essential to sustain proliferation and survival, thereby enabling durable clonal expansion of antigen-primed T cells (Bachmann and Oxenius 2007, Ross and Cantrell 2018).

In response to pathogen infection, naïve T cells can undergo asymmetric cell division (ACD) that partitions fate determinants unequally and seeds daughter cells with distinct developmental trajectories (Chang, Palanivel et al. 2007). Under strong TCR stimulation, ACD biases one daughter toward an effector program characterized by high CD25 expression and stronger IL-2 signalling, favouring short-lived effector potential with more limited self-renewal. The sibling daughter typically expresses lower CD25, thus receives lower IL-2 signalling, preferentially upregulates survival and memory-associated programs over time, such as the IL-7 receptor, and serves as a memory precursor with long-term self-renewal and the capacity to generate effectors cells upon re-stimulation. ACD is one mechanism that contributes to early fate bifurcation; its impact varies with antigen strength, co-stimulation and presence of cytokines in the environment (Chang, Wherry et al. 2014, Gräbnitz, Stark et al. 2023).

Once these early fate decisions are established, the effector pool expands and, following pathogen clearance, undergoes a contraction phase in which most progeny die by apoptosis, restoring homeostasis. Among the surviving cells, memory precursors generated during the initial asymmetric divisions contribute to the long-lived memory pool. They establish immunological memory, a defining feature of adaptive immunity: the antigen-experienced cells mount a faster, more robust response upon re-exposure to the pathogen, due to their increased frequency and acquired functional properties (Sallusto, Geginat et al. 2004, Chang, Wherry et al. 2014). Memory cells comprise heterogeneous subsets with distinct trafficking patterns, phenotypic and functional profiles. Effector memory cells (T_{EM}) are found in peripheral tissues and display immediate effector function. Central memory cells (T_{CM}) instead have limited effector function and recirculate through peripheral blood and SLOs; they express CCR7 and CD62L receptors that are also characteristic of naïve T cells, because they share the same migratory pattern. Compared to naïve T cells, T_{CM} have higher sensitivity to antigenic stimulation, are less dependent on co-stimulation and readily proliferate and differentiate into T_{EM} in response to antigen encounter. T_{EM} lose the expression of CCR7 and instead display characteristic sets of chemokine receptors and adhesion molecules that are required for

homing to inflamed tissues, such as CXCR3 and CCR5 together with integrins VLA-4 (α4β1) and LFA-1 (αLβ2) (Sallusto, Lenig et al. 1999, Sallusto, Geginat et al. 2004, Lanzavecchia and Sallusto 2005). Tissue-resident memory T cells (T_{RM}) occupy tissues without recirculating in peripheral blood, they are especially found at barrier-sites such as the dermis or the intestine. T_{RM} derive from T_{EM} that entered tissues and remained positioned there. T_{RM} exhibit different transcriptional signatures compared to the other memory subsets, they are anatomically positioned for rapid responses at sites of infections and are phenotypically and functionally heterogeneous, depending on their location and inducing stimulus (Schenkel and Masopust 2014, Mueller and Mackay 2016). Repeated antigen encounters can drive effector memory T cells to re-express CD45RA, a marker normally found on naïve T cells. These terminally differentiated $CD45RA^+$ effector-memory cells (T_{EMRA}) are especially enriched within the $CD8^+$ compartment. They retain strong effector properties, but their proliferation rate is limited (Geginat, Lanzavecchia et al. 2003, Henson, Riddell et al. 2012, Muroyama and Wherry 2021). The process from the priming of naïve T cells to the establishment of immunological memory is illustrated in **Figure 5**.

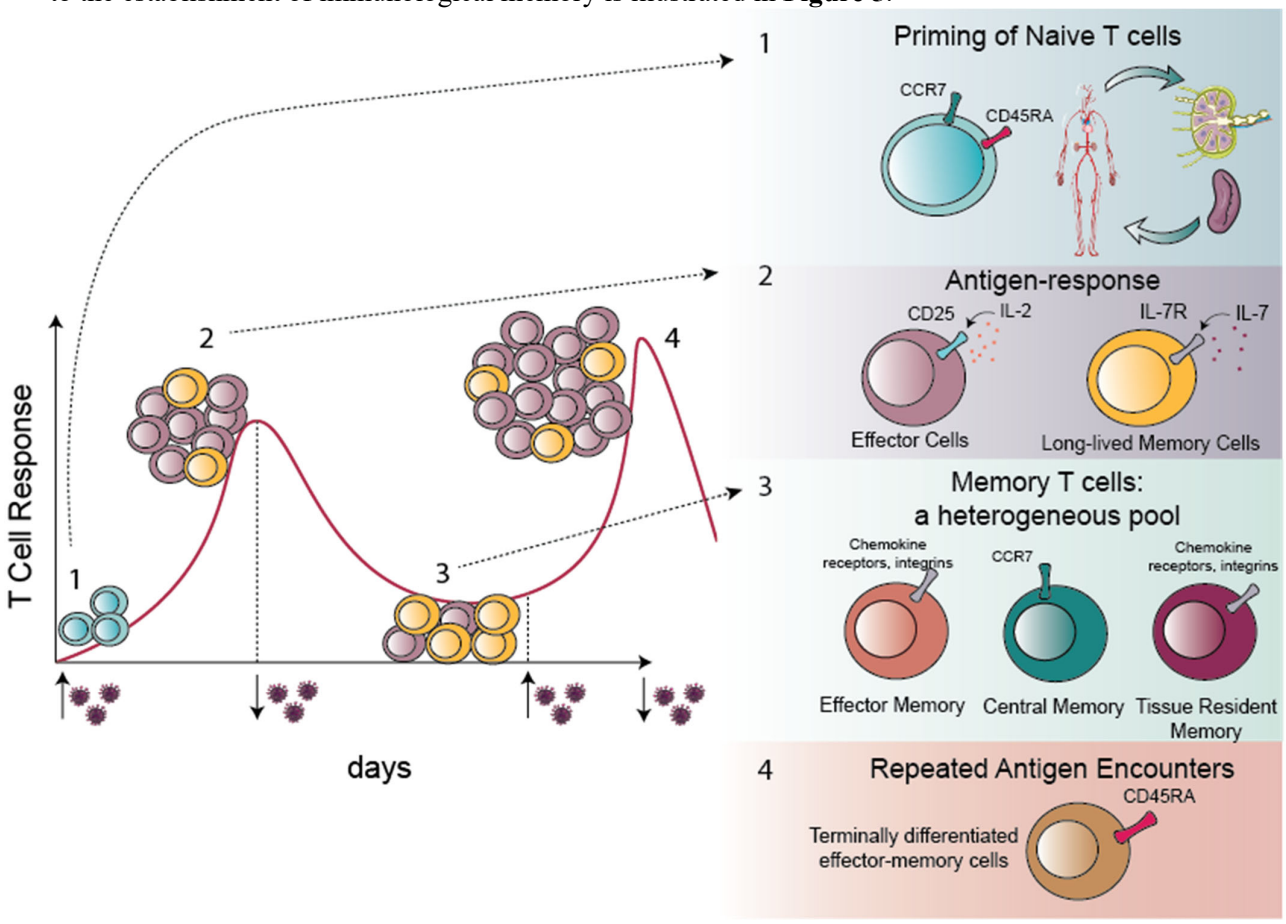

Figure 5. Legend on next page.

Figure 5. Priming of naïve T cells and establishment of immunological memory. 1) Naïve T cells recognize antigen and become activated. 2) Activated T cells undergo clonal expansion and differentiate into effector and memory subsets. 3) After antigen clearance, most effector cells undergo contraction, while memory T cells persist in a quiescent state, ready to mount rapid recall responses. 4) Repeated antigen encounters drive further differentiation, leading to terminally differentiated effector populations. Memory T cells comprise a heterogeneous pool of subsets with distinct phenotypes, gene expression profiles, and receptor repertoires.

1.5 Effector Subsets

$CD4^+$ and $CD8^+$ T lymphocytes are the two principal αβ T lymphocytes lineages. Naïve-to-effector priming, followed by clonal contraction and memory maintenance, proceeds similarly in both subsets. TCR engagement activates shared proximal signalling cascades; differences arise from the landscape of co-stimulatory molecules. For example, ICOS and OX40 expression is enriched on $CD4^+$ T cells, while CD137 is more expressed by $CD8^+$ T cells (Gramaglia, Weinberg et al. 1998, Munks, Mourich et al. 2004, Mahajan, Cervera et al. 2007). In healthy adults, $CD4^+$ T cells typically outnumber $CD8^+$ T cells (CD4/CD8 ratio is usually 1.5–2.5) (Kaech, Wherry et al. 2002, Seder and Ahmed 2003, McBride and Striker 2017).

A central role in mediating effector functions (as well as naïve-to-effector priming) is played by the immunological synapse. When $CD8^+$ and $CD4^+$ T lymphocytes recognize their cognate peptide–MHC complexes, the T cell cytoskeleton rapidly reorganizes: the microtubules polarize toward the target cell, aligning the secretory machinery at the contact site. This polarization creates a tightly regulated interface called the immunological synapse, through which effector molecules are delivered with precision, cytolytic granules (perforin and granzymes) from $CD8^+$ T cells and cytokines from $CD4^+$ T cells, leaving the neighboring healthy cells unharmed (Douanne and Griffiths 2021).

$CD4^+$ T lymphocytes

Upon activation, each naïve $CD4^+$ T cell clone has the potential to differentiate into several functionally distinct effector programs, generally referred to as a subsets. Their function is to secrete cytokines to coordinate immune responses, therefore they are also called helper T cells (T_H). The different $CD4^+$ T subsets arise depending on the cytokines secreted at the site of their activation; dendritic cells are the major source of cytokines, but also other innate immune cells can contribute. Different cytokines activate specific transcription factors that will determine $CD4^+$ cells phenotype and response. Effective eradication of pathogens requires that $CD4^+$ T cells adaptive responses are finely integrated with those of innate immunity. Broadly, the T helper effector immune module can be grouped into three main response types, each defined by specific T_H subsets and their innate partner cells, tailored to different classes of microbes and parasites (Zhu, Yamane et al. 2010, Kenneth M. Murphy 2022). Besides the T_H subsets involved in activating innate immunity there are other subsets, such as T Follicular Helper cells (T_{FH}), Tregs and $CD4^+$ cytotoxic which have a different, specialized function that will be explained below. The several subsets towards which $CD4^+$ T lymphocytes can differentiate are illustrated in **Figure 6.**

Type 1 response and T_H1 cells. The T_H1 subset was one of the first T helper subsets to be identified (Mosmann and Coffman 1989). The gene network that establishes their lineage is initiated by cytokines

IFN-γ and IL-12, that activate the master regulator of T_H1 phenotype, Tbet (Zhu and Paul 2008). The main role of T_H1 cells is to eradicate infections caused by microbes that survive within macrophages, such as viruses, intracellular bacteria and protozoans. When T_H1 cells recognize microbial antigens exposed on the surface of macrophages, they secrete IFN-γ and GM-CSF to activate them, which in turn become potent activators of inflammation (Mosmann and Coffman 1989, Szabo, Sullivan et al. 2003, Gieseck, Wilson et al. 2018).

Type 2 response and T_H2 cells. Together with T_H1 cells, the T_H2 subset was the first to be identified (Mosmann and Coffman 1989). The presence of IL-4 in the T cell microenvironment induces the expression of GATA3, the master regulator of the T_H2 phenotype (Zhu and Paul 2008). Type 2 responses control infections caused by multicellular parasites, such as helminths, through the secretion of IL-4, IL-5 and IL-13, which activate eosinophils, basophils and mast cells. These in turn release cytotoxic molecules and inflammatory mediators. Helminths size ranges from 1 mm to more than 1 meter, so their direct killing is difficult. Indeed, in Type 2 immunity T_H2 cells also promote the activity of non-immune cells, such as epithelial cells, with the major focus of expelling the worms from the body. IL-13 directly enhances the production of mucus, critical to eliminate the parasites attached to the epithelium. T_H2 cytokines can also activate macrophages, which acquire a phenotype that is distinct compared to what happens in Type 1 responses: they secrete mediators that are toxic for helminths but can promote host tissue remodelling and repair, such as ornithine (Mosmann and Coffman 1989, Ruterbusch, Pruner et al. 2020).

Type 3 response and T_H17 cells. T_H17 differentiation arises when IL-6 and IL-23 are present in the extracellular milieu; they induce the expression of RORγt, the master regulator of T_H17 cells (Zhu and Paul 2008). The T_H17 subset is physiologically present at barrier sites, with a prominent role in the intestinal mucosa. In presence of pathogenic extracellular bacteria or fungi, T_H17 cells secrete IL-17 and IL-22 to orchestrate Type 3 responses, in which neutrophils are the principal effector cells. Interestingly, IL-17 and IL-22 can induce epithelial cells to produce antimicrobial molecules that can kill bacteria, such as defensins and lectins, or bacteriostatic and fungistatic molecules (Korn, Bettelli et al. 2009).

The heterogeneity of $CD4^+$ T cells is high and additional T_H cell subsets have been described but are less characterized, such as T_H9 and T_H22 (Annunziato and Romagnani 2009, Zhu and Paul 2010). Each T_H subset produces more than one cytokine. However, individual cells within same lineage may display different patterns of cytokine production. Indeed, most of the stimulated cells express one, two or three but rarely all of the cytokines that one lineage can potentially produce (O'Shea and Paul 2010, Zhu and Paul 2010, Zhu, Yamane et al. 2010, Geginat, Paroni et al. 2014).

T Follicular Helper cells. T_{FH} play a crucial role in the trafficking of B cells within lymph nodes and in germinal center reactions. Within the lymph nodes, the germinal centers are sites of intense B cell proliferation, in which T_{FH} deliver signals to promote hypermutation of B cell antibody genes, resulting in

the production of high-affinity antibodies against antigens. CXCR5 is a key receptor expressed by T_{FH} for localization in the follicles of lymph nodes and the key transcription factor for the T_{FH} lineage is Bcl-6 (Crotty 2011).

T Regulatory cells. From the 1970s to the early 2000s, accumulating evidence delineated a subset of $CD4^+$ T cells whose function is to proactively suppress immune responses and inflammation. The pivotal discovery of immune tolerance mediated by Tregs led to the awarding of the 2025 Nobel Prize in Physiology or Medicine to Mary E. Brunkow, Fred Ramsdell, and Shimon Sakaguchi. Tregs are characterized by the constitutive expression of the high-affinity binding subunit of IL-2R, CD25, and the key transcription factor for differentiation towards their lineage is FOXP3 (Fontenot, Gavin et al. 2003). Some thymocytes are directed towards the Treg lineage already during $CD4^+$ maturation in the thymus; they are called thymic Tregs (tTregs). Alternatively, peripheral Tregs (pTregs) can originate from $CD4^+$ T cells in secondary lymphoid tissues following antigen recognition and in presence of the cytokine TGF-β. Tregs mediate immunosuppression through a diverse array of effector mechanisms, including the production of the immunosuppressive cytokines TGF-β and IL-10, the constitutive expression of CD25 which depletes IL-2 from the microenvironment, and enforcing quiescence through the CD73/CD39-mediated conversion of extracellular ATP to adenosine. Treg suppressive activity is fundamental to preserve homeostasis, and in fact mutations in the *FOXP3* gene are associated with systemic autoimmunity (Sakaguchi, Sakaguchi et al. 1995, Fontenot, Gavin et al. 2003, Hori, Nomura et al. 2003, Fontenot, Rasmussen et al. 2005, Sakaguchi, Miyara et al. 2010, Dikiy and Rudensky 2023).

Cytotoxic $CD4^+$ cells. To highlight the breadth and adaptability of $CD4^+$ T cells, studies have identified a subset with cytotoxic activity, called $CD4^+$ cytotoxic T lymphocytes (CD4 CTLs). These cells can kill target cells, mirroring and complementing the physiological cytotoxic function of $CD8^+$ T cells. They employ the same canonical mechanisms as $CD8^+$ cells: release of cytotoxic granules and Fas/FasL-mediated apoptosis. These pathways are described in detail in the section on $CD8^+$ T lymphocytes. Characteristic cytotoxic molecules expressed by CD4 CTLs include granzymes and perforin; additionally, upon activation they express CRTAM, the degranulation marker CD107a, and the transcription factor EOMES (Takeuchi, Badr Mel et al. 2016, Hoeks, Duran et al. 2022, Preglej and Ellmeier 2022). CD4 CTLs have been linked to anti-tumour immunity (Xie, Akpinarli et al. 2010, Oh, Kwek et al. 2020). Malignant cells can upregulate MHC class II and thus become susceptible to direct recognition and killing by CD4 CTLs, which then support $CD8^+$ T cells in tumour clearance (Zhou, Zha et al. 2018). The strong association between the appearance of CD4 CTLs and viral infections suggests an important role for this subset in antiviral immunity (Juno, van Bockel et al. 2017). Finally, CD4 CTLs are prominent in aging: they are enriched in the elderly and are particularly abundant in supercentenarians, in whom $CD4^+$ T cells appear to accumulate through clonal

expansion and may contribute to the elimination of senescent cells (Hashimoto, Kouno et al. 2019, Hoeks, Duran et al. 2022, Preglej and Ellmeier 2022, Hasegawa, Oka et al. 2023).

CD4$^+$ T lymphocytes plasticity. Adaptation is a key feature of T_H cells, which can change their secretory program to transition into different phenotypes contingent on changes in the local inflammatory environment: this phenomenon is called T cell plasticity. Studies published in the last decade employing high throughput techniques, such as single-cell RNA-sequencing, that measures the expression of hundreds of genes at the same time, showed that the biology of T_H responses is more complex and dynamic than previously appreciated. These findings revealed a greater functional heterogeneity in T_H subsets, and the concept that the fate of a naive CD4$^+$ T cell progeny is irreversibly defined during their priming into distinct, mutually exclusive subsets – T_H1, T_H2, T_H17, T_{FH} and so on- was reconsidered. To ensure keeping into account their broad transcriptional and functional complexity, a simpler classification of T_H cells was proposed, based on the host cells that they engage and the type of immune response that they mount (O'Shea and Paul 2010, Geginat, Paroni et al. 2014, Tortola, Jacobs et al. 2020, Kiner, Willie et al. 2021, Tuzlak, Dejean et al. 2021).

CD8$^+$ T lymphocytes

CD8$^+$ T lymphocytes are part of a different effector module of immune responses: they are capable of direct killing target cells, so effector CD8$^+$ T cells are referred to as Cytotoxic T Lymphocytes (CTLs). CD8$^+$ T lymphocytes bind peptides loaded on MHC class I molecules, which are expressed by all nucleated cells: this means that they could virtually kill any cell of the body if needed. Thanks to the immunological synapse, mechanisms of CD8$^+$ killing are powerful and accurately targeted: after completing targeted-cell killing, they disengage and migrate to the next target. Among the cytotoxic granules that they release at the interface, there are perforin, granulysin and granzymes: perforin and granulysin are membrane-perturbing molecules that help delivering contents of granules inside target cells, granzymes are serine proteases that cleave and activate caspases, that initiate apoptosis. CD8$^+$ effector T lymphocytes can kill their targets one after the other in a rapid succession because they keep cytotoxic molecules stored in an inactive state inside cytotoxic granules. When their TCR is engaged, stored cytotoxins are released rapidly, and synthesis of new cytotoxins is induced (Kenneth M. Murphy 2022, Giles, Globig et al. 2023, Koh, Lee et al. 2023). The mechanisms by which CD8$^+$ T cells effect their killing are described in detail below and illustrated in **Figure 6.**

Granzymes. Granzymes are a family of serine proteases and the main effectors of CD8$^+$ T cell-induced cell death. Humans express five family members: granzyme A, B, H, M and K. They participate in several physiological and pathological processes, and their synthesis and storage are highly regulated to avoid accidental cytotoxicity; besides caspase activation, they have been shown to be involved in other cell death

pathways, such as mitochondrion alterations and oxidative stress. Granzyme A and B are the most studied molecules, but recently interest has been increasing also towards granzyme K. Indeed, $CD8^+$ T cells expressing high levels of granzyme K were found to be the predominant subset in inflamed tissues in autoimmune or chronic disease (Donado, Theisen et al. 2025). These findings revealed that granzyme K is the main driver of the complement cascade activation, identifying a previously unknown mechanism through which granzymes molecules eventually cause death. Instead, the role of granzyme H and M is less known and explored (Ewen, Kane et al. 2012, Aubert, Jung et al. 2024, Cigalotto and Martinvalet 2024, Donado, Theisen et al. 2025).

Fas/FasL-mediated cell killing. $CD8^+$ T cells can kill their target cells also through a granule-independent mechanism mediated by the Fas/FasL interaction. In this case, the Fas ligand (FasL) expressed by cytotoxic T cells interacts with the Fas receptor (FasR) expressed by the target cells, inducing apoptosis in a way that is morphologically indistinguishable from that of granzyme-mediated cell death. FasR is upregulated in rapidly proliferating cells, not only on tumour cells but also in activated lymphocytes, so it is believed that this pathway is also needed for homeostasis maintenance and control of lymphocytes growth and differentiation (Waring and Müllbacher 1999, Hu, Lu et al. 2025).

$CD8^+$ T cells retain some degree of functional heterogeneity; although less pronounced than in $CD4^+$ T lymphocytes, and some functional subsets can be identified. The predominant population is composed of Tc1 cells, typical cytotoxic cells characterized by production of granzymes, perforin and IFN-γ at high level; EOMES is a key TF that contributes to the polarization towards this subset, together with T-bet. Alternative $CD8^+$ T cell subsets, including Tc2, Tc9, Tc17 and Tc22, have been described, but their main role remains the direct, MHC class I-restricted killing; other, minor subsets such as follicular cytotoxic T cells (Tfcs) and $CD8^+$ Tregs appear to be a support for $CD4^+$ T cells (Koh, Lee et al. 2023).

To conclude, the two different effector modules of T lymphocytes, the helper module mediated by $CD4^+$ T lymphocytes and the cytotoxic module mediate by $CD8^+$ T lymphocytes, are profoundly intertwined and are usually recruited together, their functions are complementary and both of them are needed to eliminate invading microbes effectively.

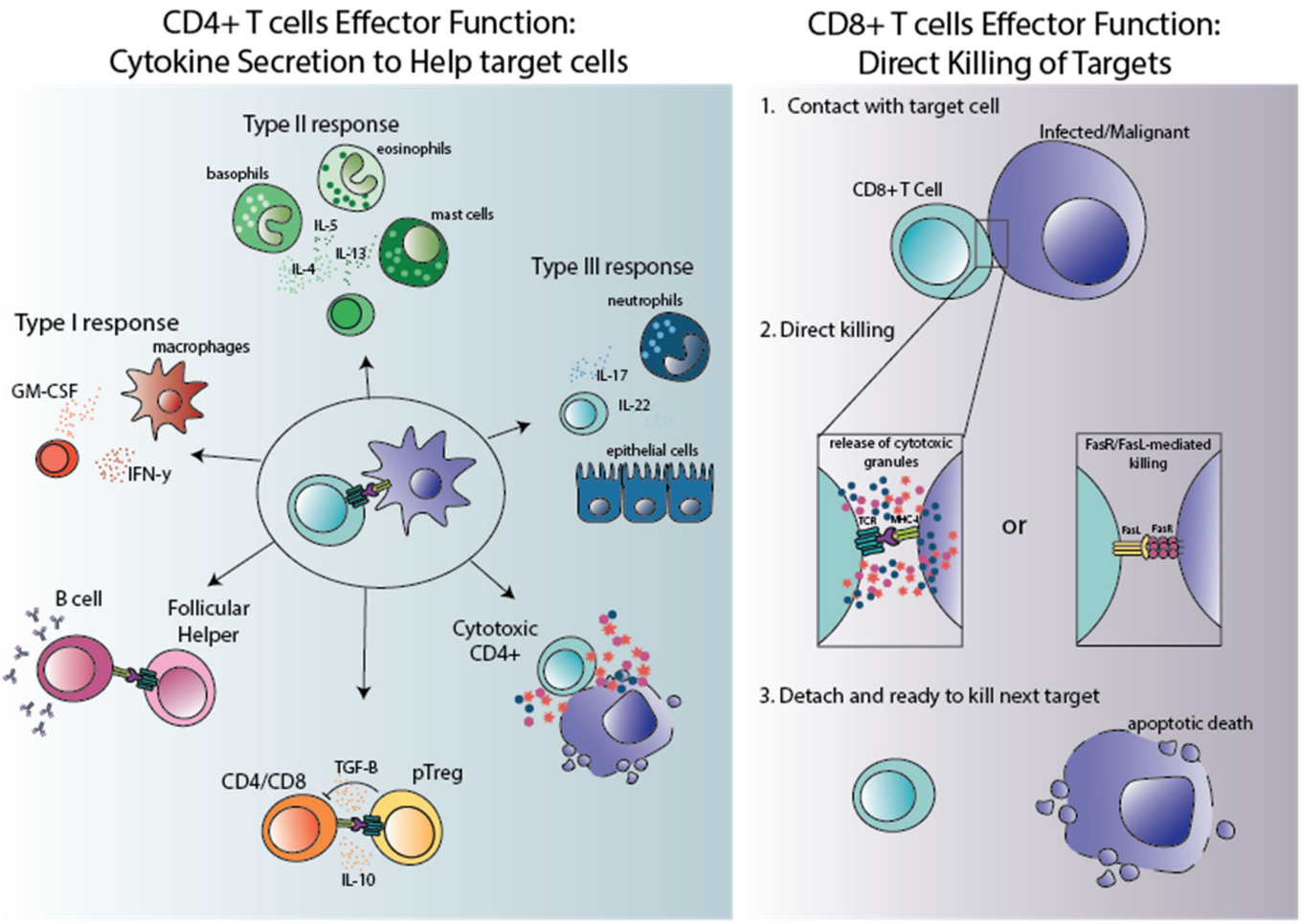

Figure 6. **Schematic representation of the effector functions of CD4$^+$ and CD8$^+$ T lymphocytes. On the left**, representative differentiation fates of CD4$^+$ T cells are illustrated. At the top, Type I responses are shown, in which effector CD4$^+$ T cells support monocytes and macrophages by secreting activating cytokines such as IFN-γ and GM-CSF. Type II responses are characterized by CD4$^+$ T cell–mediated control of infections caused by multicellular parasites through the activation of mast cells, eosinophils, and basophils. Type III responses, which occur primarily at barrier sites, involve CD4$^+$ T cell–driven recruitment and support of neutrophils and epithelial cells. In the lower section, specialized CD4$^+$ subsets are depicted: T follicular helper (TFH) cells promote the production of high-affinity antibodies by B cells; regulatory T (Treg) cells suppress the activity of other lymphocytes and immune cells; and cytotoxic CD4$^+$ T cells can acquire killing capacity, complementing CD8$^+$ T cell–mediated cytotoxicity when required. **On the right**, the primary function of CD8$^+$ T cells is illustrated: MHC class I–restricted direct cytotoxicity against target cells, either granule-mediated or FasR/FasL-mediated.

2. Regulation of T Cell Responses through Inhibitory Receptors

2.1 Exhaustion and Anergy

Multiple mechanisms regulate and restrain T cell responses: T cell unchecked activity can damage host tissues, so their activation is tightly regulated. TCR activation induces the expression of inhibitory receptors (IRs) on the cell surface that function as a negative regulatory mechanism, balancing costimulatory signals and limiting proliferation and inflammatory effector functions. Under prolonged antigen exposure or persistent inflammation, such as during chronic infection or within the tumour microenvironment (TME), sustained stimulation drives a program of progressive dysfunction termed exhaustion, a regulatory adaptation that restricts effector capacity to preserve tissue integrity (Baessler and Vignali 2024). $CD8^+$ T cell exhaustion was first described in chronic infection of mice during which virus-specific $CD8^+$ T cells persist but lack effector function (Zajac, Blattman et al. 1998). Subsequent, pioneering work has elucidated some of the underlying mechanisms of exhaustion, including the important role played by the inhibitory receptor CTLA-4 in the context of tumour immune responses and the inhibitory receptor PD-1 in regulating $CD8^+$ T cell exhaustion during chronic infection in mice. Leach and colleagues showed for the first time that blocking the inhibitory effects of CTLA-4 *in vivo* was potentiating immune responses against tumour cells (Leach, Krummel et al. 1996). Instead, Barber, Wherry and colleagues showed that blockade of the PD-1/PD-ligand pathway during chronic infection leads to recovery of T cell function and reduced viral load; these findings were replicated in humans and set the basis for checkpoint inhibitors therapy, that will be explained in Chapter 2.2 and 2.3 (Barber, Wherry et al. 2006, Wherry, Ha et al. 2007).

Exhausted cells display a spectrum of different phenotypes, which range between partial dysfunction to complete lack of effector function. A hallmark of exhaustion is the simultaneous high expression of multiple IRs, such as PD-1, TIM-3, and LAG-3, although these receptors can also be transiently expressed in activated T cells without indicating exhaustion. In general, exhausted cells have major changes in TCR and cytokine signalling pathways, display altered expression of genes involved in chemotaxis, adhesion and migration and have profound metabolic and bioenergetic deficiencies (Wherry, Ha et al. 2007).

More recently, transcriptomic and epigenomic analyses in mice and humans have shown that exhausted T cells comprise diverse subsets with distinct gene-expression programs. The establishment of exhaustion is accompanied by extensive chromatin remodelling and reorganization of transcription-factor networks, which together imprint different stable, lineage-like states (Pauken, Sammons et al. 2016, Sen, Kaminski et al. 2016, Beltra, Manne et al. 2020). Intense work has dissected the signalling pathways, transcriptional circuits, and epigenetic programs that initiate and sustain $CD8^+$ T cell exhaustion. Multiple transcription

factors, including T-bet, EOMES, BLIMP-1, the NR4A family, and FOXO1, contribute to its development and maintenance. A central node is TOX, induced downstream of calcineurin–NFAT signalling (Kallies, Zehn et al. 2020). Sustained TOX expression commits cells to the exhausted lineage and establishes a distinct transcriptional and epigenetic program (Khan, Giles et al. 2019).

Early debate about the origin of exhausted $CD8^+$ cells existed, but multiple fate-mapping, single-cell and clinical studies now support the progenitor-exhausted lineage model over the idea that terminally differentiated memory cells simply become exhausted. Specifically, exhaustion is now best understood as a distinct differentiation program initiated early during chronic antigen exposure and maintained by a subset of progenitor-exhausted cells expressing the transcription factor TCF1 (Blank, Haining et al. 2019, Baessler and Vignali 2024). Recent work showed that a niche of $TCF1^+$ progenitor-exhausted $CD8^+$ T cells emerges early during persistent antigen exposure, before the infection is resolved or becomes chronic (Chu, Wu et al. 2025). This supports the view that exhaustion can be a tissue-protective program in some settings: despite attenuated effector capacity, progenitor-exhausted cells help sustain long term pathogen control while limiting immunopathology. This niche promotes enough immunity to restrain an infection without the collateral damage that might accompany unrestrained effector responses (Chu, Wu et al. 2025).

In addition to exhaustion, T cells can also enter a distinct unresponsive state known as anergy, which arises under different conditions of activation. Specifically, anergy is a dysfunctional state that $CD4^+$ and $CD8^+$ T lymphocytes can enter when primed without co-stimulation. In this state, T cells remain hyporesponsive to subsequent stimulation, even when both TCR and co-stimulatory signals are provided. Dendritic cells are central to T-cell activation, as they efficiently prime naïve T cells. Optimal antigen presentation requires dendritic cells maturation, which enhances MHC class II expression and upregulates co-stimulatory molecules such as B7 and CD40. In contrast, immature dendritic cells express only moderate MHC class II and little or no co-stimulatory molecules, delivering suppressive rather than activating signals. This suboptimal priming induces T cell anergy, particularly in $CD4^+$ T cells. Tumours exploit this mechanism: suppressive cytokines in the TME impair dendritic cells maturation, abrogating their immunostimulatory capacity. For $CD8^+$ T cells, anergy is driven primarily by the absence of $CD4^+$ T cell help (Steinbrink, Graulich et al. 2002, Schwartz 2003, Macián, Im et al. 2004). Mechanistically, transcription factors of the NFAT family play a central role in anergy induction. When TCR engagement is accompanied by co-stimulation, NFAT and AP-1 are induced in a balanced manner, forming complexes that drive a full productive immune response. In contrast, TCR stimulation in the absence of co-stimulation preferentially activates the Ca^{2+}–calcineurin–NFAT pathway, leading NFAT to promote transcription of anergy-associated genes, as discussed in the TCR signalling Chapter 1.2 (Macián, García-Cózar et al. 2002, Macian 2005). Among the genes that are upregulated during anergy there are E3 ubiquitin ligases, which mediate the

degradation of the signalling proteins Itch and Cbl-b. Mutations or deletion of *Itch* and *Cbl-b* in mice are associated with autoimmune disease (Anandasabapathy, Ford et al. 2003, Heissmeyer, Macián et al. 2004).

Anergy, driven by incomplete priming, and exhaustion, arising from chronic stimulation, represent key states of T cell unresponsiveness. Understanding the mechanisms underlying T cell unresponsiveness is critical for the development of strategies to restore immune function. These insights have laid the foundation for immunotherapies aimed at releasing inhibitory brakes on T cells and reactivating antitumor immunity. The therapeutic implications of this will be explored in Chapter 2.3.

2.2 Inhibitory Receptors

Physiologically, IRs expression is induced upon TCR stimulation and declines as the T cell response dissipates. During chronic stimulation, T cells display sustained expression of multiple IRs, therefore these receptors are often referred to as exhaustion markers. Among the most important IRs, are those that belong to the same families as co-stimulatory molecules, including CD28-related receptors and members of the TNFRSF. Many of these receptors contain intracellular signalling domains capable of transmitting a negative signal, often through phosphatase activity or other adaptor molecules, including ITIMs and switch motifs (ITSMs) (Attanasio and Wherry 2016, Baessler and Vignali 2024). ITIMs are defined by the consensus sequence (I/V/L/S)-X-Y-X-X-(L/V/I), where X represents any amino acid. These motifs are phosphorylated by Src family kinases and recruit phosphatases, such as SH2-containing inositol phosphatase (SHIP) or SH2-containing phosphotyrosine phosphatase (SHP-1), which dephosphorylate neighbouring receptors to abrogate cellular activation (Ravetch and Lanier 2000). ITSM are defined by the consensus sequence TxYxx(V/I), which confers activating or inhibitory properties depending on the associated signalling proteins (Coxon, Geer et al. 2017). Some of the most studied inhibitory receptors include PD-1, CTLA-4, LAG-3, TIM3 and TIGIT; several IRs are targets of immune checkpoint blockade (ICB) therapies against cancer, but their clinical relevance will be best understood after introducing their mechanisms of action below.

Programmed cell death protein 1 (PD-1). PD-1, a member of the CD28 subfamily of immunoglobulin receptors, is one of the most extensively studied immune checkpoints. It was first identified by the group of Tasuku Honjo, who proposed that it plays a role in the negative regulation of immune responses (Ishida, Agata et al. 1992, H. Nishimura 1999). PD-1 is expressed on both activated $CD4^+$ and $CD8^+$ T lymphocytes, and mutations in the gene *PDCD1* are associated with the development of autoimmune disease (Ishida, Agata et al. 1992, H. Nishimura 1999). Other pioneering work on PD-1 was carried by Arlene H. Sharpe and Gordon J. Freeman, which discovered the ligands of PD-1, called PD-1 ligands 1 and 2 (PD-L1 and PD-L2); these molecules are expressed by immune and non-immune cells in response to inflammatory cytokines such as IFN-γ, to initiate a negative feedback pathway to protect host cells (Dong, Zhu et al. 1999, Freeman, Long et al. 2000, Latchman, Wood et al. 2001, Baessler and Vignali 2024). Mechanistically, PD-1 clusters near TCRs upon binding its ligand and becomes phosphorylated on its ITIM and ITSM motifs; such phosphorylation recruits protein tyrosine phosphatases, such as SHP1 and SHP2, which can subsequently dephosphorylate molecules in the TCR signalling complex, including Lck and ZAP-70, to oppose positive TCR signalling (Okazaki, Maeda et al. 2001, Patsoukis, Duke-Cohan et al. 2020, Baessler and Vignali 2024). Despite being one of the most extensively studied immune receptors, PD-1 remains a

major focus of research, as many groups continue to investigate the diverse mechanisms through which it suppresses immune signalling. For instance, recent studies have demonstrated that PD-1 inhibits actin remodelling at the immunological synapse by regulating actin cytoskeleton dynamics in a signalling motif–independent manner (Paillon, Mouro et al. 2023).

Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4). CTLA-4 is a T cell surface receptor that shares 31% amino acid identity with CD28 (Harper, Balzano et al. 1991) and binds to the same co-stimulatory ligands, B7.1 (or CD80) and B7.2 (or CD86), but with markedly increased affinity for CTLA-4 compared to CD28 (Linsley, Greene et al. 1994, Collins, Brodie et al. 2002). The critical importance of CTLA-4 in down-regulating T cell activation and maintaining immunologic homeostasis was recognized early, as mice lacking CTLA-4 develop massive proliferation of T cells and fatal multiorgan tissue destruction and die by 3-4 weeks of age (Tivol, Borriello et al. 1995, Waterhouse, Penninger et al. 1995). Few years later, the mechanism through which CTLA-4 exerts negative immune regulation was elucidated: after binding, CTLA-4 captures its ligands from antigen presenting cells through endocytosis, leading to their subsequent degradation. This depletion of B7 molecules from APCs results in impaired CD28 co-stimulation (Qureshi, Zheng et al. 2011, Kennedy, Waters et al. 2022).

Lymphocyte-activation gene 3 (LAG-3). LAG3 is a transmembrane receptor selectively expressed on activated T cells. Closely related to the CD4 molecule, it functions as a ligand for MHC class II molecules (Triebel, Jitsukawa et al. 1990, Huard, Mastrangeli et al. 1997). LAG-3 mediates inhibitory function via conserved motifs in its cytoplasmic domain, including a KIEELE motif (Workman, Dugger et al. 2002) and a RRFSALE motif, which were found to be both necessary and sufficient to mediate LAG-3–dependent inhibition of TCR signalling. Moreover, LAG-3–blocking antibodies were shown to enhance T cell activity even in the absence of LAG-3 ligands (Aigner-Radakovics, De Sousa Linhares et al. 2023). LAG-3 was shown to associate with the TCR–CD3 complex within the immunological synapse, where it disrupts the association of the CD4 (or CD8) co-receptor molecule with the tyrosine kinase Lck (Guy, Mitrea et al. 2022, Aggarwal, Workman et al. 2023). More recently, it was demonstrated that following ligand engagement, LAG-3 undergoes Cbl ligase–mediated polyubiquitination, which promotes its inhibitory function. Consistently, therapeutic LAG-3 antibodies suppress this ubiquitination, thereby unleashing the immune checkpoint activity of LAG-3 (Jiang, Dai et al. 2025).

T-cell immunoreceptor with Ig and ITIM domains (TIGIT). TIGIT is expressed on activated T cells and displays multiple inhibitory mechanisms to restrain T cell activation. It binds to CD155 (or PVR) and CD112 (or PVRL2), which are expressed on dendritic cells and tumour cells within the TME. Engagement of TIGIT on dendritic cells initiates a signalling cascade that drives the development of tolerogenic

dendritic cells, characterized by reduced IL-12 production and increased IL-10 secretion. In addition, TIGIT interferes with the activation of CD226, a costimulatory receptor broadly expressed on immune cells, including T cells. Finally, TIGIT enhances the immunosuppressive function of Tregs (Yu, Harden et al. 2009, Chauvin and Zarour 2020).

The IRs described and their ligands are summarized in **Figure 7**, along with therapeutic strategies targeting them, that will be discussed in Chapter 2.3.

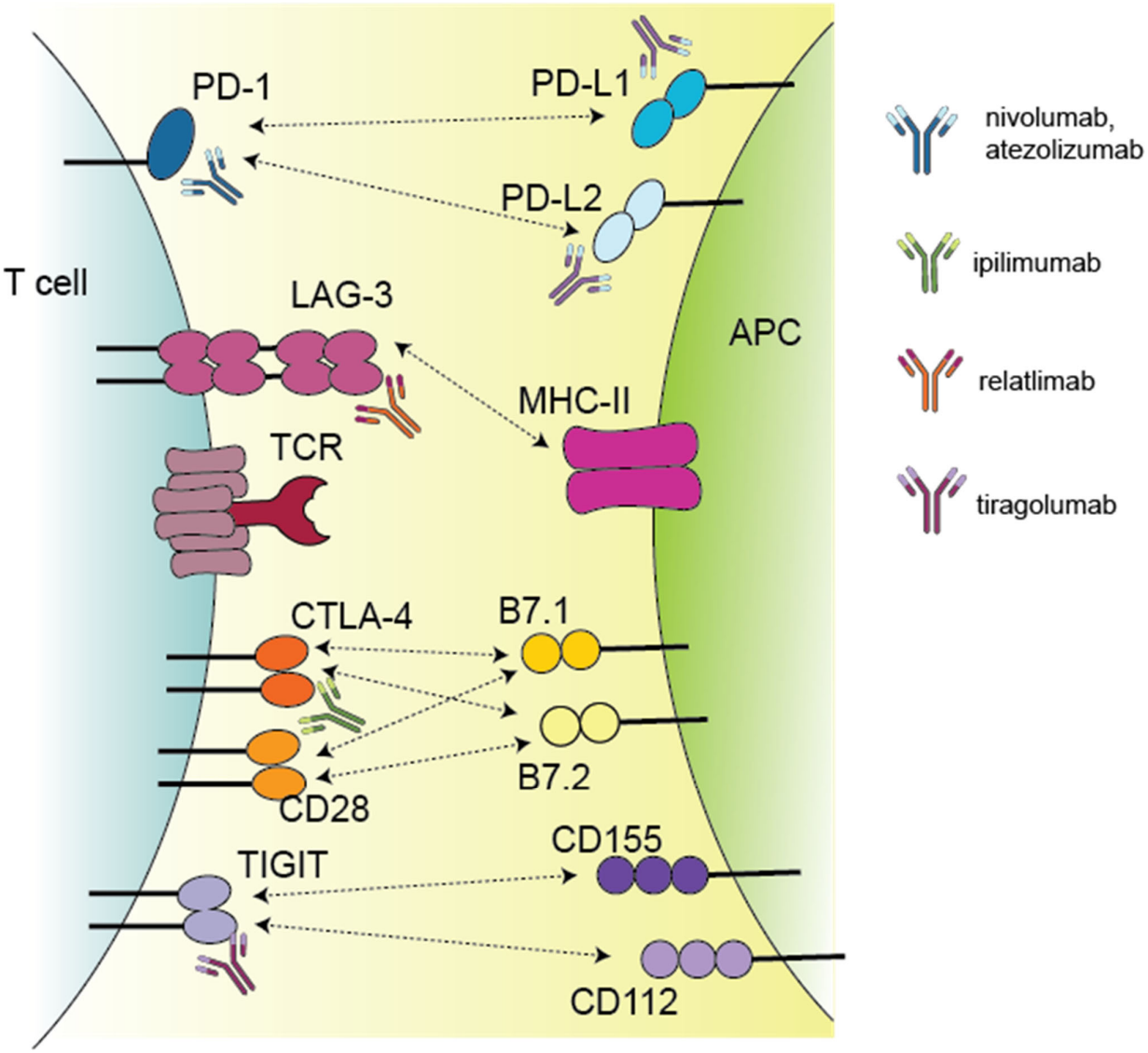

Figure 7. Schematic overview of key inhibitory immune checkpoint receptors and their ligands, along with the costimulatory receptor CD28. Clinically approved monoclonal antibodies including nivolumab, ipilimumab, atezolizumab, relatlimab, tiragolumab are shown at their respective targets, where they block inhibitory signalling pathways to enhance T cell activity. Dashed arrows indicate IR-ligand interactions. This scheme is not exhaustive, either in the range of inhibitory receptors depicted or in the monoclonal antibody therapies currently available for immunotherapy.

2.3 Immune Checkpoints in Cancer and Autoimmunity

In recognition of their pioneering studies on PD-1 and CTLA-4, Tasuku Honjo and James P. Allison were awarded the 2018 Nobel Prize in Physiology or Medicine. Their work demonstrated that blocking these inhibitory receptors could release the host's intrinsic antitumor immune activity. Important complementary contributions on PD-1 and CTLA-4 were also made by Arlene H. Sharpe and Gordon J. Freeman. These studies laid the foundation for modern cancer immunotherapy and revolutionized cancer treatment. Specifically, CTLA-4–blocking antibodies induced tumour rejection and conferred immunity against subsequent tumour challenge (Leach, Krummel et al. 1996) and ipilimumab was the first IR blockade treatment to receive FDA approval in 2011. Also blocking the PD-1/PD-L1 interaction resulted to be a powerful tool to inhibit the growth of tumour cells (Iwai, Terawaki et al. 2005), and blocking antibodies targeting PD-1 became the second FDA-approved ICB therapy in 2014. The big limitation of monotherapies targeting CTLA-4, PD-L1, or PD-1 was that only a limited subset of patients responded to therapy (Baessler and Vignali 2024). Combination of IR blocking antibodies, specifically anti-CTLA-4 antibody (ipilimumab) in combination with anti-PD-1 (nivolumab) for treatment of metastatic melanoma was more effective (Wolchok, Kluger et al. 2013). Promising results with the combination of anti-CTLA-4 and PD-L1 blocking antibodies have paved the way to the development of additional immune checkpoint inhibitor combinations with distinct mechanisms of action, aiming to enhance efficacy while reducing toxicity (Lee, Ha et al. 2021).

LAG-3 became the third checkpoint inhibitor to show efficacy when targeted in the clinic; relatlimab, an antibody targeting LAG-3, is used to treat melanoma especially in combination with nivolumab, against PD-1 (Tawbi, Schadendorf et al. 2022, Aggarwal, Workman et al. 2023). Interestingly, it was previously shown that synergistic actions of LAG-3 and PD-1 are critical for the prevention of autoimmunity in mice, thereby supporting the rationale that dual targeting of these immune receptors may represent an effective therapeutic strategy for both autoimmune disease and tumour immunity (Okazaki, Okazaki et al. 2011). Also, clinical trials targeting TIGIT in cancer gave promising initial results, especially when administered in combination with anti-PD-1 therapy. Tiragolumab, against TIGIT, plus atezolizumab, against PD-1, showed a clinically meaningful improvement in patients with non-small-cell lung cancer (NSCLC) (Cho, Abreu et al. 2022). Anti-TIGIT combined with anti-PD-1/PD-L1 and chemotherapy is currently being investigated in phase 2 and 3 trials in lung, liver, renal, and gastrointestinal cancers (Joller, Anderson et al. 2024).

At present, novel checkpoint molecules continue to be identified and explored as therapeutic targets. Approximately two-thirds of oncology trials are focused on T cell–targeted immunomodulatory strategies,

with more than 3,000 active clinical studies worldwide, testing inhibitors alone or in combination with chemotherapy (Robert 2020, Lee, Ha et al. 2021).

Impairment of physiological immune function underpins both cancer and autoimmunity, conditions at opposite ends of the immune spectrum. In cancer, inadequate antitumour immunity permits tumour growth, this is why removing the brake to T cell activity proved to be effective; conversely, breakdown of T-cell–mediated self-tolerance drives autoimmunity. Blockade of pathways that control T cell activation inevitably unleash their inflammatory potential, causing autoimmune-like toxicities (Mangani, Yang et al. 2023). Indeed, ICB is most effective when two distinct checkpoints are co-inhibited; a key limitation, however, is that combinatory therapies led to a substantial rise in damage to the host tissue, with patients experiencing immune-related adverse events (irAEs), autoimmune conditions which can affect any organ in the body (Conroy and Naidoo 2022). **Figure 8** illustrates the balance of T cell activity required for effective control of infections and malignancies, as well as the two pathological outcomes that can result from T cell dysregulation: cancer and autoimmunity (Mangani, Yang et al. 2023).

Interestingly, it was shown that resident gut bacteria can affect patient responses to cancer immunotherapy. For instance, antibiotics are associated with poor response to PD-1 blockade (Routy, Le Chatelier et al. 2018) and a significant association was observed between commensal microbial composition and clinical response. Abundance of bacteria of the *Ruminococcaceae* family was found in the guts of melanoma patients responding to PD-1 blockade; non-responders instead had an imbalance in gut flora composition, which correlated with impaired immune cell activity. Thus, maintaining healthy gut flora could help patients combat cancer (Gopalakrishnan, Spencer et al. 2018, Matson, Fessler et al. 2018). Therefore, interest is now also focused on understanding whether modulation of the microbiome could be used to prevent irAEs (Conroy and Naidoo 2022). Greater attention is also being addressed in identifying biomarkers that can predict irAEs occurrence. For example, specific HLA alleles have been associated with the development of irAEs, including ulcerative colitis. This suggests a role for HLAs in stratifying the risk of irAEs, identifying a group that requires more intensive monitoring (Hasan Ali, Berner et al. 2019).
Overall, comprehensive delineation of immune pathways will be essential to refine and individualize anticancer therapy based on each patient's clinical history and immunobiologic context, thereby optimizing therapeutic benefit and limiting collateral damage.

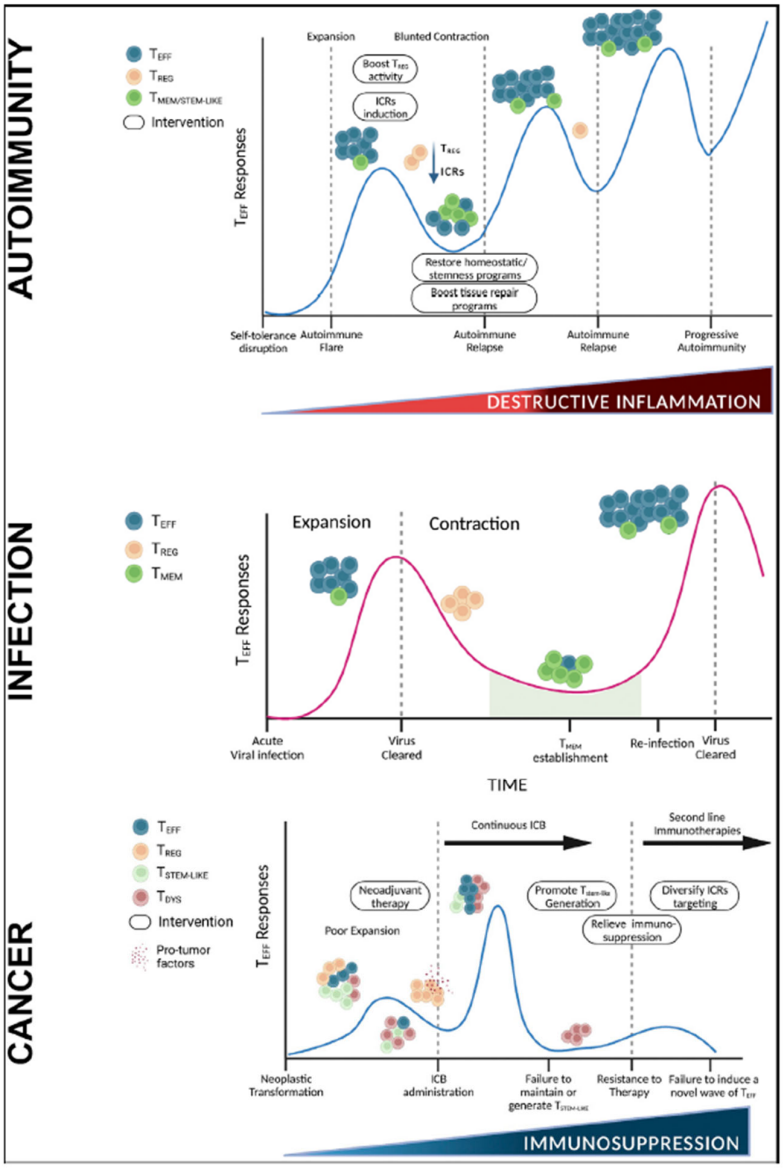

Figure 8. The (im)balance of T cell activity. Upper panel, autoimmunity: Disruption of self tolerance initiates the expansion of self-reactive effector T cells that comprise the first autoimmune flare. The inflammatory state is further accentuated by reduced regulatory T cell suppressive capacity and low immune checkpoint expression by self-reactive effector T cells. Therapeutic strategies are aimed at boosting Treg activity and immune checkpoint expression in self-reactive T cells. Eventually, autoimmune memory T cells expand again, leading to autoimmune relapses and development of progressive autoimmunity. **Middle panel, infection:** acute viral infections trigger an effector T cell expansion. Upon virus clearance, effector T cells undergo clonal contraction. Memory T cells are ready to trigger a response of bigger magnitude upon antigen re-encounters. **Bottom panel, cancer:** upon neoplastic transformation, tumour antigens are presented within a poorly inflamed environment, leading to poor expansion of tumour-reactive effector T cells. Tregs presence in the tumour microenvironment further dampens effector T cell responses. Immune checkpoint blockade (ICB) re-invigorates anti-tumour responses by eliciting a wave of T effector cells. However, continuous ICB post-surgery may hinder the establishment of long-term memory and lead to the reduction of the memory cell pool, which in turn may result in a sharp decline in the effector T cell population and hinder the generation of novel waves of effector T cells upon second-line immunotherapies, such as therapies targeting different immune checkpoint receptors (ICRs).

2.4 Fc-Receptor Like proteins

Fc-Receptor Like proteins family. Fc receptor-like (FCRL) proteins constitute a diverse multigene family phylogenetically related to classical Fc receptors. Unlike canonical Fc receptors, which are expressed mostly on innate immune cells and mediate antibody binding, FCRL proteins exhibit distinct immunomodulatory signalling functions and notably, the ligand of most FCRL proteins remains unidentified (Davis 2007, Wilson, Fuchs et al. 2012). In humans, the FCRL gene family encodes six transmembrane receptors, FCRL1-6, and two cytoplasmic proteins, FCRLA-B. The expression of most FCRL proteins is confined to the B cell lineage, with distinct patterns across different stages of differentiation. FCRL3 and FCRL6 are expressed also by T lymphocytes, including $CD4^+$, $CD8^+$, and Tregs, as well as on natural killer (NK) cells of the innate immune system (Davis 2007, Schreeder 2008). Because most FCRL members are prominently expressed in B cells, this cell type has been the primary focus for studies on their functions (Rostamzadeh, Kazemi et al. 2018). FCRL1-6 proteins contain one or more tyrosine-based motifs within their cytoplasmic tails, including ITIMs, which mediate the inhibition of TCR signalling (Ravetch and Lanier 2000), and/or ITAMs, which mediate transduction of TCR activation signals (Fodor, Jakus et al. 2006). The presence of ITAMs and ITIMs within their cytoplasmic region suggest that FCRL proteins coordinate immune responses and help in maintaining the balance required to initiate or terminate effector functions while minimizing peripheral damage to the host. Dysregulated expression or imbalance in the activity of these molecules could lead to severe biological consequences, including autoimmunity, infection and malignancy (Davis 2007).

Among the FCRL proteins expressed in humans, FCRL3 is distinguished by its unique expression pattern on T lymphocytes and NK cells, in addition to B cells, making it one of the few FCRL family members expressed beyond the B cell lineage. **Figure 9** summarizes the current state of knowledge about FCRL3, which is also detailed below.

Association of FCRL3 Genetic Variants with Autoimmunity. Notably, a single nucleotide polymorphism (SNP) within the promoter region of the *FCRL3* gene has been associated to autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus and autoimmune thyroid disease (Kochi, Yamada et al. 2005, Bajpai, Swainson et al. 2012). This SNP involves a variant at position -169 relative to the *FCRL3* transcriptional start site (rs7528684). Specifically, the major allele contains a thymine (T), whereas the minor allele contains a cytosine (C). Importantly, the transcription factor NF-κB exhibits significantly greater binding affinity to the *FCRL3* promoter when the -169C allele is present, while its affinity is reduced in the presence of the -169T allele. Correspondingly, the expression of *FCRL3* transcript is elevated in individuals carrying the -169C allele (Kochi, Yamada et al. 2005). Although *FCRL3* has been identified as an autoimmune disease-susceptibility risk allele also in other studies (Simmonds, Heward et al. 2006), evidence from the literature is inconsistent. Several reports indicate that the association between

the *FCRL3* -169C variant and disease susceptibility is significant only within specific ethnic groups (Choi, Kang et al. 2006, Newman, Zhang et al. 2006, Gibson, Li et al. 2009). Furthermore, other research suggests that the *FCRL3* -196C allele may be protective in diseases such as multiple sclerosis (MS) (Martínez, Mas et al. 2007, Matesanz, Fernández et al. 2008) and Addison's Disease (AD) (Owen, Kelly et al. 2007). Collectively, all these data underscore the importance of elucidating the signalling properties of the FCRL3 receptor to better understand its potential role in mediating autoimmune disease.

FCRL3 in cancer and infections. Interestingly, *FCRL3* is among the genes upregulated in tumour-infiltrating T lymphocytes in melanoma and NSCLC (Li, van der Leun et al. 2019). Elevated *FCRL3* is also observed in tumour T cells from patients with Sézary syndrome (Wysocka, Kossenkov et al. 2014, Anzengruber, Ignatova et al. 2019). Beyond cancer, increased *FCRL3* transcript expression has been reported in B cells and T_{FH} cells during chronic infections such as hepatitis B virus (HBV) and malaria (Portugal, Obeng-Adjei et al. 2017, Poonia, Ayithan et al. 2018). This means that *FCRL3* expression is a recurring feature of lymphocytes exposed to persistent antigen stimulation. Therefore, FCRL3 receptor may identify a subset of T cells marked by prolonged antigenic stimulation and diminished effector capacity, but whether its expression associates with favorable or adverse prognosis has not yet been established.

FCRL3 in B lymphocytes. The function of FCRL3 has been investigated in B lymphocytes, revealing a complex regulatory role. It was suggested that FCRL3 possesses inhibitory potential on BCR-mediated signalling; however, this observation derived from an artificial experimental system. In that setup, chimeric proteins bearing the extracellular domain of murine FcγRIIB and the intracellular domain of FCRL3 were crosslinked to the BCR using anti-IgM antibodies, which resulted in inhibition of BCR signalling (Kochi, Myouzen et al. 2009). In contrast, it was reported that FCRL3 expression on B lymphocytes is upregulated following Toll-Like Receptor 9 (TLR-9) activation, involved in innate immunity signalling, while concurrently suppressing immunoglobulin production (Li, Schreeder et al. 2013). This potential disparity reveals the nuanced and context-dependent functions of FCRL3 in B cell biology and illustrates how experimental design can shape interpretation. These observations highlight the necessity for further comprehensive studies to fully elucidate the mechanistic roles of FCRL3.

FCRL3 in Tregs. FCRL3 is highly expressed also on Tregs. Extensive functional analyses have demonstrated that both FCRL3-positive and FCRL3-negative Treg subsets exhibit typical suppressive activity, effectively inhibiting the proliferation of effector T cells to a comparable extent. The primary distinction between these subsets lies in their proliferative capacity *in vitro*: upon TCR stimulation in the presence of IL-2, FCRL3-negative Tregs are capable of proliferation, whereas FCRL3-positive Tregs remain unresponsive (Nagata, Ise et al. 2009). Further studies on Tregs showed that FCRL3 and TIGIT co-expression characterizes a subset of $Helios^{+}FOXP3^{+}$ Tregs that is highly enriched for suppressive clones

(Bin Dhuban, d'Hennezel et al. 2015), consistent with the observation that Helios is a marker of stable, thymically-derived Tregs exhibiting potent suppressive capacity (Thornton and Shevach 2019). Finally, it was shown that FCRL3 stimulation in Tregs promotes a T_H17-like phenotype, characterized by increased expression of the cytokines IL-17, IL-26, IFN-γ, as well as elevated levels of the transcription factor RORγt (Agarwal, Kraus et al. 2020). As for the ligand of FCRL3, mechanistic *in vitro* studies found that FCRL3 is capable of binding Secretory IgAs (SIgAs), crucial mediators of mucosal immunity, and engagement of FCRL3 by SIgA was shown to impair the suppressive ability of Tregs (Agarwal, Kraus et al. 2020, Kraus, Birla et al. 2025). However, *in vitro* the interaction FCRL3-SIgA exhibited an affinity of 0.45 μM, whereas SIgA concentrations in human serum are approximately 0.03 μM (Delacroix and Vaerman 1981), indicating that the physiological relevance of this interaction *in vivo* remains uncertain and warrants further investigation.

FCRL3 in conventional T lymphocytes. The function and signalling role of FCRL3 in conventional T lymphocytes has not yet been investigated. In a previous study from our laboratory, we found that within $CD4^+$ T lymphocytes separated by their capacity to secrete the pro-inflammatory cytokine GM-CSF, *FCRL3* was among the most differentially expressed genes in the subset of $CD4^+$ effector memory cells that did not produce GM-CSF (Emming, Bianchi et al. 2020). This population exhibited upregulation of multiple genes associated with the negative regulation of T cell activation, including *FOXP3*, *CTLA4*, and *TIGIT*. In contrast, genes typically linked to an exhausted phenotype, such as *PDCD1* (encoding PD-1) and *LAG3*, were not upregulated, indicating that GM-CSF–non-producing effector memory $CD4^+$ T lymphocytes are not fully dysfunctional (Emming, Bianchi et al. 2020). Moreover, ATAC-seq revealed that the *FCRL3* locus was selectively accessible in GM-CSF–negative cells, indicating that *FCRL3* transcription is restricted to this subset which has been linked to negative regulation of T cell activation (Emming, Bianchi et al. 2020). The detection of FCRL3, a receptor with immunomodulatory potential, in a population displaying increased expression of selected inhibitory molecules suggests a possible role in modulating T lymphocyte responses, particularly given that it is one of only two FCRL proteins expressed in T cells, together with FCRL6. Elucidating how FCRL3 regulates responses across distinct effector T lymphocyte subsets and determining whether its regulatory polymorphisms perturb its biology in ways that contribute to disease pathogenesis, remain important areas of investigation, with the potential to advance our understanding and treatment of immune-mediated disorders.

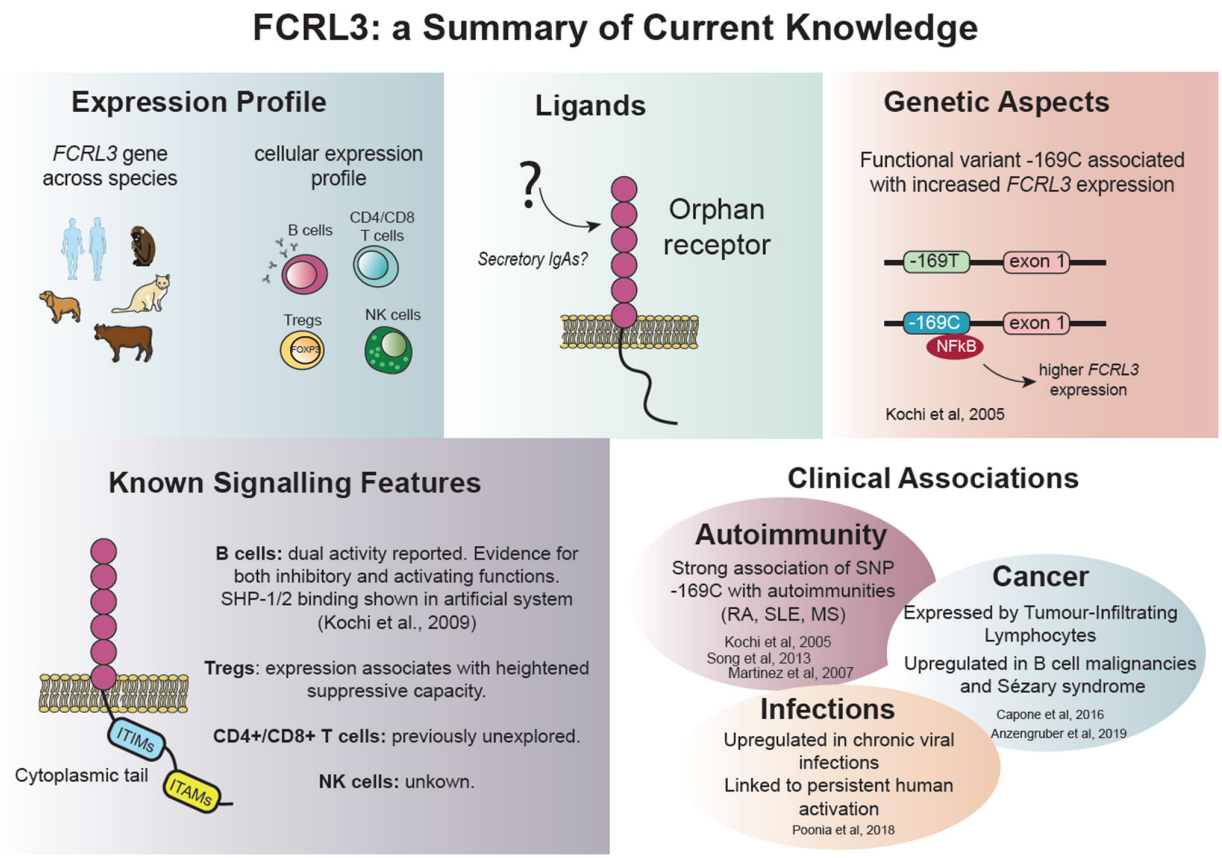

Figure 9. FCRL3: summary of current knowledge. The figure integrates expression patterns, signalling functions, and disease associations of FCRL3, highlighting both established findings and unresolved questions. The panels illustrate: immune cell types expressing *FCRL3* (expression profile, top left); its status as an orphan receptor (top middle); promoter region polymorphism (-169 T/C SNP) increasing NF-κB binding and *FCRL3* expression (top right); reported signalling activity in different cell types (bottom left); and clinical associations with immune-mediated diseases (bottom right).

3. Hypothesis and aims of the thesis

Failure of the mechanisms that maintain immune homeostasis can result in sustained or excessive T cell activity, leading to pathological conditions such as chronic inflammation and autoimmunity. Although FCRL3 has been implicated in immune regulation, its precise function and mechanistic contribution to T cell biology remain incompletely defined. Its immunomodulatory potential, together with the strong association of a functional variant of *FCRL3* with several autoimmune diseases, suggests that FCRL3 may play a key role in regulating T cell responses.

Based on this rationale, the objectives of this thesis are the following:

1. **To functionally characterize FCRL3$^+$ T lymphocytes** by assessing whether this subset is functionally competent and defining core T cell properties, including activation, proliferative capacity, and cytotoxic activity.
2. **To identify the signals that induce FCRL3 expression** in T lymphocytes.
3. **To determine the FCRL3-mediated functions** by investigating whether FCRL3 itself contributes to the functional properties of FCRL3$^+$ T cells.

4. Manuscript

This chapter is based on the manuscript accepted in the Journal of Experimental Medicine (Bianchi, Foli et al. 2025), titled "FCRL3 is an immunoregulatory receptor that restrains the activation of human memory T lymphocytes".

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In this manuscript, we investigated the role of FCRL3 in T lymphocytes. We performed an extensive functional characterization of the subsets of T lymphocytes expressing FCRL3. Our results across conventional T lymphocytes revealed that FCRL3 marks highly differentiated T cells with reduced proliferative and activation capacity but enhanced cytotoxic potential. Furthermore, using CRISPR-Cas9–mediated gene editing and ectopic expression approaches, we identified a direct role for FCRL3 in the negative regulation of T cell activation.

In this manuscript I contributed to every figure except for Figure 6 and Supplementary Figure 5.

JEM Journal of Experimental Medicine

ARTICLE

FCRL3 is an immunoregulatory receptor that restrains the activation of human memory T lymphocytes

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Genetic variants in the *FCRL3* gene are linked to autoimmune disorders. However, the functional properties of FCRL3-expressing T lymphocytes, and the regulation and functional impact of FCRL3 expression remain understudied. Here, we performed a multiomic and functional analysis of human T lymphocytes expressing FCRL3. FCRL3 expression correlated with reduced capacity of T cells to undergo activation and was accompanied by functional specialization toward a cytotoxic phenotype, resembling cytotoxic CD4⁺ T lymphocytes and CD8⁺ effector memory T_{EMRA} cells. FCRL3 expression was induced upon repetitive TCR engagement, and sufficed to attenuate T cell responses, indicating a role as a negative regulator of the activation of differentiated T cell subsets with high cytotoxic capacity. Mechanistically, the cytoplasmic domain of FCRL3 engaged inhibitory molecules, suggesting a direct role in limiting activating signals. Overall, our study establishes FCRL3 as a functional immunoregulatory receptor that restrains the activation of highly specialized human memory T cells.

Introduction

The dynamic interplay between costimulatory and coinhibitory receptors during T cell activation plays a critical role in shaping immune responses and in maintaining immune homeostasis. Disruption of such regulatory circuits on the one hand contributes to the pathogenesis of autoimmune diseases through the dysregulation of immune tolerance, and on the other can be harnessed to enhance cancer immunotherapy. Hence, identification of molecular players involved in such processes is essential not only to understand regulatory circuits underlying immune cell activation and regulation, but also to identify actionable therapeutic targets. Fc receptor–like protein 3 (FCRL3) is a type I transmembrane protein expressed by T and B lymphocytes and NK cells (Li et al., 2013; Schmiedel et al., 2022). It displays high homology to Fcγ receptors and contains extracellular Ig-like domains, as well as a cytoplasmic tail comprising four putative immunoreceptor tyrosine–based inhibitory motifs (ITIMs), short sequences that by recruiting inhibitory signal transducers such as protein phosphatases counteract immune cell activation (Davis, 2007; Xu et al., 2002). The immunomodulatory potential of FCRL3 is indicated by the observation that polymorphisms in the *FCRL3* gene are associated with several autoimmune disorders, including multiple sclerosis (Martínez et al., 2007; Matesanz et al., 2008), autoimmune Addison's disease (Owen et al., 2007), and rheumatoid arthritis (Kochi et al., 2005). For instance, a specific polymorphism in the *FCRL3* promoter leads to enhanced gene expression, owing to the formation of an NF-kB binding site with increased affinity for its cognate transcription factor (Kochi et al., 2005). However, the mechanistic links between this polymorphism, lymphocyte functionality, and disease predisposition remain to be understood. Consistent with a possible regulatory role, chimeric proteins composed of murine FcγRIIB fused to human FCRL3 were shown to inhibit B cell receptor–mediated signaling in B lymphocytes, an effect that was at least in part linked to the recruitment of the phosphatase SHP1 to the FCRL3 ITIMs (Kochi et al., 2009). However, the identity of FCRL3 ligand(s) is still unknown. Indeed, although FCRL3 binds secretory IgA antibodies *in vitro* (Agarwal et al., 2020), its ability to bind antibodies *in vivo* and the potential presence of other physiological ligands for this receptor are yet to be established.

Among T lymphocytes, FCRL3 is highly expressed by a subset of Treg cells (Bin Dhuban et al., 2015; Nagata et al., 2009; Swainson et al., 2010), where it was shown to modulate cytokine production and to limit suppressive capacity (Agarwal et al., 2020). As regards conventional memory CD4⁺ and CD8⁺ T lymphocytes, we recently found that the expression of the *FCRL3*

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gene was increased in a subset of human effector memory T lymphocytes characterized by their limited capacity of producing inflammatory cytokines (Emming et al., 2020). However, no functional role of this transmembrane protein in T lymphocytes other than Tregs has been identified. Notably, in keeping with the extensive evolutionary divergence of FCRL family members, FCRL3 has no ortholog gene in the mouse (Davis, 2007; Li et al., 2014). The lack of animal models has insofar hampered a complete understanding of FCRL3 functions in T lymphocytes.

In this study, we aimed to determine the regulation and functional impact of FCRL3 in human T lymphocyte subsets using comprehensive molecular profiling coupled with mechanistic and functional analyses. FCRL3 expression correlated with reduced T cell activation and proliferation, as well as with increased specialization toward a cytotoxic phenotype. FCRL3 expression was induced by repetitive T cell receptor (TCR) stimulation, and the ectopic expression of the full-length receptor and even of its cytoplasmic tail was sufficient to limit T cell activation, pointing toward a direct role of FCRL3 in modulating TCR signaling. Consistent with this model, the FCRL3 protein interactome included adaptor proteins involved in the inhibition of TCR-mediated signaling. Overall, our study identifies FCRL3 as an immunoregulatory receptor induced by repetitive stimulation and capable of limiting the activation of highly differentiated memory T cells.

Results

FCRL3 expression modulates activation of human memory T cells

Within the T cell compartment in the peripheral blood of healthy donors, FCRL3 was expressed by a large proportion of naïve and memory CD25$^+$ Treg cells, as well as by memory CD8$^+$ T cells, while expression by conventional memory CD4$^+$ and naïve T cells was more limited (Fig. 1, A and B; gating strategies in Fig. S1 A). Within the CD8$^+$ memory compartment, the highest expression was observed in the subpopulation of terminally differentiated CCR7$^-$ CD45RA$^+$ T_{EMRA} cells (T effector memory cells expressing CD45RA), compared with effector memory (CCR7$^-$ CD45RA$^-$, T_{EM}) and central memory (CCR7$^+$ CD45RA$^-$, T_{CM}) cells (Fig. S1 B). However, FCRL3 expression did not simply correlate with the proportion of T_{EMRA} cells in these donors (Fig. S1 C), consistent with its expression also by other subsets, including T_{EM} and T_{CM} cells (Fig. S1 B). No correlation was observed with the age and/or gender of the donors, pointing toward a general mechanism that regulates FCRL3 expression independent of sex- and aging-related processes (Fig. S1 D). Additionally, FCRL3$^+$ CD8$^+$ cells were not enriched for either polarized subset of Tc1 or Tc2 cells, indicating limited specialization (Fig. S1 E). FCRL3 expression was previously shown to be directly controlled by a T/C single-nucleotide polymorphism (SNP) in the *FCRL3* promoter, with the minor allelic variant (C) increasing the affinity of the transcription factor NF-kB for a cognate DNA binding motif (Gibson et al., 2009; Kochi et al., 2005; Swainson et al., 2010). Since the percentage of memory T lymphocytes expressing FCRL3 showed high donor-to-donor variability, ranging from 1 to >50% (Fig. 1 B), we hypothesized that part of this variability could be explained by allelic variation at such polymorphic site. Genotyping of the donors confirmed that the highest percentage of FCRL3 expression was indeed associated with homozygosity for the minor allele, which, however, did not significantly alter the levels of FCRL3 expression in individual cells within the FCRL3$^+$ subset (Fig. S2, A–D). Consistent with their differentiated phenotype, FCRL3$^+$ CD4$^+$ and CD8$^+$ T cells showed comparatively reduced activation in activation-induced marker (AIM) assays (Poloni et al., 2023) upon 48-h stimulation with plate-bound anti-CD3 and anti-CD28 antibodies (hereafter anti-CD3/CD28) (Fig. 1, C–E). Cells did not express these markers before activation (Fig. S2 E). Upon stimulation, the AIMs CD25, OX40, and CD40L were upregulated in both cell subsets; however, their expression was significantly attenuated in FCRL3$^+$ cells (Fig. 1, C–E and Fig. S3 A), pointing toward altered activation thresholds in these cells. Additionally, FCRL3$^+$ cells displayed enhanced activation-induced cell death and reduced proliferation capacities (Fig. S3, B and C), which is consistent with a differentiated T cell phenotype.

Since FCRL3-expressing cells were mostly contained within more terminally differentiated T cell compartments, we investigated whether repetitive TCR stimulation or costimulation was sufficient to induce FCRL3 expression in memory T cells. First, we selected donors with low or no expression of FCRL3, and then, we compared full stimulation with anti-CD3/CD28 with the repetitive stimulation using anti-CD3 alone. Two consecutive stimulations of memory T cells with anti-CD3 antibody were indeed sufficient to induce FCRL3 expression (Fig. 2, A–C), suggesting that FCRL3$^+$ cells arise *in vivo* through repetitive encounters with recurrent or common antigens.

Since FCRL3$^+$ T cells showed reduced AIM expression after TCR engagement (Fig. 1, C and D), we asked whether FCRL3 expression was causally associated with reduced T cell activation. First, we optimized an experimental system to delete FCRL3 in primary T cells by CRISPR/Cas9 and to detect the effect of its loss on T cell activation. FCRL3$^+$ T cells were enriched from the peripheral blood and transfected with ribonucleoprotein (RNP) complexes containing recombinant Cas9 and sgRNAs against *FCRL3*, after which cell viability was maintained with the addition of recombinant human IL-7 and IL-15 (Fig. 2, D and E) (Albanese et al., 2022). After repetitive stimulation of these cells with anti-CD3 antibody, surface FCRL3 expression was observed on control cells, but not on cells transfected with *FCRL3* sgRNAs (Fig. 2 F). Deletion of FCRL3 was sufficient to enhance the expression of the measured AIMs (CD25, CD137), thus pointing toward a causal association between FCRL3 expression and reduced T cell activation (Fig. 2, G and H).

To confirm these findings with an orthogonal experimental approach, we ectopically expressed FCRL3 by lentiviral transduction in memory CD8$^+$ T cells not expressing FCRL3 (Fig. 2 I). Since T cells needed to be preactivated for efficient lentiviral transduction and selection, in this system we could not monitor early activation events. However, we found that the ectopic expression of FCRL3 in cells preactivated with plate-bound anti-CD3/CD28 was sufficient to reduce CD25 expression at later time points (day 10) after the initial activation (Fig. 2, J and K), further

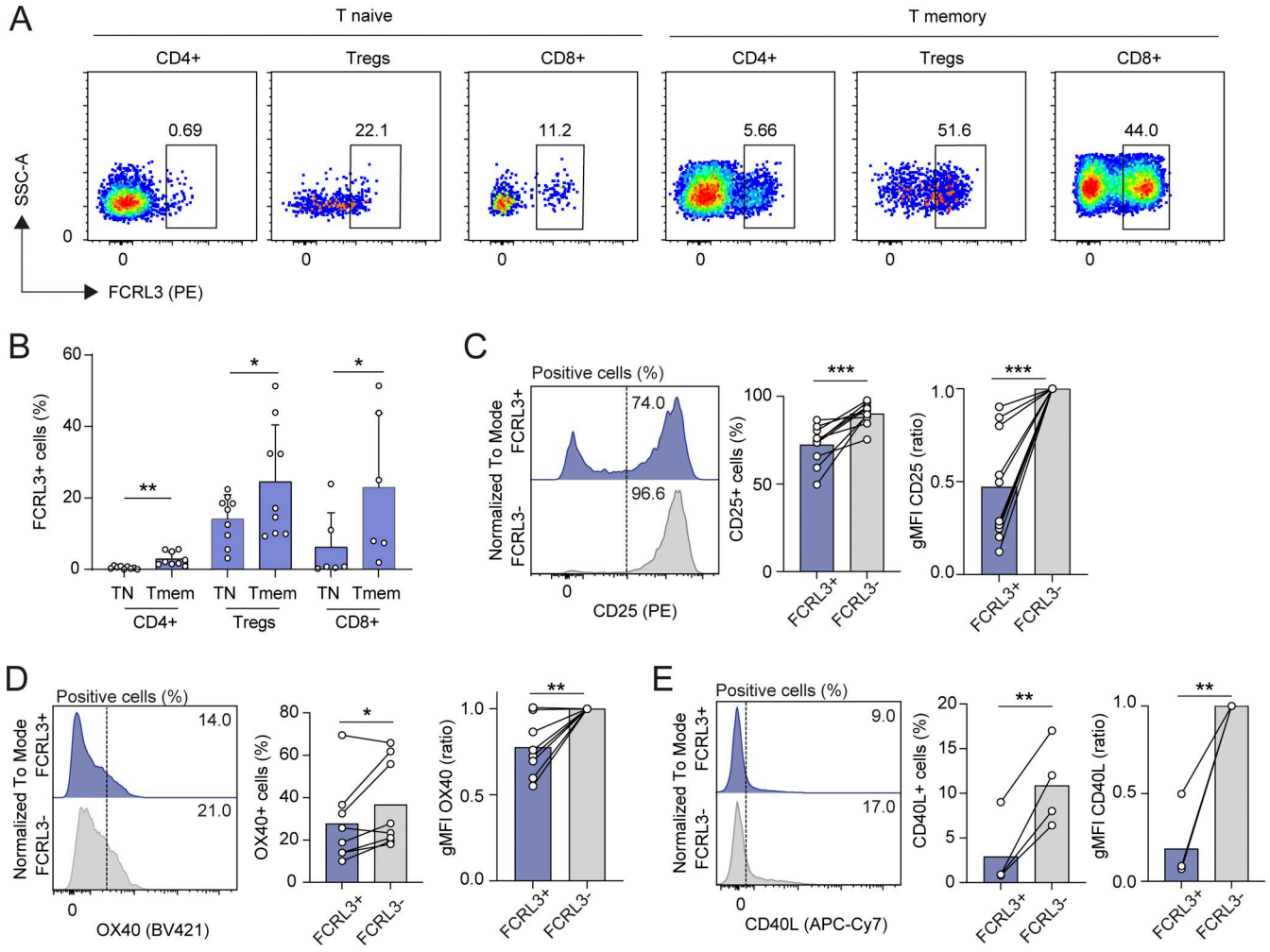

Figure 1. **Reduced activation of FCRL3⁺ T cells. (A)** Surface FCRL3 expression measured by FACS in human naïve and memory CD4⁺ T helper, Tregs, and CD8⁺ T lymphocytes freshly isolated from peripheral blood of one representative healthy donor. **(B)** FCRL3 expression in different T cell populations measured as in A. Each dot represents one donor. *N* = 6–9; mean ± SD; paired *t* test, two-tailed. From left to right: **P = 0.0033, *P = 0.0303, *P = 0.0255. **(C–E)** Surface staining for CD25 (C), OX40 (D), and CD40L (E) expression in sorted FCRL3⁺ and FCRL3⁻CD8⁺ memory T cells after stimulation with plate-bound anti-CD3/CD28 for 48 h. Each dot represents a different donor, *N* = 4–10. Mean, paired *t* test or ratio paired *t* test, two-tailed. From left to right: (C)***P = 0.0008, ***P = 0.0003, (D)*P = 0.048, **P = 0.0071, (E)**P = 0.0064, **P = 0.0044. Data underlying this figure can be found in Data S4.

underscoring the causal link between FCRL3 expression and dampened T cell activation.

Overall, FCRL3 is expressed by a subpopulation of memory T cells upon iterative TCR stimulation, and its expression is associated with reduced activation.

Functional specialization of the FCRL3⁺ T cell subset

To further characterize the FCRL3⁺ T cell subset, we performed RNA-seq of memory CD8⁺ T lymphocytes separated from the peripheral blood of *N* = 5 healthy donors, stimulated for 3 h with phorbol 12-myristate 13-acetate (PMA) and ionomycin to induce cytokine expression. For comparison, we also performed RNA-seq of memory CD4⁺ and total Treg cells obtained from the same donors. Using a false discovery rate (FDR) ≤0.05 and $\log_2$ fold change ≥0.5, we found that in CD8⁺ T cells, 44 transcripts were preferentially expressed by FCRL3⁻ cells and 45 were preferentially expressed by FCRL3⁺ cells (Fig. 3, A and B; Fig. S3 D; and Data S1). Among these, *IL17A* and *IL17F* were prominently less expressed by FCRL3⁺ cells, while *TOX* and *CXCR5* were expressed at higher levels. Preferential CXCR5 expression by FCRL3⁺ cells was confirmed by surface staining, showing that indeed CXCR5⁺ cells are mostly contained within the FCRL3⁺ subset (Fig. S3 E). In CD4⁺ T lymphocytes, 104 genes had significantly reduced expression in FCRL3⁺ cells, including transcripts for inflammatory cytokines such as *IL17A*, *IL17F*, and *IL23A*, consistent with the results obtained in CD8⁺ T cells (Fig. S4, A–C; and Data S1). Interestingly, among the transcripts enriched in both CD8⁺ and CD4⁺ FCRL3⁺ cells, markers of both cytotoxicity and terminal differentiation emerged, including the transcription factors *TOX* and, to a lesser extent, Eomesodermin (*EOMES*). Increased *TOX* expression is consistent with repetitive *in vivo* TCR stimulation of these cells, since TOX is typically associated with chronic T cell stimulation (Sekine et al., 2020; Soerens et al., 2023). Specific for CD4⁺ T cells, CRTAM emerged as a marker of a cytotoxic phenotype (CD4⁺ CTLs) (Fig. S4, A–C; and Data S1). This is in agreement with the fact that EOMES directly enhances IFN-γ

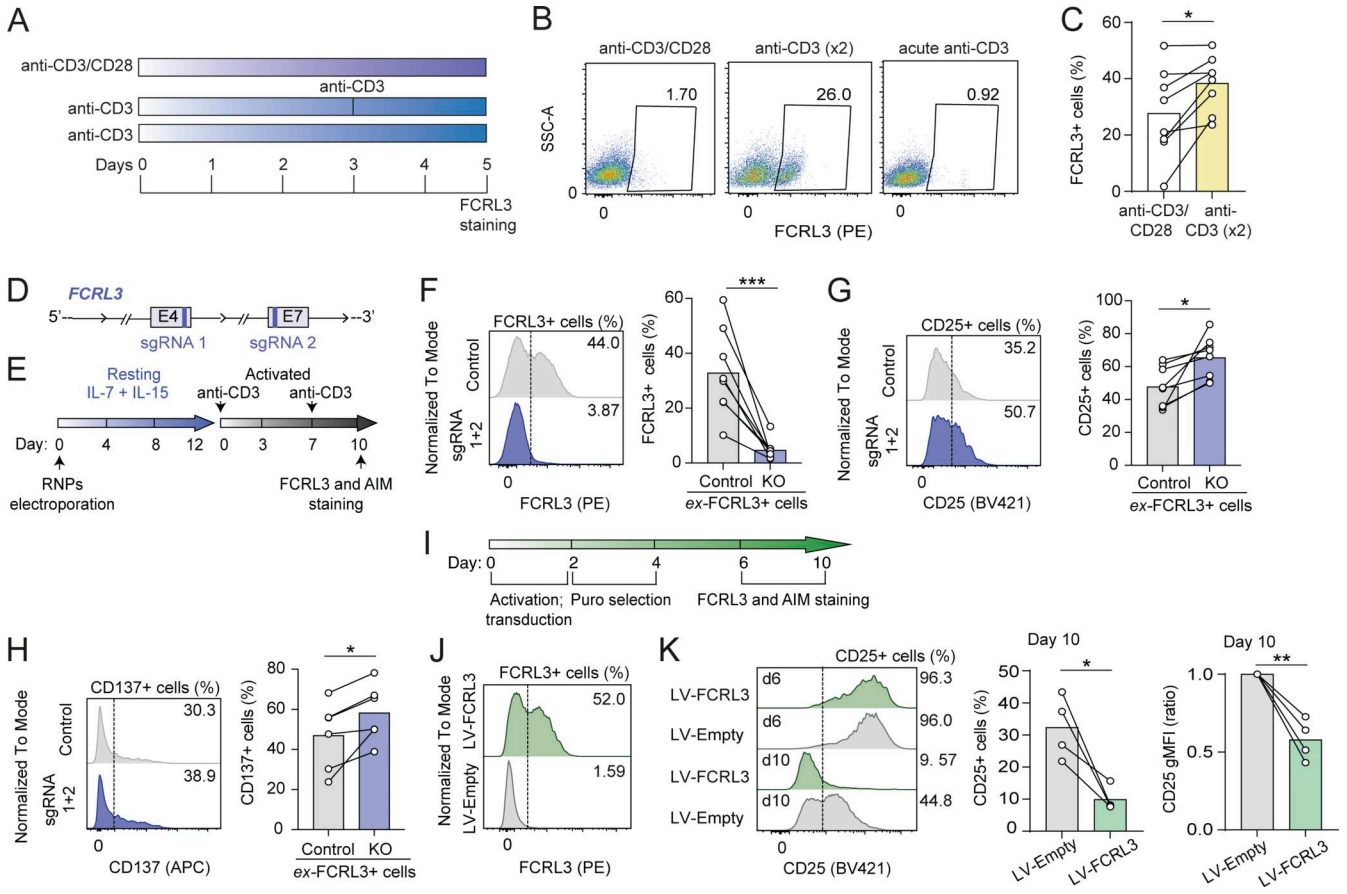

Figure 2. **FCRL3 expression is sufficient to modulate T cell activation. (A)** Schematic representation of the experimental setup for repetitive T cell stimulation. Memory CD8⁺ T cells isolated from peripheral blood were activated on plate-bound anti-CD3 antibody with or without costimulation with an anti-CD28 antibody or restimulation. **(B and C)** Surface expression of FCRL3 measured 5 days after activation with anti-CD3/CD28 or with two consecutive stimulations (day 0 and day 3) with anti-CD3 antibody. One representative donor (B) and $N = 8$ independent experiments (C) are shown. Each dot represents one donor. Mean; paired *t* test, two-tailed. *P = 0.0147. **(D)** Schematic representation of the *FCRL3* locus, with the exons (4 and 7) targeted by the selected sgRNAs. Only the exons targeted by the sgRNAs are shown. **(E)** Experimental workflow for FCRL3 CRISPR-KO in FCRL3⁺ CD8⁺ T cells. **(F–H)** FCRL3 (F), CD25 (G), and CD137 (H) expression on CD8⁺ FCRL3⁺ cells, transfected with RNPs containing sgRNAs targeting the *FCRL3* gene, or control. Surface expression was monitored 10 days after reactivation with anti-CD3. $N = 6–8$ independent experiments. Mean; paired *t* test, two-tailed. From left to right: (F) ***P = 0.001, (G) *P = 0.0145, (H) *P = 0.0171. **(I)** Experimental workflow for the ectopic expression of FCRL3 in CD8⁺ T cells, by lentiviral transduction. After activation with plate-bound anti-CD3/CD28 and transduction, transduced cells are selected by puromycin treatment, followed by recovery and surface staining for FCRL3 and AIMs. **(J)** Surface expression of FCRL3 in memory CD8⁺ T cells transduced with FCRL3-encoding lentivirus or empty lentivirus as a control. One representative experiment of $N = 4$. **(K)** Surface CD25 expression in FCRL3-transduced memory CD8⁺ T cells, 6 and 10 days after the initial activation. The left panel shows the result for one representative donors, while the bar graphs represent the results for $N = 4$ independent donors at day 10. The gMFI and percentage of CD25⁺ cells are both shown. Mean ± SD; paired *t* test, two-tailed. *P = 0.0306, **P = 0.0076. Data underlying this figure can be found in Data S4.

production, counteracts production of type-17 cytokines, and is required for the terminal differentiation of CD4⁺ CTLs, especially under conditions of chronic infection or prolonged immune activation (Geginat et al., 2023; Malyshkina et al., 2023; Pearce et al., 2003), while CRTAM is an adhesion molecule contributing to the enhancement of cytotoxic functions by CD4⁺ CTLs (Takeuchi and Saito, 2017). Consistent with this observation, we also found enhanced expression of granzyme transcripts (Fig. S4 C), and intracellular staining for granzyme B confirmed its increased expression in FCRL3⁺CD4⁺ cells (Fig. S4 D). Analysis of Treg cells from the same donors revealed reduced expression of all cytokine genes in FCRL3⁺ Treg cells, but otherwise limited overlap with the gene expression profile of conventional memory CD4⁺ T cells (Fig. S4, E and F). Although *FOXP3* emerged as a differentially expressed gene in memory CD4⁺ cells, its expression was lower than in Tregs, consistent with the observation that conventional memory human CD4⁺ T cells are able to express FOXP3 at levels that are usually insufficient to cause suppression (Sakaguchi et al., 2010) (Fig. S4 G). Indeed, both CD4⁺ and CD8⁺ FCRL3⁺ T cells maintained high expression of IFN-γ, to levels that were at least comparable to those of FCRL3⁻ cells (Fig. S4 H), suggesting reduced expression of selected cytokines but not dysfunction, and consistent with an overall CD8⁺ T_{EMRA} and CD4⁺ CTL phenotype. Our results are also concordant with single-cell RNA-seq data of tumor-infiltrating human CD4⁺ and CD8⁺ T lymphocytes, showing that in the CD4⁺ compartment, FCRL3 expression is primarily confined to Tregs and cytotoxic CD4⁺ CTLs, while within the CD8⁺ compartment, expression is more widespread but mostly localized to T_{EMRA} cells (Andreatta et al., 2022).

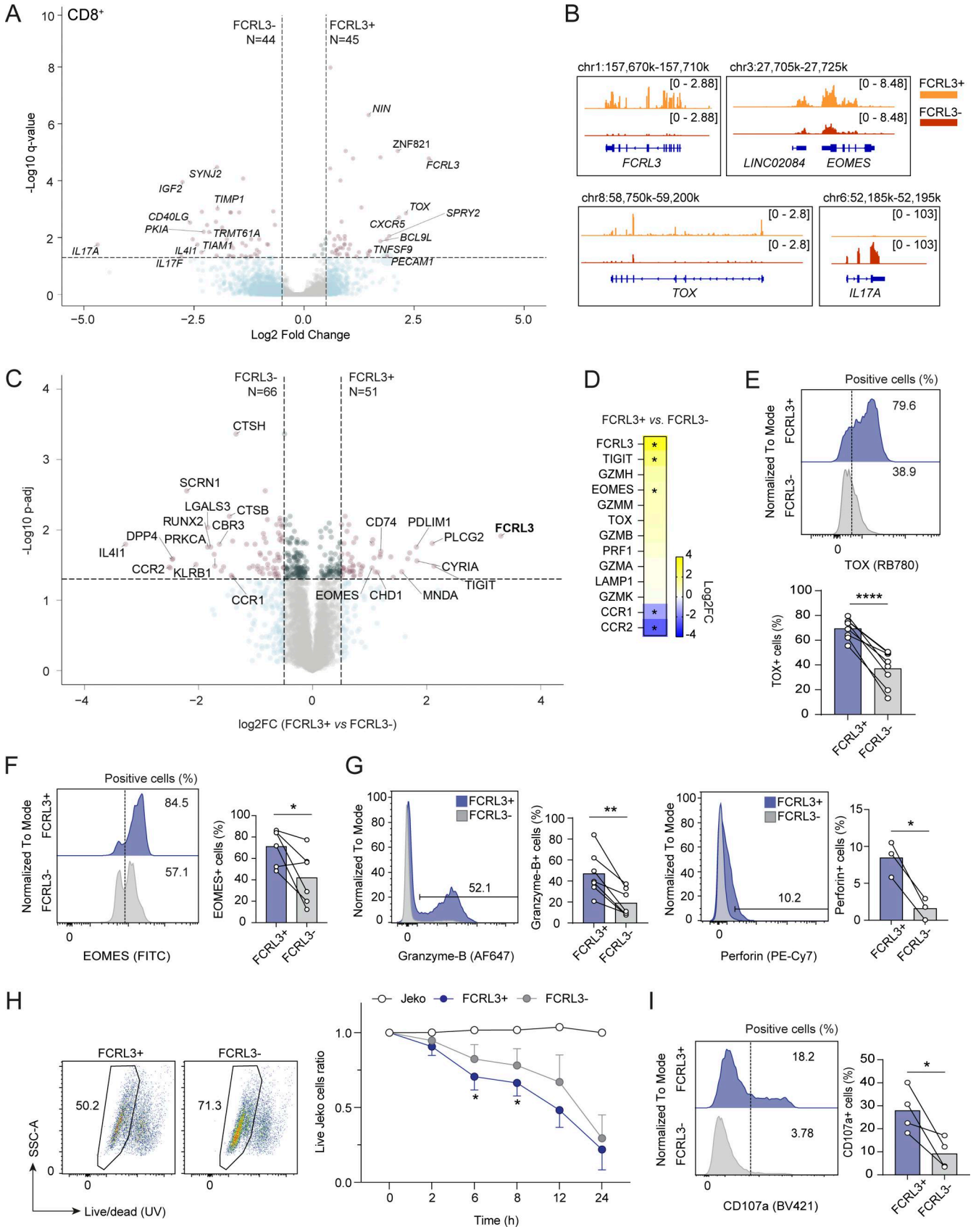

Figure 3. **Characterization of the CD8⁺ FCRL3⁺ T cell subset. (A)** Differentially expressed genes in FCRL3⁺ versus FCRL3⁻ memory CD8⁺ T cells sorted from the peripheral blood of $N = 5$ healthy donors and analyzed by RNA-seq (FDR ≤ 0.05 and $\log_2$FC ≥|0.5|). **(B)** RNA-seq tracks for selected transcripts enriched in FCRL3⁺ or FCRL3⁻ cells. **(C)** Differentially expressed proteins in sorted CD8⁺ FCRL3⁺ versus CD8⁺ FCRL3⁻ memory T cells, measured by shotgun mass

spectrometry ($log_2FC \geq |0.5|$; FDR [Benjamini and Hochberg multiple *t* test correction] ≤0.05; *N* = 7 independent donors). **(D)** Heatmap showing the differential expression of selected proteins from C. **(E and F)** TOX (E) and EOMES (F) intracellular staining in sorted FCRL3$^+$ and FCRL3$^-$ memory T cells. One representative experiment and the results from *N* = 8 (TOX) or *N* = 6 (EOMES) independent donors are shown; each dot represents one donor. Paired *t* test, two-tailed. **(E)** ****P < 0.0001, (F) *P = 0.0352. **(G)** Granzyme B (left) and perforin (right) expression in sorted CD8$^+$ FCRL3$^+$ and CD8$^+$ FCRL3$^-$ memory T cells as determined by intracellular staining. The histograms show the results from different donors (*N* = 7 for granzyme and *N* = 3 for perforin). Each dot represents one donor. Mean ± SD; paired *t* test, two-tailed. From left to right: **P = 0.0018, *P = 0.0182. **(H)** Cytotoxicity of CD8$^+$ FCRL3$^+$ and FCRL3$^-$ memory T cells. CD8$^+$ FCRL3$^+$ and FCRL3$^-$ memory T cells were sorted and cocultured with the JeKo B cell line preincubated with the bispecific antibody blinatumomab. JeKo cell viability was then monitored with Live/Dead staining at the indicated time points. The FACS plots on the left are representative of the 8-h time point. The graph on the right shows the percentage of live JeKo cells normalized at time 0 h. *N* = 4–9 biological replicates (independent donors), mean ± SD, paired *t* test. From left to right: *P = 0.0260, *P = 0.0185. **(I)** Degranulation assay in sorted FCRL3$^+$ and FCRL3$^-$ memory CD8$^+$ T cells. T cells were stimulated for 5 h with PMA and ionomycin, and surface CD107A (LAMP-1) expression was measured. One representative experiment and the results from *N* = 4 independent experiments are shown. Each dot represents one donor. Paired *t* test, two-tailed. *P = 0.0408. FC, fold change. Data underlying this figure can be found in Data S4.

To further characterize the FCRL3$^+$ T cell subset, we carried out shotgun proteomic analysis on FCRL3$^+$ and FCRL3$^-$ CD8$^+$ cells obtained from *N* = 7 healthy donors. CD4$^+$ T cells were not further characterized because of their lower numbers, and because their overall terminally differentiated phenotype was consistent with that of CD8$^+$ T cells. By proteomic analysis, we found that 66 proteins were significantly depleted, while 51 proteins were significantly enriched in FCRL3$^+$ cells (Fig. 3, C and D; and Data S2). Among the FCRL3$^+$ enriched proteins, the coinhibitory receptor TIGIT and the transcription factor EOMES stood out once again for their described roles in T cell regulation. Consistent with the significant upregulation of EOMES, which especially in the context of chronic stimulation directly induces IFN-γ and granzyme expression (Geginat et al., 2023; Malyshkina et al., 2023; Pearce et al., 2003), FCRL3$^+$ cells also showed enrichment of most components of the cytotoxic machinery, as well as of TOX, although below the statistical threshold (Fig. 3 D). Further analysis of TOX, EOMES, granzyme B, and perforin expression by intracellular staining of freshly isolated FCRL3$^+$ CD8$^+$ cells confirmed significantly increased expression of these proteins in this subset (Fig. 3, E–G).

High IFN-γ expression and granzyme expression are potentially associated with an increased ability of CD8$^+$ T cells to perform efficient killing, which is also a feature of T_{EMRA} cells (Sallusto et al., 2004). We therefore investigated the killing potential of FCRL3$^+$ and FCRL3$^-$ CD8$^+$ T cells against a target JeKo B cell line. To facilitate T cell activation and engagement with the target cells, we preincubated the JeKo cells with the bispecific T cell engager (BiTE) blinatumomab, which simultaneously binds CD19 and CD3. In pilot experiments, we tested a range of effector:target ratios and different concentrations of BiTE, and we monitored the viability of JeKo cells over time using Live/Dead staining. We then selected a concentration of BiTE (2–3 ng/ml) that led to only partial killing and a 1:1 effector:target ratio, followed by measurement of JeKo cell viability over time. We found that FCRL3$^+$ cells were capable of enhanced killing compared with FCRL3$^-$ T cells (Fig. 3 H), consistent with the increased expression of components of the cytolytic machinery. Similar results were obtained by staining stimulated FCRL3$^+$ and FCRL3$^-$ cells for the degranulation marker CD107a (LAMP-1), confirming significantly enhanced degranulation in FCRL3$^+$ cells (Fig. 3 I).

Overall, our data indicate that FCRL3$^+$ human T cells resemble CD8$^+$ T_{EMRA} cells and acquire increased cytotoxic capacity.

FCRL3$^+$ and FCRL3$^-$ cells share a common origin

We found that repetitive TCR stimulation was sufficient to induce FCRL3 expression by CD8$^+$ T cells, consistent with their terminally differentiated phenotype and suggesting that these T cells have undergone multiple encounters with prevalent antigens *in vivo*. To determine whether FCRL3$^+$ cells derive from FCRL3$^-$ cells and/or represent a clonally expanded population of cells that had undergone extensive stimulation *in vivo*, we performed TCR Vβ sequencing of CD8$^+$ FCRL3$^+$ and FCRL3$^-$ cells from three donors. We measured thousands of productive TCR Vβ rearrangements in both subsets, spanning from 2544 to 5181 TCR Vβ clonotypes in FCRL3$^+$ cells and 7493 to 8592 TCR Vβ clonotypes in the FCRL3$^-$ subset. Analysis of the diversity (richness) and evenness (Simpson's clonality) of TCR sequences showed no significant differences between FCRL3$^+$ cells and FCRL3$^-$ cells (Fig. 4 A). Analysis of Vβ gene family usage revealed an overall comparable distribution in the two subsets and across all three donors (Fig. 4 B), suggesting a broad repertoire without missing or overrepresented families. Furthermore, analysis of the length of CDR3β sequences showed similar distributions in both subsets (Fig. 4 C), excluding any skewing of the repertoire. To gain insights into the TCR Vβ repertoire overlap between FCRL3$^+$ and FCRL3$^-$ subsets, we performed pairwise comparisons of TCR Vβ frequency distribution. Within each donor, this analysis revealed a high level of clonal overlap between the two repertoires, with the most expanded clonotypes present in both FCRL3$^+$ and FCRL3$^-$ cells (Fig. 4 D). On average, the shared TCR Vβ clonotypes represented 10% of the total clonotypes (range 8–11%) and accounted for >70% of the sequenced templates in each subset (range 67–76%) (Fig. 4 E), indicating a substantial repertoire overlap based on reference datasets (Hu et al., 2023).

Collectively, these data indicate that FCRL3$^+$ and FCRL3$^-$ memory CD8$^+$ T cells have rich and complete TCR Vβ repertoires that overlap extensively, suggesting a common origin followed by intraclonal diversification and FCRL3 expression.

The intracellular portion of the FCRL3 receptor is sufficient to attenuate T cell activation

Data shown above indicate that FCRL3 expression was causally associated with reduced T cell activation, since its deletion led to increased AIM expression upon TCR stimulation, while its overexpression had the opposite effect (Fig. 2). Additionally, CRISPR/Cas9-mediated deletion of FCRL3 was sufficient to reproducibly reduce the expression of two key markers of these

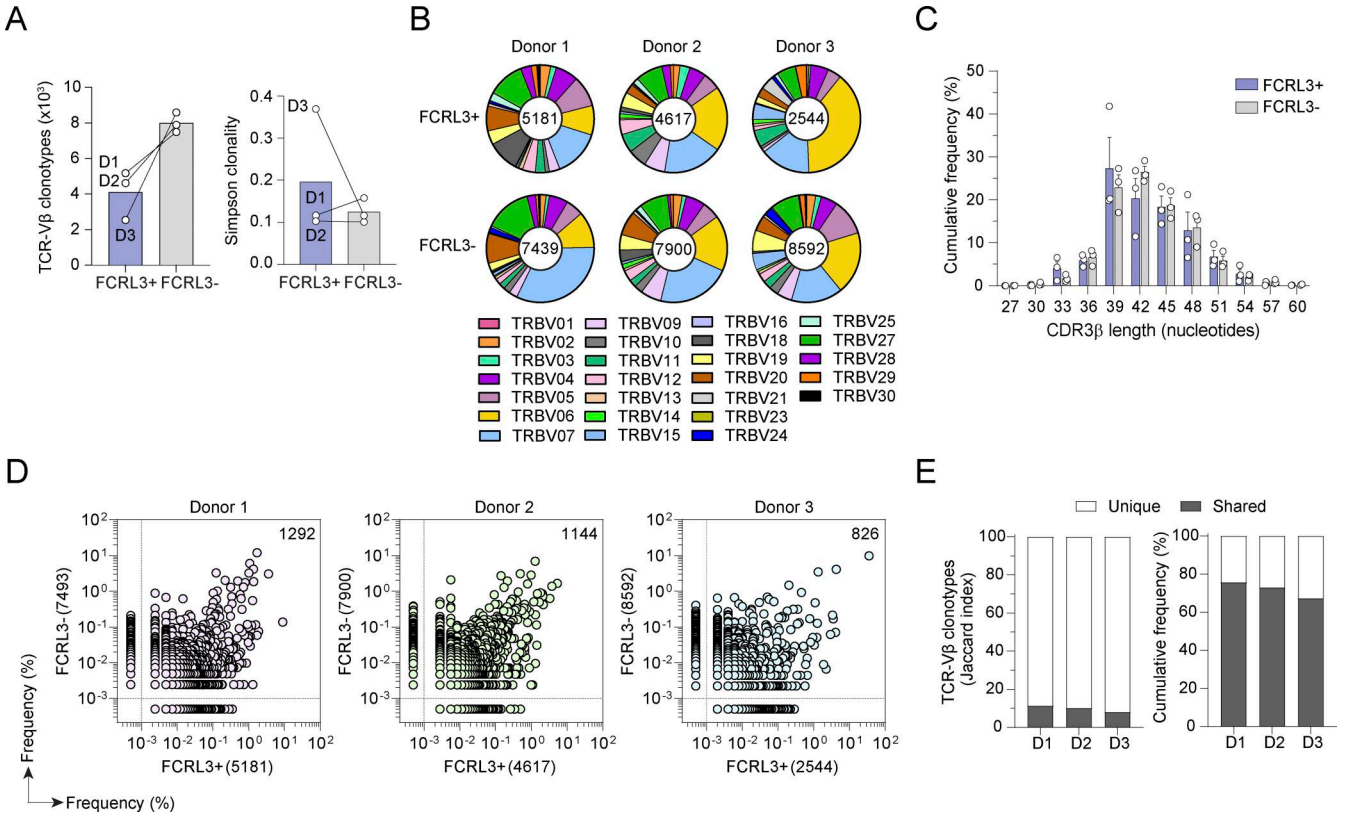

Figure 4. **Overlapping TCR repertoire in FCRL3⁺ and FCRL3⁻ cells. (A)** Number of unique productive TCR Vβ rearrangements (left) and Simpson's clonality index (right) in sorted CD8⁺ FCRL3⁺ and CD8⁺ FCRL3⁻ memory T cell subsets from three healthy donors (D1–D3) (initial input, 5 × 10^5 cells per subset). Each dot represents one donor (N = 3). Mean ± SD. **(B)** TCR Vβ gene family usage by CD8⁺ FCRL3⁺ (upper panel) and CD8⁺ FCRL3⁻ memory T cells (lower panel) in three healthy donors. Slices in the chart represent different Vβ gene families, and their size is proportional to the frequency of clonotypes using that segment. The color-coded legend is reported for the 26 different Vβ gene family. The total number of clonotypes is indicated at the center of the pie chart. **(C)** Percentage of clonotypes bearing the same CDR3β length defined by the number of nucleotides. The CDR3β length of TCR Vβ clonotypes from CD8⁺ FCRL3⁺ and CD8⁺ FCRL3⁻ memory T cells is shown in lavender and gray, respectively. Each dot represents one donor (N = 3). Mean ± SD. **(D)** Pairwise comparison of TCR Vβ clonotype frequency distribution in CD8⁺ FCRL3⁺ memory T cells (x axis) and CD8⁺ FCRL3⁻ memory T cells (y axis) from N = 3 donors. Frequencies are shown as a percentage of productive templates. Each dot indicates a unique TCR Vβ clonotype. Dots outside the dashed lines represent clonotypes that were found in only one of the two samples and that were assigned an arbitrary frequency value for graphical purposes. The total number of clonotypes is indicated in the x and y axes. Values in the upper right corner represent the number of clonotypes shared between two samples. **(E)** Unique and shared TCR Vβ clonotypes between CD8⁺ FCRL3⁺ and CD8⁺ FCRL3⁻ memory T cells in N = 3 donors. Shown are the percentage of clonotypes based on the Jaccard index (left) and their cumulative frequency (right). Data underlying this figure can be found in Data S4.

cells, namely, the transcription factors *TOX* and *EOMES* (Fig. 5 A), raising the possibility that the modulation of TCR signaling by FCRL3 contributes to the phenotype acquired by differentiated memory T cells.

To investigate the mechanism underpinning these findings, we ectopically expressed FCRL3 in Jurkat T cells that do not express FCRL3 (Fig. 5 B). The surface expression of the TCR complex (CD3ε) and CD28 was not significantly affected by ectopic FCRL3 expression. However, upon TCR stimulation using anti-CD3/CD28-coated beads for 24 h, FCRL3⁺ Jurkat T cells showed on average a ∼31.1% reduction in CD25⁺ cells compared with control cells (Fig. 5, C and D), recapitulating the phenotype observed using primary T cells and further pointing toward an intrinsic effect of FCRL3 in the modulation of T cell activation.

To determine whether the expression of the intracellular tail of FCRL3 was sufficient to modulate TCR signaling, we generated a chimeric protein containing the intracellular and transmembrane portions of FCRL3 fused to the extracellular domain of the EGF receptor (EGFR) (Desai et al., 1993). We engineered versions of this chimera containing the full-length intracellular tail (140 amino acids, 595–734), a truncation mutant lacking about half of the C-terminal distal intracellular portion (93 aa long, 595–687), and a shorter version containing only the membrane-proximal region (50 aa, 595–644) (Fig. 5, E and F). The location and number of tyrosine residues belonging to previously identified putative ITIMs are also indicated (Kochi et al., 2009). Surface expression of these chimeric proteins in Jurkat T cells was robust and comparable across samples (Fig. 5 G). Acute stimulation with anti-CD3/CD28-coated beads led to a significant reduction in CD25 expression (∼35.0% reduction) in cells expressing the C140 full-length construct, recapitulating the effect observed with the full-length FCRL3 protein (Fig. 5, H and I). This effect on CD25 expression was gradually lost with increasing truncations of the C-terminal tail, with the largest and intermediate truncations showing minor (11.2% reduction) and intermediate (18.7% reduction) effects, respectively. This indicates that the

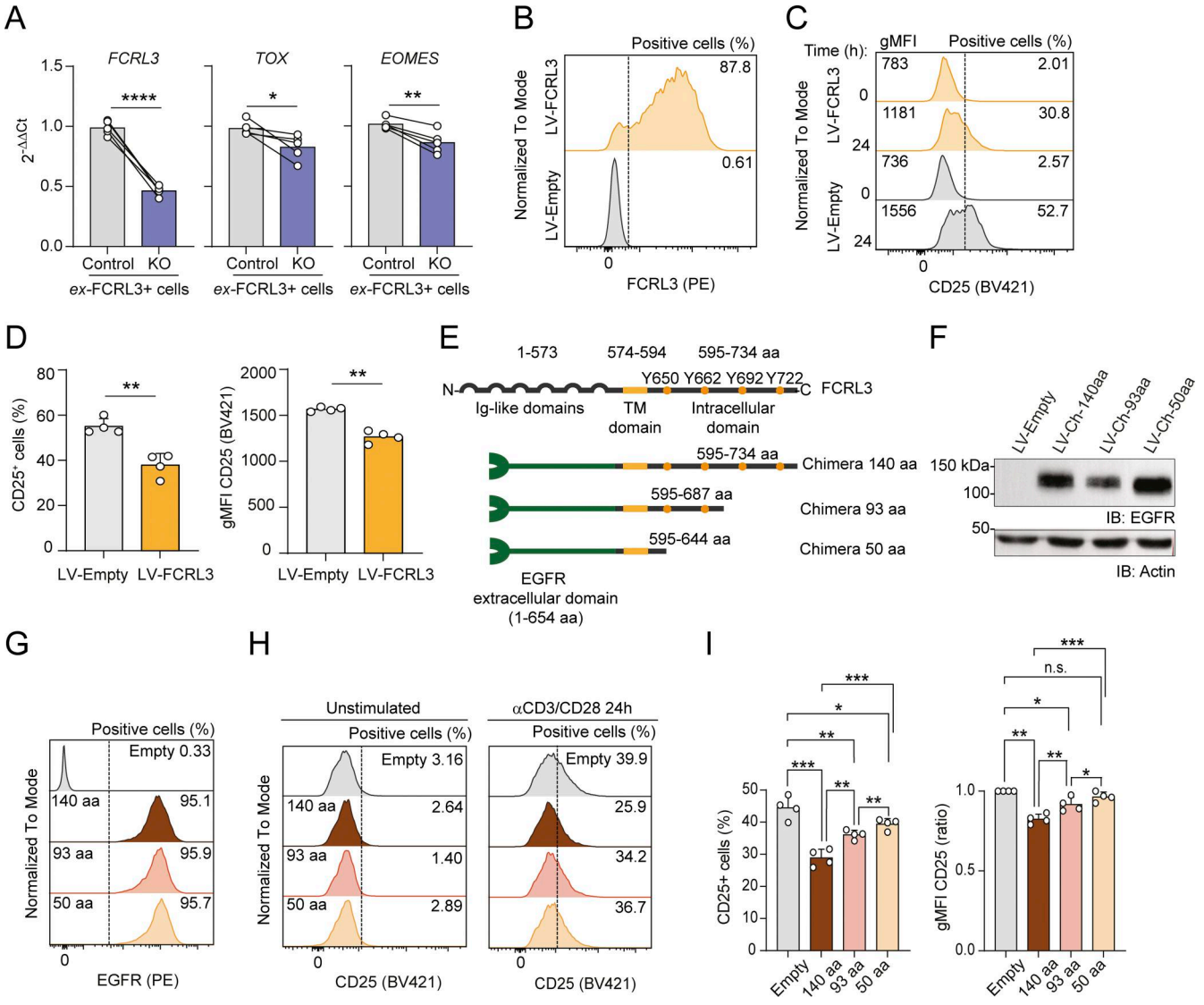

Figure 5. **FCRL3 expression is causally associated with reduced T cell activation. (A)** Expression of *FCRL3*, *TOX*, and *EOMES* transcripts was measured in FCRL3-KO and control cells by RT-qPCR. Transcript expression was normalized to the housekeeping gene *UBE2D2* and is shown relative to control samples. *N* = 5 independent experiments; paired *t* test, two-tailed. From left to right: ****P < 0.0001, *P = 0.0475, **P = 0.0040. **(B)** Surface expression of FCRL3 in Jurkat T cells transduced with FCRL3-lentivirus or empty lentivirus as a control. One representative experiment of *N* = 4 is shown. **(C and D)** Surface CD25 expression in FCRL3-transduced Jurkat cells. Cells were stimulated for 24 h with anti-CD3/CD28-coated beads. One representative experiment is shown in C, while the bar graphs in D show the compiled results of *N* = 4 independent experiments. The gMFI and percentage of $CD25^+$ cells are both shown. Mean ± SEM; paired *t* test, two-tailed. From left to right: **P = 0.0052, **P = 0.0033. **(E)** Schematic representation of the EGFR-FCRL3 chimeric proteins. **(F)** Western blot showing the expression of the different truncated chimeric proteins in Jurkat T cells, using an anti-EGFR antibody. Data are representative of *N* = 2 experiments. **(G)** Surface expression of the EGFR in Jurkat T cells transduced to ectopically express the EGFR-FCRL3 chimeras. Data are representative of *N* = 4 independent experiments. **(H)** Surface expression of CD25 in Jurkat T cells expressing the EGFR-FCRL3 chimeras. Cells were stimulated for 24 h with anti-CD3/CD28-coated beads. One representative experiment is shown. **(I)** Surface expression of CD25 in stimulated (24 h) Jurkat T cells expressing the EGFR-FCRL3 chimeras. The compiled results of *N* = 4 independent experiments as in E are shown. Mean ± SEM; paired *t* test, two-tailed. Left panel, from top to bottom: ***P = 0.0007, *P = 0.0151, **P = 0.0046, ***P = 0.0002, **P = 0.0030, **P = 0.0013. Right panel, from top to bottom: ***P = 0.0001, n.s. (not significant), P = 0.1005, *P = 0.0326, **P = 0.0014, **P = 0.0050, *P = 0.0157. Data underlying this figure can be found in Data S4. Source data are available for this figure: SourceData F5.

entire length of the intracellular FCRL3 domain is required for its maximal effect.

The FCRL3 intracellular tail interacts with negative regulators of TCR signaling

To explore the signaling molecules that may relay FCRL3 signals inside the cell, we set out to identify proteins proximal to FCRL3 in transduced Jurkat T cells. To this aim, we used a high-stringency proximity labeling approach based on the detection of FCRL3 with a specific monoclonal antibody, followed by its binding with a fusion protein consisting of protein A and TurboID, a highly efficient promiscuous biotin ligase, and the subsequent biotinylation of proteins within a 20-nm radius (Santos-Barriopedro et al., 2021) (Fig. 6, A and B). After streptavidin pull-down and mass spectrometry, we recovered FCRL3 itself and the antibody used for biotinylation, indicating

successful antibody recognition and biotinylation reaction. Among the additional proteins that were significantly enriched in FCRL3-expressing samples compared with empty vector controls, we identified the transmembrane protein FAT atypical cadherin 1 (FAT1), a previously reported interactor of FCRL3 (Oughtred et al., 2021), thus further validating our dataset (Fig. 6 C). These identified proteins were not differentially expressed between FCRL3$^+$ and FCRL3$^-$ T cells, indicating that their proximity with FCRL3 is independent of their expression level and only depends on the expression of FCRL3 itself.

Among the biotinylated proteins proximal to FCRL3, a few prominent negative regulators of TCR signaling were recovered, including the phosphatase ubiquitin-associated and Src-homology 3 (SH3) domain containing A (UBASH3A) and SHP2-interacting adaptor protein 1, a transmembrane protein adaptor capable of limiting T cell activation by recruiting the SH2 domain–containing tyrosine phosphatase SHP2 (Marie-Cardine et al., 1999; Simeoni et al., 2005) (Fig. 6 C). In particular, UBASH3A stood out because of its high enrichment, as well as its established importance as a negative regulatory of T cell activation (Carpino et al., 2004; Ge et al., 2019; Shifrut et al., 2018), further highlighted by the identification of genetic variants in the *UBASH3A* gene that are associated with autoimmune disease, including type 1 diabetes, rheumatoid arthritis, systemic lupus erythematosus, celiac disease, and others (Smyth et al., 2008; Tsygankov, 2018). While the closely related protein UBASH3B is widely expressed, UBASH3A is highly enriched in lymphoid tissues, and specifically in T lymphocytes (Carpino et al., 2004). Both UBASH3A and UBASH3B contain an ubiquitin-associated (UBA) domain, a SH3 domain, and a phosphatase domain, which is a key functional domain capable of dephosphorylating a variety of substrates, most notably ZAP70, hence dampening TCR signaling and limiting T cell activation (Mikhailik et al., 2007; San Luis et al., 2011; Tsygankov, 2018). In addition to its direct interactions with ZAP70, UBASH3A was also reported to interact with the CD3 chains of the TCR complex, as well as with CBL and CBL-B, E3 ubiquitin ligases that act as negative regulators of T cell activation (Ge et al., 2019; Liu and Gu, 2002). The SH3 domain of UBASH3A is primarily involved in the recognition of proline-rich sequences, and this was reported to be the mode of UBASH3A recognition at least for CBL (Feshchenko et al., 2004) and CD3ε (Ge et al., 2019), while the UBA domain may interact with other target proteins depending on their ubiquitination status. Concordant with a role in suppressing T cell functions, *Ubash3a* deletion in mice led to exacerbated experimental autoimmune encephalomyelitis (Carpino et al., 2004), and deletion of the *UBASH3A* gene in primary human CD8$^+$ T lymphocytes led to increased cell proliferation (Shifrut et al., 2018).

To further validate the interaction between UBASH3A and FCRL3, we used Jurkat cells ectopically expressing FCRL3 (or empty vector control cells) to immunoprecipitate FCRL3, followed by mass spectrometry (IP-MS). IP-MS of FCRL3 was efficient (Fig. 6 D), and recovered significantly enriched levels of FAT1 and UBASH3A, confirming the interaction (direct or indirect) of these proteins with FCRL3 (Fig. 6 E). Importantly, UBASH3A was retrieved using two different experimental systems based on two different antibodies targeting FCRL3 (a mouse monoclonal for proximity labeling and MS, and a rabbit polyclonal antibody for IP-MS), indicating high-confidence and robust FCRL3-UBASH3A proximity. Additional interactors involved in TCR signaling included the TCRβ chain and CD3ε. Co-IP of FCRL3 and UBASH3A was also independently confirmed in HEK cells transiently transfected with these two proteins (Fig. 6 F). A version of FCRL3 tagged with a 3xFLAG sequence at the intracellular C terminus was also generated and used for IP-MS using anti-FLAG–conjugated beads, in comparison with untagged FCRL3 (Fig. S5, A–C). Mass spectrometry of the immunoprecipitated proteins provided additional independent confirmation of the interaction of FCRL3 with UBASH3A, CD3ε, and FAT1, among other proteins (Fig. S5 D). Given the established importance of UBASH3A as a negative regulator of TCR signaling, we also set out to determine its interactome. Jurkat T cells ectopically expressing either FCRL3 or the full-length EGFR-FCRL3 C140 aa chimera (or LV-empty controls) were used for proximity biotinylation of UBASH3A, followed by mass spectrometry (schematic in Fig. S5 E). Many proteins were recovered after streptavidin pull-down and tandem MS/MS, consistent with high UBASH3A abundance in T cells and promiscuous interactions. Several interactors of UBASH3A reported in the BioGRID database were recovered, including UBASH3B, as well as components of the TCR complex (CD3ε) and regulators of TCR signaling (ZAP70, CBL) (Data S3). Importantly, FCRL3 peptides were recovered in the FCRL3- and chimera-expressing samples, while peptides corresponding to EGFR were recovered from the chimera-expressing samples, further validating the proximity of UBASH3A with the intracellular portion of FCRL3 in T cells (Fig. S5 F).

To identify the region required for the putative interaction between FCRL3 and UBASH3A, we generated truncated versions of FCRL3, containing progressively shorter intracellular regions. All proteins were efficiently expressed in Jurkat T cells (Fig. 6 G). IP-MS of full-length FCRL3 and its truncations confirmed once again the interaction between FCRL3 and UBASH3A and, additionally, revealed progressive loss of such interaction with the decreasing length of the intracellular tail, particularly in the region between aa 50 and 93 (Fig. 6 H).

Overall, these data indicate that FCRL3 expression in T cells is causally associated with reduced activation capabilities by these cells, mediated at least in part by the interaction of the FCRL3 intracellular portion with negative regulators of TCR signaling.

Discussion

In this work, we characterized human FCRL3$^+$ T lymphocytes as a subset of CD4$^+$ and CD8$^+$ human memory T cells characterized by a highly differentiated effector phenotype with features of cytotoxic activity. FCRL3 expression itself was induced by repetitive TCR stimulation, and its intracellular domain was sufficient to limit T cell activation. Our data indicate an immunoregulatory function for this receptor in T lymphocytes, which we suggest being mediated at least in part by its interplay with UBASH3A, a validated negative regulator of TCR signaling involved in the dephosphorylation of ZAP70 (Mikhailik et al., 2007; San Luis et al., 2011; Tsygankov, 2018). The unifying

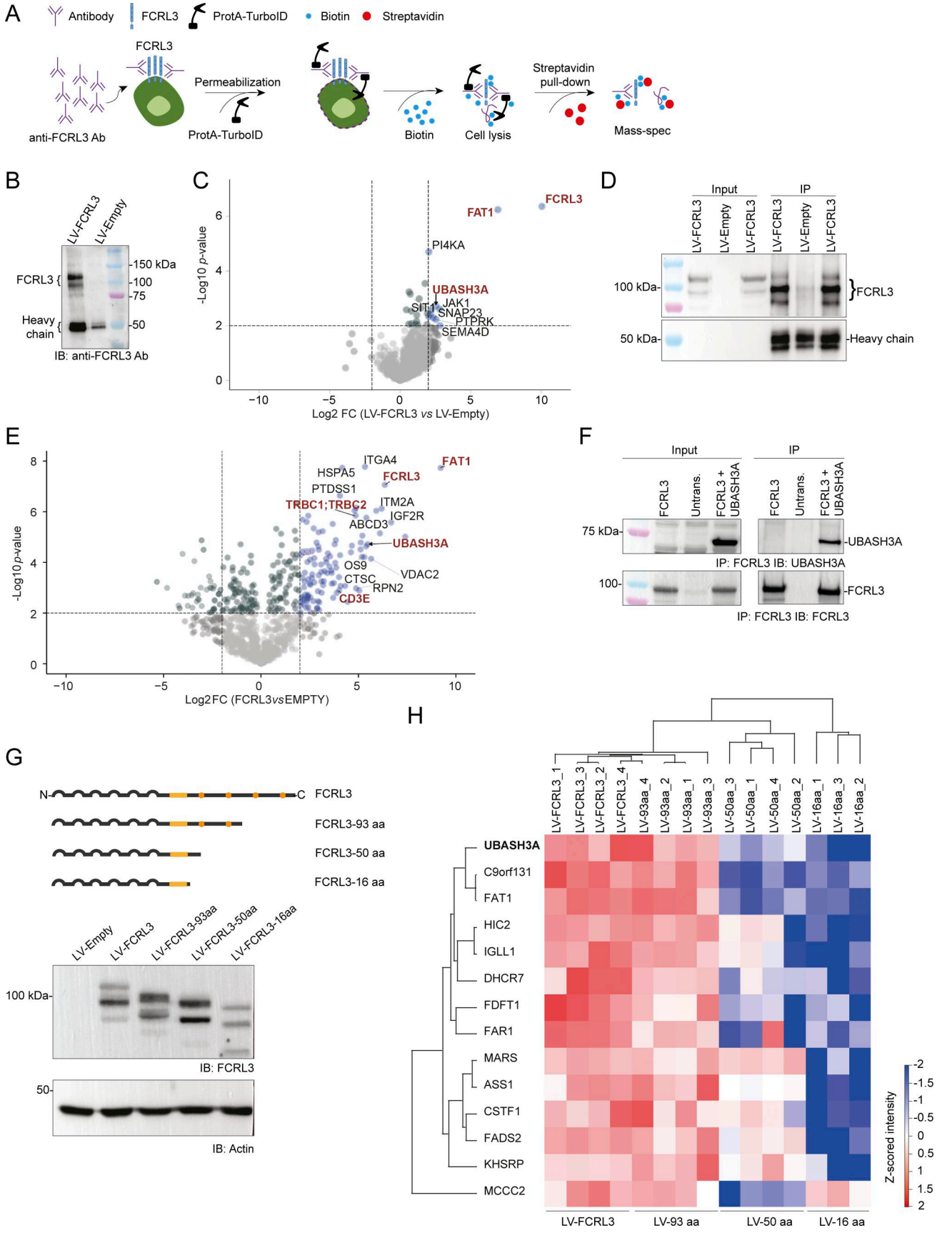

Figure 6. **Intracellular portion of FCRL3 interacts with UBASH3A. (A)** Schematic representation of the Protein A-TurboID experimental workflow. An anti-FCRL3 antibody was added to FCRL3-expressing or nonexpressing Jurkat cells followed by the addition of Protein A-TurboID fusion protein and biotin. After

biotinylation and extensive washing, biotinylated proteins were recovered using streptavidin-conjugated beads and subjected to mass spectrometry analysis. **(B)** Example of Protein A-TurboID experiment. After incubation of FCRL3-expressing Jurkat cells (or empty vector control cells) with a mouse monoclonal anti-FCRL3 antibody together with recombinant Protein A-TurboID, biotinylated proteins were recovered by streptavidin pull-down, followed by western blot to confirm enrichment of FCRL3. The antibody used for immunoblot was a rabbit polyclonal anti-FCRL3. **(C)** Differentially retrieved proteins following FCRL3 Protein A-TurboID and mass spectrometry of FCRL3-expressing versus nonexpressing cells ($log_2FC \geq |2|$; $P \leq 0.01$; $N = 5$ replicates). **(D)** Example of FCRL3 IP. Jurkat cells expressing FCRL3 (or empty vector control) were lysed, and 1.5 mg of protein extract was used for IP with a rabbit polyclonal anti-FCRL3 antibody, followed by western blot with the same antibody. Two independent representative FCRL3 IPs are shown. **(E)** Differentially retrieved proteins following FCRL3 IP-MS of FCRL3-expressing versus nonexpressing cells ($log_2FC \geq |2|$; $P \leq 0.01$; $N = 4$ replicates). **(F)** Co-IP of FCRL3 with UBASH3A. HEK cells were transfected with the indicated plasmids, followed by IP of FCRL3 using a rabbit polyclonal anti-FCRL3 antibody and immunoblot for either UBASH3A (top) or FCRL3 itself (bottom). Data are representative of $N = 2$ independent experiments. **(G)** Schematic representation of the FCRL3 protein and C-terminal truncations (top). All proteins were efficiently expressed, as shown by western blot (bottom). **(H)** Heatmap showing the differentially enriched proteins by IP-MS of Jurkat cells transduced with full-length FCRL3 and its truncations. $N = 3–4$ independent samples. Significant differences between multiple experimental conditions were assessed with an ANOVA multiple-sample test (0.01 permutation-based FDR cut-off, 250 randomizations). Significant proteins were filtered, and Z-score normalization was applied to each protein across all samples. Unsupervised hierarchical clustering was employed to visualize on a heatmap proteins with a positive Z-score value for all replicates of the conditions LV-FCRL3 and LV-FCRL3-93 aa. IP, immunoprecipitation. Data underlying this figure can be found in Data S4. Source data are available for this figure: SourceData F6.

features of FCRL3$^+$ cells across conventional memory CD4$^+$ and CD8$^+$ cells include a cytotoxic profile associated with high IFN-γ expression and reduced activation and proliferation capacity, resembling highly differentiated effector memory cells deriving from repetitive antigen stimulation. Consistent with our observations, an expanded population of human CD4$^+$ CTLs characterized by perforin and granzyme expression has been identified especially in the context of chronic viral infections (Preglej and Ellmeier, 2022; Takeuchi and Saito, 2017). These cells are antigen-experienced, display a terminally differentiated functional profile (Appay et al., 2002), and were recently shown to target cytomegalovirus antigens in fibroblasts, contributing to the elimination of senescent cells in the aging human skin (Hasegawa et al., 2023). In fact, elevated levels of circulating CD4$^+$ CTL cells are a feature of ultra-centenarian people (Hashimoto et al., 2019). Similarly, FCRL3$^+$ cells are likely to derive from repetitive encounters with common viral/microbial antigens. The fact that FCRL3$^+$ cells may derive from memory T lymphocytes undergoing multiple rounds of stimulation *in vivo* is also suggested by the increased expression of TOX, which was shown to be induced in T cells retaining effector functions even after many rounds of repetitive *in vivo* stimulation (Soerens et al., 2023). In these conditions, TOX is likely to promote cellular longevity, rather than exhaustion (Baessler and Vignali, 2024). Despite the expression of some inhibitory receptors, most notably TIGIT, FCRL3$^+$ cells do not present prominent features of exhaustion, since effector functions such as IFN-γ production and killing capacity were fully maintained or even enhanced. Furthermore, the expression of other inhibitor receptors was not significantly increased. Instead, their phenotype is consistent with that of highly differentiated effector cells deriving from repetitive TCR stimulation. Consistent with this observation, we found that surface expression of FCRL3 could be induced in human T cells *in vitro* upon repetitive exposure to anti-CD3 antibody. Our data are also in line with single-cell RNA-seq data from tumor-infiltrating human CD4$^+$ and CD8$^+$ T lymphocytes (Andreatta et al., 2022) and with single-cell profiling of human memory CD8$^+$ T cell subsets (Galletti et al., 2020). In both instances, FCRL3 expression was shown to be primarily confined to Tregs and cytotoxic CTLs in the CD4$^+$ compartment, while within the CD8$^+$ compartment, FCRL3 expression was more widespread to T_{EM} and T_{EMRA} lymphocytes. These observations suggest that FCRL3 expression may also in part contribute to dysfunction or exhaustion. Notably, deletion of FCRL3 reduces *TOX* expression in CD8$^+$ T cell, suggesting a direct role in modulating the expression of this key transcription factor required for the exhaustion program and also implying that FCRL3 expression may negatively regulate T cell activation in tumors. However, at this stage the relative stability of FCRL3$^+$ expression *in vivo* remains to be fully understood, and the possibility remains that acquisition of FCRL3 expression represents a relatively transient state for memory T cells, which can be eventually reverted upon complete removal of the stimulus.

Identifying the mechanism underlying the effect of an immunomodulatory receptor in immune cells is no trivial matter. For example, the phosphorylation of tyrosines in the ITIMs of PD-1 is followed by the recruitment of phosphatases that limit T cell proximal signaling and costimulation (Hui et al., 2017; Kamphorst et al., 2017), while the local acidification mediated by glutamic acid and proline-rich regions of LAG3 induces the dissociation of LCK from the CD4 and CD8 coreceptor molecules (Guy et al., 2022). The FCRL3 intracellular tail contains 11 prolines within the first 70 amino acids of the membrane-proximal region, but whether a similar mechanism could also be at play for FCRL3, and at least in part contribute to its effect on T cell activation, remains unknown and difficult to dissect, especially considering the unstructured nature of this part of the protein. Even well-studied receptors like PD-1 still hold surprises, since for instance it was recently shown to modulate cytoskeletal reorganization in T cells independently of its ITIMs (Paillon et al., 2023), while LAG3 was shown to require ligand-induced ubiquitination to release its cytoplasmic tail from the membrane, enabling signaling (Jiang et al., 2025). As for FCRL3, proximity labeling experiments identified several negative regulators of TCR signaling, including UBASH3A. However, these proteins are not differentially expressed by FCRL3$^+$ versus FCRL3$^-$ T cells, suggesting that FCRL3 expression may contribute to increasing the local concentration of negative regulators of TCR signaling. Indeed, mouse Ubash3a was shown to be part of a TCR-inducible CD6 signalosome important in the regulation of T cell activation (Mori et al., 2021).

Since the expression of the FCRL3 intracellular tail was sufficient to diminish T cell activation, it is likely that the

expression of this receptor is induced concomitantly to the acquisition of functional properties by T lymphocytes that are potentially highly damaging for healthy tissues, thereby balancing immune responses. In fact, polymorphisms in the *FCRL3* gene are associated with many autoimmune diseases. As confirmed also by our data, the presence of the minor "C" allele associates with higher percentages of FCRL3 expression by T cells. However, this was true for all T cell subsets, including Tregs, in which FCRL3 was shown to affect suppressive capacity (Agarwal et al., 2020). Therefore, although induction of FCRL3 expression (and the consequent effect on T cell signaling) appears to be a normal, common process in humans, it is unclear whether the association of this gene with autoimmunity may be linked to a dysfunction of Tregs or other $CD4^+$ and $CD8^+$ T cell subsets, or even to other cell types on which this receptor is highly expressed, like B lymphocytes and NK cells. One additional remaining question is also linked to the ligand recognized by this receptor *in vivo*. Although FCRL3 was shown to bind sIgAs *in vitro* (Agarwal et al., 2020), at this stage it is difficult to envision a situation that might require recognition of sIgAs by a large proportion of circulating memory $CD8^+$ T lymphocytes. Having firmly established the groundwork on the role of FCRL3 in human T cell subsets, the focus of future investigations will center around the possibility of identifying its physiological ligand(s) and the possibility of targeting it for therapeutic interventions.

Limitations of this study

Although our study establishes the impact of FCRL3 expression in T cells, it remains difficult to dissect the overall impact of FCRL3 expression *in vivo* in humans, also because this receptor is expressed by additional immune cells such as NK and B cells. For instance, while FCRL3 expression is associated with a protective role in multiple sclerosis (Yu et al., 2024), the cell type predominantly responsible for this protective effect may range from Tregs, to B cells and other immune cell subsets. An additional limitation relates to the identification of the precise mechanistic details of the interaction between FCRL3 and UBASH3A. The possibility remains that some of the more labile, dynamic, or condition-specific interactions may have not been caught in our experimental systems. In particular, whether part of the negative effect of FCRL3 on signaling is also in part mediated by tyrosine phosphorylation upon T cell activation remains to be fully explored. Finally, whether FCRL3 expression represents a relatively transient state of memory T cells, or it can be "fixed" in the memory population will be the subject of future studies.

Materials and methods

Ethics statement

Peripheral blood from healthy donors was obtained from the Swiss Blood Donation Centers of Lugano and Basel (Switzerland), with informed consent (authorization number CE 3428 from the Comitato Etico Canton Ticino).

Primary human T cell separation

Peripheral blood mononuclear cells (PBMCs) were separated by gradient centrifugation (Ficoll-Paque Plus; GE Healthcare), followed by positive selection of $CD4^+$ or $CD8^+$ T lymphocytes using magnetic beads (Miltenyi Biotec). Naïve and memory $CD4^+$ T cell subsets were further separated using a FACSAria or a SORP FACSymphony S6 (BD Biosciences) as follows: naïve: $CD4^+CD25^-CD45RA^+CCR7^+$; total memory: $CD4^+CD25^-CD45RA^-CD127^{+/-}$; Tregs naïve: $CD4^+CD25^{hi}CD127^-CD45RA^+CCR7^+$; Tregs memory: $CD4^+CD25^{hi}CD127^-CD45RA^-CCR7^{+/-}$. $CD8^+$ T lymphocytes were sorted as follows: naïve: $CD8^+$ and CD45RA/CCR7 double-positive; memory: $CD8^+CD45RA^{+/-}CCR7^{+/-}$. When cells were separated in the subsets $FCRL3^+$ and $FCRL3^-$, an anti-FCRL3 antibody (clone H5; BioLegend) was also added to the sorting. All antibodies used in this study are described in Table S1.

T cell stimulation and culture

Acute activation of sorted cells was performed as follows: cells were stimulated for 2 days with plate-bound recombinant anti-CD3 (0.7 µg/ml; clone TR66, in-house production) (Lanzavecchia and Scheidegger, 1987) and anti-CD28 (1 µg/ml) antibodies, followed by expansion in complete medium (RPMI-1640 supplemented with 5% human serum, 1% nonessential amino acids, 1%, sodium pyruvate, 1% glutamine, penicillin, streptomycin, and 50 µM β-mercaptoethanol). In repetitive stimulation experiments, $CD8^+$ memory T lymphocytes were activated with anti-CD3 antibody alone and restimulated after 3 days with anti-CD3 antibody. For experiments no longer than 5 days, cells were kept in culture without IL-2. Otherwise, recombinant human IL-2 was added at 50–100 U/ml after 3 or 5 days from activation. FCRL3 surface staining was performed after 5 or 10 days with conjugated antibody (FCRL3-PE). All antibodies and reagents used in this study are described in Table S1.

Intracellular and surface staining

For intracellular cytokine staining, $CD4^+$ T cells were stimulated for 5 h with PMA (200 nM) and ionomycin (1 µg/ml). For $CD8^+$ T cells, 100 nM PMA and 0.5 µg/ml ionomycin were used. For the last 2.5 h of stimulation, brefeldin A (10 µg/ml) was added to the cells. When specified, instead of PMA and ionomycin, cells were stimulated for 6 h with plate-bound anti-CD3 and anti-CD28 antibodies, and brefeldin A (10 µg/ml) was added to the cells for the last 2 h of stimulation. After fixation (paraformaldehyde, 4%) and permeabilization (0.5% BSA and saponin in Dulbecco's phosphate-buffered saline [DPBS]), the staining was performed with the conjugated antibodies listed in Table S1. Intracellular staining for granzyme B, perforin, TOX, and EOMES was performed on unstimulated cells, fixed, and permeabilized using eBioscience Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) according to the manufacturer's instructions. Staining of surface molecules was performed in MACS buffer with the conjugated antibodies listed in Table S1. Live/Dead staining was performed before fixation, after washing cells with DPBS; either Aqua Dead or Blue Dead dyes (Invitrogen) were used. To stain surface receptors (FCRL3, CD28, CD3ε), 2×10^5 cells were washed once with 1X DPBS, and incubated with a primary anti-CD28 antibody (1:50) for 15 min at 4°C, followed by staining with an AF750-conjugated secondary antibody (1:300) for 30 min at 4°C. After washing, cells were stained with anti-FCRL3-PE (1:50) and anti-CD3ε-FITC (1:20)

antibodies for 30 min. For FLAG detection, cells were fixed and permeabilized using IntraSure Kit (BD Biosciences), then stained intracellularly with a Brilliant Violet 421 anti-DYKDDDDK Tag antibody (1:50) for 30 min. All samples were acquired on Fortessa Flow Cytometer, FACSymphony A3 or A5 (BD Biosciences), and data were analyzed with FlowJo software.

Cell proliferation and viability

Cell proliferation was measured using APC-BrdU Flow Kit from BD Biosciences, following the manufacturer's instructions. After 2 days of stimulation, cells incorporated BrdU for 1 h at 37°C. Cell viability was measured using a Live/Dead staining (Invitrogen) following the manufacturer's instructions.

Plasmids and cloning

Plasmids were generated and modified using standard cloning techniques. To generate the pLVX-EF1α-IRES-PURO, the ZsGreen1 sequence of pLVX-EF1α-IRES-ZsGreen1 (Clontech) was substituted with a puromycin resistance gene (PuroR) from Addgene plasmid n.99636 (Montagner et al., 2016). First, AsiSI and BlpI restriction sites were added to flank the ZsGreen1 sequence of the pLVX-EF1α-IRES-ZsGreen1 by site-directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis Kit, Agilent Technologies), and then used to excise the ZsGreen1 sequence. The PuroR sequence was amplified by PCR with primers containing same restriction sites and subcloned into the pLVX backbone. The *FCRL3* gene was amplified from cDNA from the Daudi cell line (CCL-213; ATCC) and then cloned into pLVX-IRES-PURO using the SpeI and NotI restriction sites. The pLVX-EF1α-EGFR-FCRL3-140 aa-chimera-puro plasmid was generated using overlap extension PCR (Higuchi et al., 1988). First, the sequence corresponding to the extracellular region of EGFR was amplified from Addgene plasmid # 11011 (Greulich et al., 2005). Then, the sequence corresponding to the transmembrane–intracellular domain of FCRL3 was amplified from pLVX-EF1α-FCRL3-IRES-puro with a forward primer containing an additional sequence complementary to the 3′ end of the newly generated extracellular EGFR amplicon. The two generated PCR products were then combined into an overlap extension PCR. In this PCR, the first 8 cycles were run without primers, allowing the added overlapping sequence of the transmembrane–intracellular domain of FCRL3 amplicon to bind pairwise to the EGFR extracellular region and start the extension, generating the full chimera. After 8 cycles, two outer primers containing the restriction sites for XbaI and NotI were added to amplify the full-length EGFR-FCRL3-140aa chimera, and the PCR was restarted for additional 27 cycles, adjusting the extension time. Finally, this amplicon was used for cloning into pLVX-EF1α-IRES-PURO using the XbaI and NotI restriction sites. For the 93 and 50 aa truncated versions of the EGFR-FCRL3 chimera, the sequences were amplified from pLVX-EF1α-EGFR-FCRL3-140 aa-chimera-puro plasmid using reverse primers containing NotI restriction sites together with the XbaI-containing forward primer used for 140 aa chimera generation. Cloning was then performed using XbaI and NotI restriction enzymes. All PCRs were performed using Platinum SuperFi II DNA Polymerase (Thermo Fisher Scientific). WT-long-UBASH3A-V5-6xHis was a gift from Patrick Concannon, Unversity of Florida, Gainesville, FL, USA (plasmid # 192101; Addgene). To generate the versions of FCRL3 truncated in the intracellular domain, the plasmid pLVX-EF1α-FCRL3-IRES-puro served as the template for site-directed mutagenesis. Mutagenesis was performed with QuikChange II XL Site-Directed Mutagenesis Kit (Agilent) following a PCR-based approach according to the manufacturer's guidelines. After PCR, parental methylated and hemi-methylated DNA was digested with DpnI endonuclease for 1 h at 37°C. The resulting PCR-amplified plasmid containing the mutations of interest was transformed into Stbl3-competent bacteria. Plasmid DNA was purified using E.Z.N.A. Plasmid DNA Mini Kit I (Omega Bio-Tek). To generate the FLAG-tagged version of FCRL3, a 3×FLAG epitope tag was fused in-frame to the C terminus of the FCRL3 coding sequence in the pLVX-FCRL3-puro plasmid using In-Fusion Snap Assembly Master Mix (Takara Bio) following the manufacturer's instructions. Primer sequences used for cloning are listed in Table S1. All plasmids were first screened by restriction enzyme digestion and then verified by Sanger or next-generation sequencing (Microsynth).

Cell transfection and T cell transduction

HEK293T cells were seeded at 8×10^6 per T75 flask and cultured in DMEM supplemented with 10% FBS, penicillin, streptomycin, and 1% sodium pyruvate. After 20–24 h, HEK293T cells were transfected using polyethylenimine or Lipofectamine 3000 (Invitrogen) with the packaging vectors pMD2.G and psPAX (# 12259 and 12260; Addgene), together with a lentiviral vector encoding the gene of interest. Medium was changed 8 h after transfection. After 24–48 h, the supernatant containing lentiviral particles was filtered and PEG-8000 and NaCl were added to a final concentration of 10% and 0.3 M. After mixing at 4°C for 12–18 h, the suspension was centrifuged (1,600 × *g*, 1 h), and the pelleted lentiviral particles were resuspended in PBS. To transduce primary T cells, 1.5×10^5 cells were seeded in flat-bottom 96-well plates coated with anti-CD3 anti-CD28 antibodies, and 5–10 µl of lentivirus was added to each well. 48 h after activation and transduction, cells were removed from stimuli. For vectors containing the PuroR, puromycin (2 µg/ml) was added for 48 h, followed by removal and replacement with fresh medium containing recombinant human IL-2 (50 U/ml). All experiments were performed at least 48 h after the removal of puromycin. For Jurkat cells, 5×10^5 cells were plated in a 48-well plate and 5 µl of lentivirus was added to each well. Transduced cells were selected with puromycin (2 µg/ml) for 48–72 h.

Genotyping

Genomic DNA (gDNA) was isolated from human PBMCs using the DNeasy Blood & Tissue Kit (Qiagen). PCR primers were designed to amplify the promoter region (–169 from the transcription start site) containing a T/C SNP. Primer sequences are listed in Table S1. PCR amplification was performed using KOD Hot Start DNA Polymerase (Sigma-Aldrich); 20 ng of gDNA was used as a template, and two independent PCRs were performed to amplify either the T or the minor C allele. The annealing temperature was set at 60.5°C (T allele) or 61°C (C allele).

Reverse transcription–quantitative PCR

T cells were lysed in TRI Reagent (Molecular Research Center), and total RNA was extracted using a Direct-zol RNA MicroPrep kit (Zymo Research). cDNA was synthesized using qScript cDNA SuperMix (Quanta Bioscience). SYBR Green FastMix (Quanta Bioscience) was used to amplify target genes in QuantStudio 3 Real-Time PCR System (Thermo Fisher Scientific). For data analysis, the gene expression level between different samples was calculated using the $2^{-\Delta\Delta CT}$ method; normalizing gene expression to the housekeeping gene *UBE2D2*. All primers used for RT-qPCR experiments are listed in Table S1.

CRISPR/Cas9 gene editing

CRISPR/Cas9-mediated editing of the *FCRL3* gene was performed as described with some modifications (Emming et al., 2020; Leoni et al., 2021). Briefly, freshly isolated, resting CD8$^+$ T cells were transfected with Cas9-gRNA RNPs using the Amaxa 4D-Nucleofector (Lonza). For RNP preparation, equal amounts (400 pmol) of crRNA and tracrRNA were mixed with nuclease-free duplex buffer and annealed by boiling at 95°C for 5 min followed by cooling down to room temperature. Two crRNAs targeting the *FCRL3* gene were selected, along with a nontargeting crRNA (scrambled) as a control, all obtained from Integrated DNA Technologies (IDT; sequences are listed in Table S1). Guides targeting the coding region of the *FCRL3* gene were selected based on the ON- and OFF-target scores predicted *in silico* using both the IDT-design tool and CHOP-CHOP (Labun et al., 2019). To further limit the risk of off-target effects, both *FCRL3*-targeting sgRNAs were selected to contain a minimum of four mismatches with any possible off-target genomic coding regions. The RNPs were prepared by mixing 1.5 µl of TrueCut Cas9 Protein v2 (5 µg/µl; Thermo Fisher Scientific) with 1.5 µl of annealed gRNAs. To increase transfection efficiency, polyglutamic acid (100 mg/ml; Sigma-Aldrich) was added in a ratio of gRNA:PGA 1:0.8. The mix was incubated for 20 min at room temperature. 1×10^6 cells were resuspended in Lonza P3 buffer, with its supplement added at a ratio of 4.5:1, in a final volume of 22 µl. The Cas9/gRNA/PGA mixture, along with Alt-R Electroporation Enhancer (10.8 µM; IDT), was added to the transfection mix. The cell/RNP suspension was transferred to nucleofection wells, and 1×10^6 T cells were electroporated using the program EH-115. Transfected cells were kept in antibiotic-free medium for 48 h; recombinant human IL-7 and IL-15 were added at 2 ng/ml for 10 min after electroporation and supplemented to the culture medium every three days to maintain cell viability. After 12 days, cells were activated with plate-bound anti-CD3 antibody (0.7 µg/ml; clone TR66, in-house production).

RNA sequencing

Memory CD8$^+$, CD4$^+$ helper, and CD4$^+$ Treg populations of primary human T cells obtained from $N = 5$ independent donors were sorted and separated for FCRL3 expression as described above. Following overnight incubation at 37°C in complete medium, 1–2 $\times 10^6$ FCRL3$^+$ and FCRL3$^-$ cells were stimulated for 3 h with PMA (200 nM) and ionomycin (1 µg/ml). For CD8$^+$ T cells, 100 nM PMA and 0.5 µg/ml ionomycin were used. After lysis in TRI reagent (MRC), RNA extraction was performed using the Direct-Zol RNA Miniprep kit (Zymo Research). RNA-seq was carried out using the SMART-seq2 protocol (Picelli et al., 2014b) with minor modifications. Briefly, 5 ng of total RNA (RIN > 8) was reverse-transcribed with template switching using oligo(dT) primers and an LNA-containing template-switching oligo. The resulting cDNA was preamplified, purified, and tagmented with Tn5 transposase produced in-house using a described protocol (Picelli et al., 2014a). cDNA fragments generated after tagmentation were gap-repaired, enriched by PCR, and purified to create the final cDNA library. All the samples have been paired-end-sequenced on an Illumina NOVASeq 6000 platform. RNA-seq data are available at Gene Expression Omnibus (GEO) with the accession number GSE241004.

RNA-sequencing analysis

Read quality control was performed using FastQC v0.11.9 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Adaptor sequences were removed using Trimmomatic v0.39 (Bolger et al., 2014). Reads were subsequently mapped to the human genome (GENCODE, version GRCh38) using HISAT2 v2.1.0 (Kim et al., 2019). HTSeq-count v2.0.2 (Anders et al., 2015) was then used to generate the table of counts containing the number of reads mapping to each feature in each sequencing sample. Differential expression analysis was performed using DESeq2 with a threshold of counts >10. For track visualization in Integrative Genomics Viewer, BigWig files were generated using the *BamCoverage* function and normalized to bins per million using the Galaxy Community (2024).

TCR sequencing

gDNA was extracted from sorted memory CD8$^+$ FCRL3$^+$ and FCRL3$^-$ cells from $N = 3$ independent donors using QIAamp DNA Micro Kit (Qiagen), according to the manufacturer's instructions. gDNA quantity and purity were assessed spectrophotometrically. Sequencing of TCR Vβ CDR3 was performed by Adaptive Biotechnologies using the ImmunoSEQ assay. The assay was performed at the survey level (detection sensitivity, 1 cell in 40,000). Each clonotype was defined as a unique productively rearranged TCR Vβ DNA nucleotide sequence; data processing was done using the productive frequency of templates provided by ImmunoSEQ Analyzer V.3.0. The Simpson clonality index was used to determine the evenness of the TCR repertoire. The unique productive rearrangements were used to determine the richness of the TCR repertoire. The percentage of shared clonotypes was calculated using the Jaccard index [$J = (A \cap B)/(A \cup B)$] as the number of shared clonotypes between two subsets divided by the total number of clonotypes present in the same subset and normalized to 100.

Cell killing and degranulation assays

After sorting CD8$^+$ memory T cells based on the expression of FCRL3, cells were cocultured with JeKo cells at different ratios. Specifically, 5×10^4 CD8$^+$ T cells were incubated with 5×10^4 JeKo cells (1:1 ratio). For the 1:5 ratio, 5×10^4 JeKo cells were incubated with 1×10^4 T cells, and for the ratio 1:10, 5×10^4 JeKo cells were incubated with 5×10^3 T cells. Prior to the coculture, JeKo cells were preincubated with the bispecific antibody against the

human CD19 and CD3 antigens (InvivoGen) at a concentration of 2–3 ng/ml for 30 min at 37°C. JeKo viability was monitored with Blue Dead staining (Invitrogen) at different time points. To discriminate T cells from JeKo cells, surface staining with conjugated antibodies CD8-FITC was added. As a control for JeKo viability, JeKo cells were cultured without T cells and the experiment was performed in JeKo cell medium for optimal viability (complete RPMI with 20% FBS). All samples were acquired on a FACSymphony A5 (BD Biosciences) cytometer, and data were analyzed with FlowJo software. For CD107A (LAMP-1) degranulation assay, FCRL3$^+$ and FCRL3$^-$ CD8$^+$ T cells were sorted and rested one night in the incubator. Cells were then activated with PMA (200 nM) and ionomycin (1 µg/ml) for 5 h. Anti-CD107A-BV421 antibody (1:400) and the protein transport inhibitor monensin (1:1,000; BD Biosciences) were also added at the same time as the stimuli. Live/Dead staining was also performed before FACS acquisition. All reagents are described in Table S1.

Shotgun comparative proteomics

Ex vivo–sorted memory CD8$^+$ FCRL3$^+$ and FCRL3$^-$ T cells (0.5 × 10^6 cells) from $N = 7$ independent donors (all genders) were incubated overnight at 37°C. Cells were then washed twice with PBS and lysed in 8 M urea in 100 mM Tris-HCl (pH 8.0) supplemented with a cocktail of protease inhibitors (Sigma-Aldrich) and phosphatase inhibitors (PhosSTOP, Roche). Protein concentration was determined by Qubit 4 Fluorometer following the manufacturer's instructions. Mass spectrometry was performed at the Core Facility Proteomics & Mass Spectrometry of the University of Bern (Switzerland). For Spectronaut (SN) intensity analysis, protein-level imputation was performed if there was a minimum of 2 detections in at least one group. If there were at most one nonzero values in the group for a protein, then the remaining missing values were imputed by a left-censored method (Gaussian draw). This was done on a per sample basis, drawing values from a Gaussian distribution of width 0.3× sample standard deviation centered at the sample distribution mean minus 2.5× sample standard deviation. Any remaining missing values were imputed by the maximum likelihood estimation method. Imputation was repeated 20 times. Differential expression tests were performed by applying paired Student's *t* test on imputed SN intensities, and an associated adjusted P value (FDR-controlled Benjamini and Hochberg multiple test correction) was calculated. For downstream analysis, only the proteins detected in at least 4 out of 7 donors were used.

Recombinant Protein A-TurboID protein purification

The plasmid encoding the recombinant Protein A-TurboID enzyme was kindly provided by Michiel Vermeulen, Radboud University, Nijmegen, The Netherlands, and the recombinant protein was expressed and purified as previously described with few modifications (Santos-Barriopedro et al., 2021). Briefly, the plasmid was transformed into *Escherichia coli* strain C3013 and cultured on LB agar supplemented with 50 µg/ml kanamycin. After overnight incubation, six colonies were selected and inoculated into 50 ml LB medium with 50 µg/ml kanamycin, forming the starter culture. For large-scale purification, 4 Liters of LB was inoculated with 40 ml of the starter culture. Cultures were grown at 37°C until reaching an OD_{600} of 0.6, followed by the addition of 2 mM isopropyl 1-thio-β-d-galactopyranoside (Sigma-Aldrich). After an additional 3 h of growth, cells were harvested by centrifugation at 4,600 × *g* for 10 min at 4°C. The resulting cell pellet was resuspended in 160 ml lysis buffer (20 mM HEPES, pH 8.0, 2 mM dithiothreitol (DTT), 500 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% NP-40, 1 mM PMSF, 1× protease inhibitor cocktail, and 10 mM imidazole). The cell suspension underwent sonication (6 cycles at 70% amplitude, 40 s ON, and 2 min OFF) and was clarified by centrifugation at 29,000 × *g* for 1 h at 4°C. The clarified lysate passed through Pierce Disposable Column (10 ml; Thermo Fisher Scientific) containing 4 ml of Ni-NTA agarose beads (Qiagen) via gravity flow. The column was washed with 30 ml of lysis buffer and then twice with 30 ml of wash buffer (10 mM Tris, pH 7.5, 500 mM NaCl, 500 µM EDTA, and 20 mM imidazole). The bound protein was eluted in 1 ml fractions with 20 ml of elution buffer 1 (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 100 mM imidazole) and 10 ml of elution buffer 2 (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 200 mM imidazole). Aliquots of the collected fractions were loaded onto an SDS-PAGE gel and stained with Imperial Protein Staining (Thermo Fisher Scientific) following the manufacturer's instructions to determine the amount and purity of the enzyme. Fractions containing Protein A-TurboID protein were pooled and concentrated using a 10-kDa MWCO spin concentrator (Amicon Ultra-15, Millipore) and dialyzed overnight in storage buffer (20 mM HEPES, pH 8.0, 1 mM DTT, 150 mM NaCl, 0.1 mM EDTA). The purified protein was filter-sterilized using a 0.22-µm Millex-GP filter (Millipore) and stored in aliquots at −80°C.

Proximity labeling and mass spectrometry

FCRL3-expressing and control cells (20 × 10^6 cells for each sample) were stained with a mouse monoclonal anti-FCRL3 antibody (1:50, Ultra-LEAF Purified IgG2b; BioLegend) for 15 min in PBS. After removing the unbound antibody through washing with wash buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mM spermidine, 1× proteinase inhibitor cocktail [Sigma-Aldrich], and 1× phosphatase inhibitors [Sigma-Aldrich]), Jurkat cells were treated with digitonin buffer (0.04% digitonin in wash buffer) for 10–15 min using a rotator, and permeabilization of >80% of the cells was verified using trypan blue staining. The permeabilized cells were then incubated with 4 µg of Protein A-TurboID ligase per sample in digitonin buffer, for 45 min at 4°C using a rotator. After three washes with digitonin buffer, cells were resuspended in biotinylation reaction buffer (5 mM $MgCl_2$, 20 µM biotin, 1 mM ATP in digitonin buffer) and incubated for 30 min by gentle shaking at room temperature. The cells were then pelleted at 500 × *g* for 15 min at room temperature, lysed in 200 µl RIPA buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP-40), and sonicated in cycles of 15 s on/10 s off in an NGS Bioruptor sonicator until the samples became almost transparent. The samples were then centrifuged at 4°C for 10 min at maximum speed to clear the lysate. The cleared lysates were incubated with 30 µl of streptavidin beads (Sigma-Aldrich) overnight, and biotinylated

proteins were pulled down at 1,500 × *g* at 4°C for 2 min. Subsequently, the beads were washed four times, including two washes with RIPA buffer and two washes with PBS, to remove residual contaminants such as detergent. A similar protocol was used for UBASH3A proximity labeling, except that after washing once with wash buffer, the cells were fixed with reagent A (BD IntraSure Kit) for 5 min at room temperature, before permeabilization in digitonin buffer. The UBASH3A protein was targeted by incubating the cells with 4 µg of UBASH3A antibody (Proteintech) for 35 min in digitonin buffer. After removal of the unbound antibody with two washes in digitonin buffer, cells were pelleted at 500 × *g* for 15 min at room temperature and incubated with digitonin buffer containing 4 µg of Protein A-TurboID ligase per sample for 45 min at 4°C using a rotator. Following this step, cells underwent two washes using digitonin buffer, pelleting down at 500 × *g* for 15 min at room temperature after each washing cycle. After the second wash, cells were resuspended in the biotinylation reaction buffer, followed by lysis and sonication as described above. All the buffers used in this protocol were supplemented with 1× proteinase inhibitor cocktail (Sigma-Aldrich) and 1× phosphatase inhibitor (Sigma-Aldrich).

Sample preparation for mass spectrometry was performed exactly as described previously (Bataclan et al., 2024). Briefly, for on-bead digestion of pulled-down proteins beads were resuspended in 8 M urea, 50 mM ammonium bicarbonate buffer, followed by a reduction reaction with 10 mM DTT for 60 min at 37°C and alkylation with 50 mM iodoacetamide for 30 min at room temperature. Digestion was performed with 1 µg of Lys-C (FUJIFILM Wako Chemicals) in 8 M urea, 50 mM ammonium bicarbonate buffer for 2 h at 37°C, followed by dilution to final 2 M urea with 50 mM ammonium bicarbonate, addition of 1 µg of trypsin (Promega), and overnight digestion at 37°C, with shaking. After addition of acetonitrile to 2% and trifluoroacetic acid to 0.3% to stop the digestion reaction, digested peptides were purified with C18 StageTips (Rappsilber et al., 2007), and eluted with 80% acetonitrile, 0.5% acetic acid. The elution buffer was eliminated by vacuum centrifugation, and the purified peptides were resolved in 2% acetonitrile, 0.5% acetic acid, 0.1% trifluoroacetic acid for single-shot LC-MS/MS measurements.

Co-immunoprecipitation and mass spectrometry

Jurkat cells either ectopically expressing FCRL3 or an empty control vector were used. After washing with 1X PBS, cells were lysed in lysis buffer (25 mM Tris-HCl, pH 8.0, 250 mM NaCl, 0.2% NP-40, 0.5 mM EDTA, 0.5 mM EGTA), supplemented with 1× proteinase inhibitor cocktail (Sigma-Aldrich) and 1X phosphatase inhibitor (Sigma-Aldrich) for 30 min on ice. The lysate was then cleared through centrifugation at 4°C at 13,000 × *g*. Protein concentration was determined with a BCA assay (Thermo Fisher Scientific, Pierce BCA Protein Assay Kit), and 2 mg of proteins was used for immunoprecipitation. To minimize nonspecific binding, the extracted protein was initially incubated with 10 µl of protein A magnetic beads (Thermo Fisher Scientific) for 1 h at 4°C using a rotator. Subsequently, the protein extract was incubated with 50 µl of protein A magnetic beads (previously washed with lysis buffer) and 2.1 µg of anti-FCRL3 antibody (HPA048022-100UL; Sigma-Aldrich) overnight at 4°C in a total volume of 400 µl. The beads were then separated using a magnetic rack and washed twice with lysis buffer and twice with 1X PBS supplemented with 1× proteinase inhibitor cocktail (Sigma-Aldrich) and 1× phosphatase inhibitor (Sigma-Aldrich). For immunoprecipitation using anti-FLAG agarose beads, Jurkat cells ectopically expressing FCRL3 or FCRL3-3×FLAG were lysed in buffer containing 25 mM Tris-HCl (pH 8.0), 250 mM NaCl, 0.2% NP-40, 0.5 mM EDTA, and 0.5 mM EGTA, supplemented with 1× protease inhibitor cocktail (Sigma-Aldrich) and 1× phosphatase inhibitor (Sigma-Aldrich). A total of 25 µl of anti-FLAG agarose beads (ChromoTek) was added to 2 mg of total cell lysate and incubated overnight at 4°C in a final volume of 500 µl. After incubation, beads were washed twice with lysis buffer and twice with 1× DPBS. The immunoprecipitated material was then directly subjected to in-solution protein digestion for mass spectrometry analysis. Sample preparation for mass spectrometry was performed exactly as described above (Bataclan et al., 2024).

Tandem mass spectrometry and data analysis

LC-MS/MS and data analyses were performed exactly as described previously (Bataclan et al., 2024). Briefly, peptides were separated on an EASY-nLC 1200 HPLC system (Thermo Fisher Scientific) coupled online via a nanoelectrospray source (Thermo Fisher Scientific) to a Q Exactive HF mass spectrometer (Thermo Fisher Scientific). MS/MS spectra were acquired with a resolution of 15,000 at 200 m/z, maximum injection time of 55 ms, and AGC target of 1e5. Dynamic exclusion was set to 30 s to avoid repeated sequencing. The mass spectrometry raw files were processed using MaxQuant software v.1.6.7.0 (Cox and Mann, 2008). Peptides and proteins were identified with a 0.01 FDR using the integrated Andromeda search engine (Cox et al., 2011) to search spectra against the June 2019 Human UniProt database (FCRL3 immunoprecipitation) or February 2024 (UBASH3A immunoprecipitations) and a common contaminants database (247 entries). Enzyme specificity was set as "Trypsin/P" with a maximum of two missed cleavages and minimum length of seven amino acids. N-terminal protein acetylation, methionine oxidation, and lysine biotinylation (226.0776 Da) were set as variable modifications, and cysteine carbamidomethylation was set as a fixed modification. In the case of the FCRL3 immunoprecipitation experiment, to transfer identifications across samples based on mass and normalized retention times, match between runs was enabled, using a matching time window of 0.7 min and an alignment time window of 20 min. Label-free protein quantification (LFQ) was performed with MaxLFQ (Cox et al., 2014) with a minimum required peptide ratio count of 1. Data analysis was performed using Perseus software v.1.6.2.3 (Tyanova et al., 2016). After $\log_2$ transformation of LFQ intensities, biological replicates were grouped, and proteins were filtered for a minimum of four valid values in at least one group. Missing data points were then replaced by imputation from a normal distribution with 0.3 width and 1.8 downshift, and a two-sided two-samples *t* test was used to identify significant changes of protein intensity between each immunoprecipitation experiment and its corresponding isotype control.

Immunoprecipitation and western blots

HEK cells were transfected with plasmids to express FCRL3 and UBASH3A. After 48 h, cells were lysed in lysis buffer (25 mM Tris-HCl, pH 8.0, 250 mM NaCl, 0.2% NP-40, 0.5 mM EDTA, and 0.5 mM EGTA), supplemented with 1× proteinase inhibitor cocktail (Sigma-Aldrich) and 1× phosphatase inhibitor (Sigma-Aldrich) for 30 min on ice. The lysate was then cleared by centrifugation at 4°C at 13,000 × *g*. Protein concentration was determined using a BCA assay (Thermo Fisher Scientific, Pierce BCA Protein Assay Kit), and 700 µg of proteins was used for immunoprecipitation. To minimize nonspecific binding, the extracted protein was initially incubated with 10 µl of protein A magnetic beads (Thermo Fisher Scientific) for 1 h at 4°C using a rotator. Subsequently, the protein extract was incubated with 30 µl of protein A magnetic beads (previously washed with lysis buffer) and 1.5 µg of polyclonal rabbit anti-FCRL3 antibody (HPA048022-100UL; Sigma-Aldrich) overnight at 4°C in a total volume of 400 µl. The beads were then separated magnetically and washed twice with lysis buffer and twice with PBS. For western blot analysis, 50 µg of protein extract (input) or the entire immunoprecipitation was loaded on an 8% polyacrylamide gel after the addition of Laemmli buffer and boiling for 5 min. Blotting on a polyvinylidene difluoride membrane was performed using a methanol-based transfer buffer (20 mM Tris, 150 mM glycine, and 20% methanol). Blocking was performed with 5% milk in TBST (5 mM Tris, pH 7.3, 150 mM NaCl, and 0.1% Tween-20) for 60 min at room temperature with gentle shaking. Membranes were then incubated with primary antibodies at 4°C, followed by development with HRP-conjugated secondary antibodies. Analysis was performed with a Vilber Fusion FX blot imager.

Data analysis

Statistical analysis was performed with Prism version 10 (GraphPad). Data visualization was performed using R (version 4.3.0). Venn diagrams were generated using Venny 2.0 (Oliveros, 2007).

Online supplemental material

Fig. S1 provides additional characterization of human FCRL3$^+$ T cells, Fig. S2 shows the association between the genotype of the donors and FCRL3 expression, Fig. S3 shows additional functional properties of FCRL3$^+$ T cells, Fig. S4 shows data pertaining specifically to CD4$^+$FCRL3$^+$ T cells, both conventional and Tregs, Fig. S5 contains additional controls and mass-spec datasets for the FCRL3-UBASH3A interaction. Table S1 contains detailed information about the materials used in this study. Data S1 contains the RNA-seq datasets, Data S2 and S3 contain all mass-spec datasets, and Data S4 provides data underlying the figures in this study.

Data availability

Materials are available in Addgene (https://www.addgene.org/Silvia_Monticelli/) or upon request. The Protein A-TurboID construct belongs to Michiel Vermeulen. All data are available in the main text, in the supplementary materials, or in GEO with the accession number GSE241004. TCR Vβ sequences are shared through the immuneACCESS data portal (https://clients.adaptivebiotech.com/pub/bianchi-2025-jem).

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Supplemental material

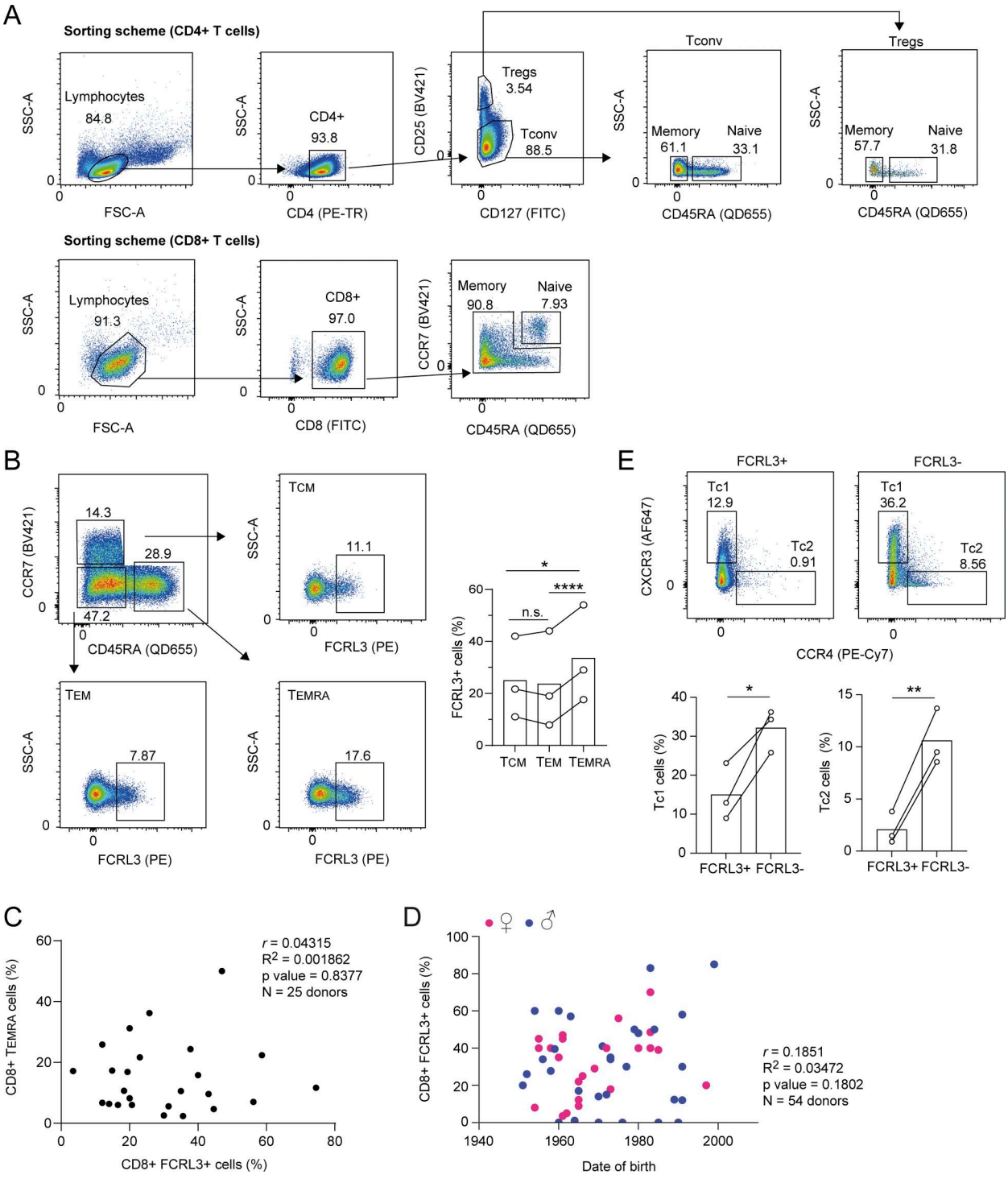

Figure S1. **Characterization of human FCRL3⁺ cells. (A)** Gating strategies (sorting scheme) for the separation of human CD4⁺ T cells (conventional memory and naïve T cells and Tregs, top) and CD8⁺ lymphocytes (bottom) from peripheral blood. For conventional CD4⁺ cells, naïve T cells were defined as $CD4^+CD25^-CD45RA^+CCR7^+$, while memory cells were $CD4^+CD25^-CD45RA^-CD127^{+/-}$. Naïve Tregs were defined as $CD4^+CD25^{hi}CD127^-CD45RA^+CCR7^+$, while memory Tregs were $CD4^+CD25^{hi}CD127^-CD45RA^-CCR7^+$. Naïve CD8⁺ T lymphocytes were sorted as CD8⁺ and CD45RA/CCR7 double-positive, while memory cells were $CD8^+CD45RA^{+/-}CCR7^{+/-}$. **(B)** FCRL3 expression in different CD8⁺ T cell subpopulations. Among the CD8⁺ memory cells $CD8^+CD45RA^{+/-}CCR7^{+/-}$, the subpopulations were defined as T_{CM} $CD8^+CCR7^+CD45^-$, T_{EM} $CD8^+CCR7^-CD45^-$, and T effector memory reexpressing CD45RA (T_{EMRA}) $CD8^+CCR7^-CD45^+$. Each dot represents one donor. *N* = 3; paired *t* test, two-tailed. From top to bottom: *P = 0.0365, ****P < 0.0001, n.s. (not significant), P = 0.5185. **(C)** Pearson's correlation coefficient between the percentage of CD8⁺ FCRL3⁺ memory T cells and the percentage of CD8⁺ T_{EMRA} cells across donors. Each dot represents one donor (*N* = 25). **(D)** Pearson's correlation coefficient between the percentage of CD8⁺ FCRL3⁺ memory T cells and the date of birth of the donors. Each dot represents one donor (*N* = 54). **(E)** FCRL3 expression in the CD8⁺ T cell effector subsets Tc1 and Tc2. $CD8^+$ memory cells $CXCR3^+CCR4^-$ were defined as Tc1, and $CXCR3^-CCR4^+$ cells were defined as Tc2. Each dot represents one donor. *N* = 3; paired *t* test, two-tailed. From left to right: *P = 0.0393, **P = 0.0066. T_{CM}, T central memory; T_{EM}, T effector memory. Data underlying this figure can be found in Data S4.

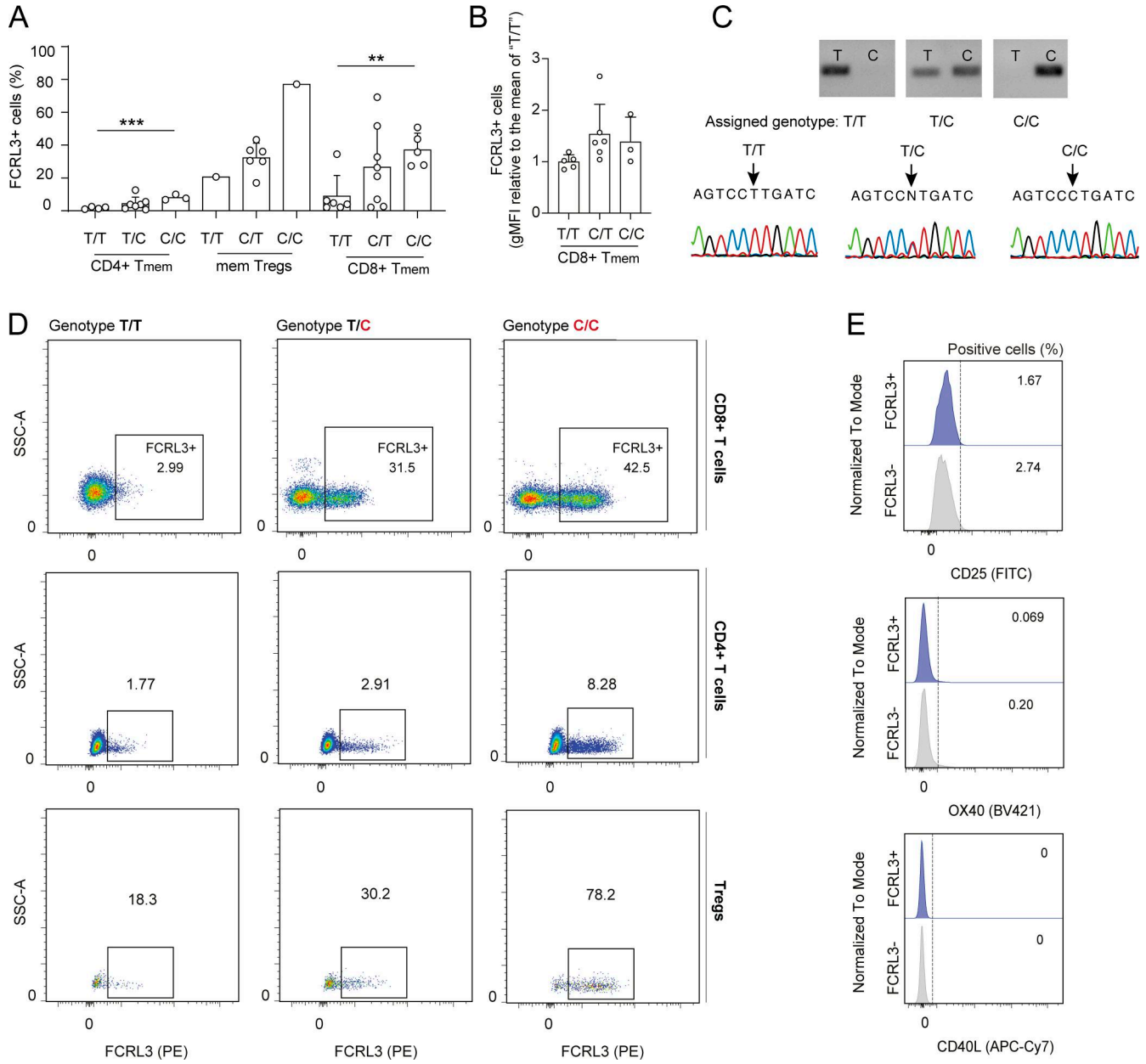

Figure S2. **FCRL3 expression is associated with the donors' genotype. (A)** Percentage of surface FCRL3 expression on sorted T cell subsets from genotyped healthy donors. Each dot represents one donor. Mean ± SD; unpaired *t* test, two-tailed. ***P = 0.0009, **P = 0.003. **(B)** gMFI of gated FCRL3$^+$ CD8$^+$ T memory cells from genotyped healthy donors. Each dot represents one donor. Results were normalized to the average of "T/T" donors. Mean ± SD; unpaired *t* test, two-tailed. No difference was statistically significant. **(C)** Examples of genotyping results by PCR and Sanger sequencing. **(D)** Examples of FCRL3 expression in donors carrying the indicated SNP variants in the *FCRL3* promoter region (−169 bp from the transcription start site). For every donor, FCRL3 expression is shown in CD8$^+$ and CD4$^+$ T memory cells and Tregs. **(E)** Surface staining for the indicated AIMs (CD25, OX40, CD40L) in unstimulated CD8$^+$ FCRL3$^+$ and FCRL3$^-$ memory T cells. gMFI, geometric mean fluorescence intensity. Data underlying this figure can be found in Data S4. Source data are available for this figure: SourceData FS2.

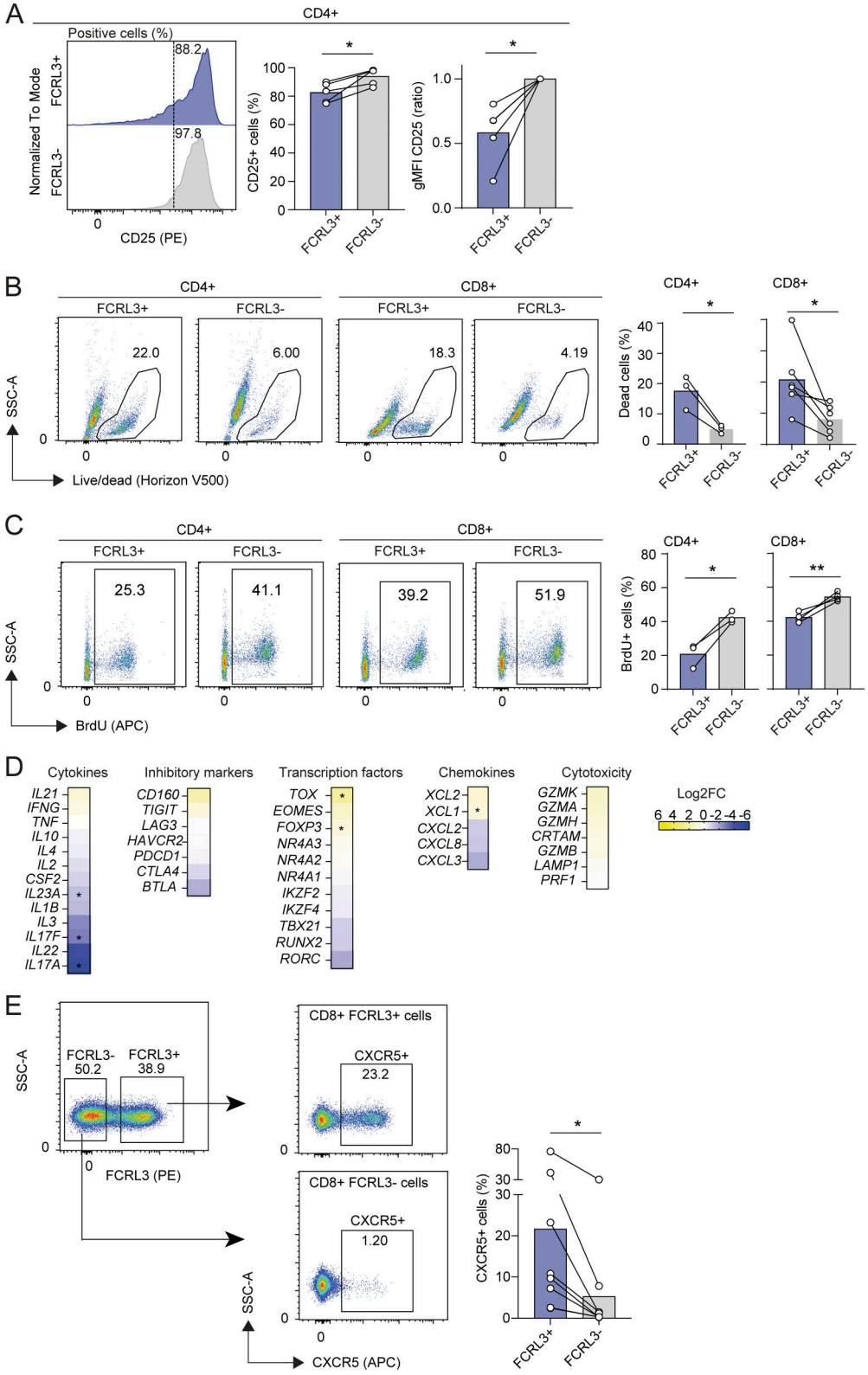

Figure S3. **Phenotypic characterization of FCRL3⁺ T cells. (A)** Surface staining for CD25 expression in FCRL3⁺ and FCRL3⁻ CD4⁺ memory T cells. Cells were stimulated with plate-bound anti-CD3/CD28 for 48 h. Each dot represents one donor, *N* = 5. Mean ± SD, paired *t* test, two-tailed. From left to right: *P = 0.0174, *P = 0.015. **(B)** Live/Dead staining showing viability of sorted FCRL3⁺ and FCRL3⁻ CD4⁺ and CD8⁺ memory T cells, 3 days after activation. Each dot represents a different donor, *N* = 3–6. Mean ± SD, paired *t* test, two-tailed. From left to right: *P = 0.0369, *P = 0.0272. **(C)** BrdU incorporation assay to measure the proliferation of sorted FCRL3⁺ and FCRL3⁻ CD4⁺ and CD8⁺ memory T cells, 3 days after activation. Each dot represents a different donor, *N* = 3–4. Mean ± SD, paired *t* test, two-tailed. *P = 0.0226, **P = 0.0093. **(D)** Heatmaps showing the differential expression of selected genes from Fig. 3 A. **(E)** Surface staining for CXCR5 expression in FCRL3⁺ and FCRL3⁻ CD8⁺ memory T cells. Each dot represents one donor, *N* = 8. Mean ± SD, paired *t* test, two-tailed. *P = 0.0221.

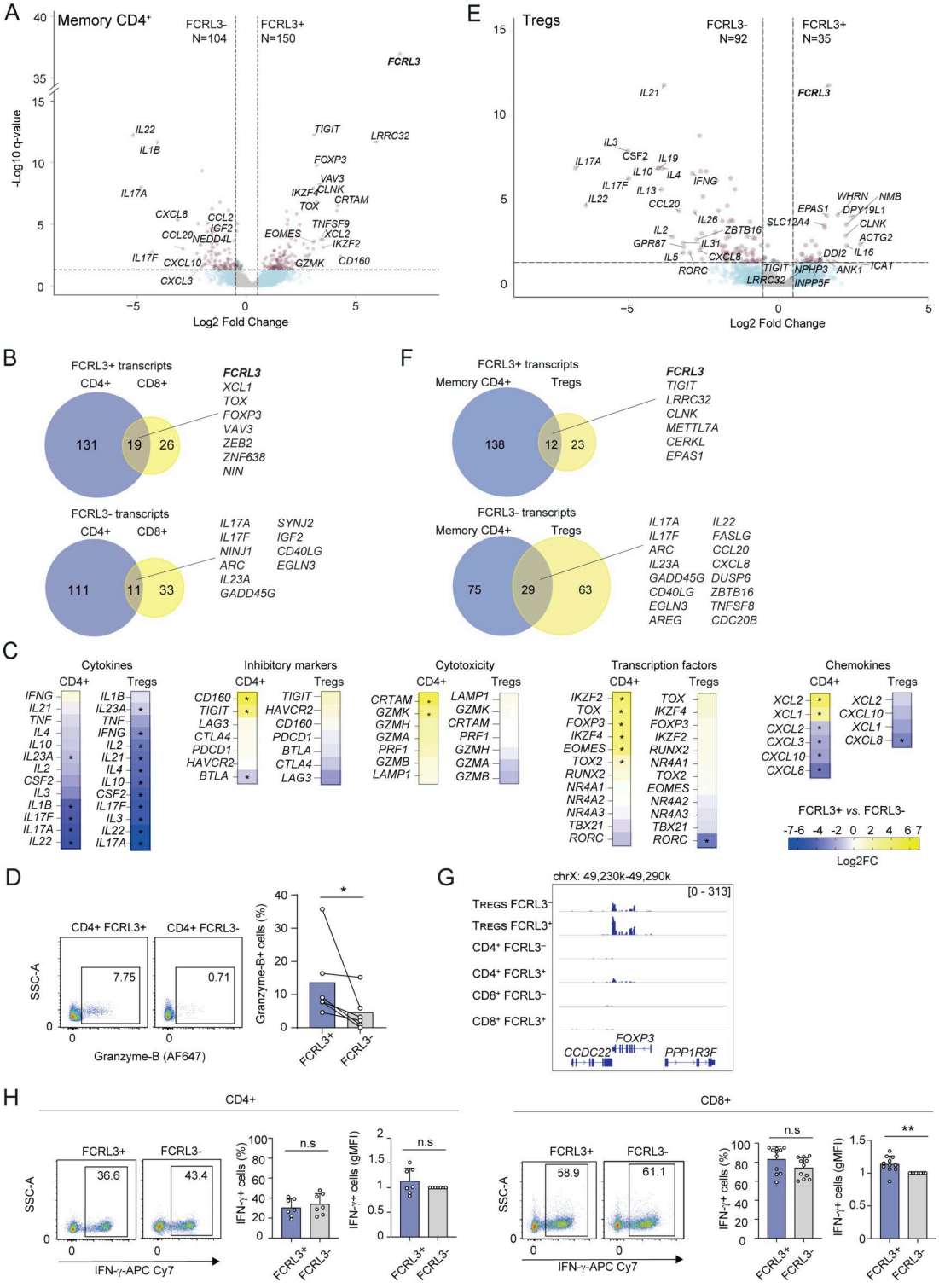

Figure S4. **Characterization of human CD4⁺ FCRL3⁺ T cells. (A)** Volcano plot showing the differentially expressed genes for FCRL3⁺ versus FCRL3⁻ memory CD4⁺ T cells from *N* = 5 independent donors, analyzed by RNA-seq (FDR ≤ 0.05 and $\log_2$FC ≥|0.5|). **(B)** Venn diagrams showing the intersection of the genes differentially expressed in memory CD4⁺ and CD8⁺ T cells. **(C)** Heatmaps showing the $\log_2$FC of selected genes from (A and E). **(D)** Granzyme B expression (by intracellular staining) in sorted CD4⁺ FCRL3⁺ and FCRL3⁻ memory T cells. *N* = 6, mean ± SD; paired *t* test, two-tailed. *P = 0.0419. **(E)** Volcano plot showing the differentially expressed genes for FCRL3⁺ versus FCRL3⁻ Treg cells from *N* = 5 independent donors, analyzed by RNA-seq (FDR ≤ 0.05 and $\log_2$FC ≥|0.5|). **(F)** Venn diagrams showing the intersection of the genes differentially expressed in memory conventional CD4⁺ T cells and Tregs. **(G)** Screenshot of *FOXP3* expression in FCRL3⁺ and FCRL3⁻ Tregs, conventional memory CD4⁺ and CD8⁺ T cells, by RNA-seq. **(H)** IFN-γ production by FCRL3⁺ and FCRL3⁻ CD8⁺ or CD4⁺ memory T cells. Intracellular cytokine staining was performed upon stimulation with PMA and ionomycin for 5 h. The dot plots are from one representative donor; the histograms show the results from different donors (*N* = 5–11), with each dot representing one donor; mean ± SD; paired *t* test, two-tailed. n.s.: not significant, **P = 0.004. FC, fold change. Data underlying this figure can be found in Data S4.

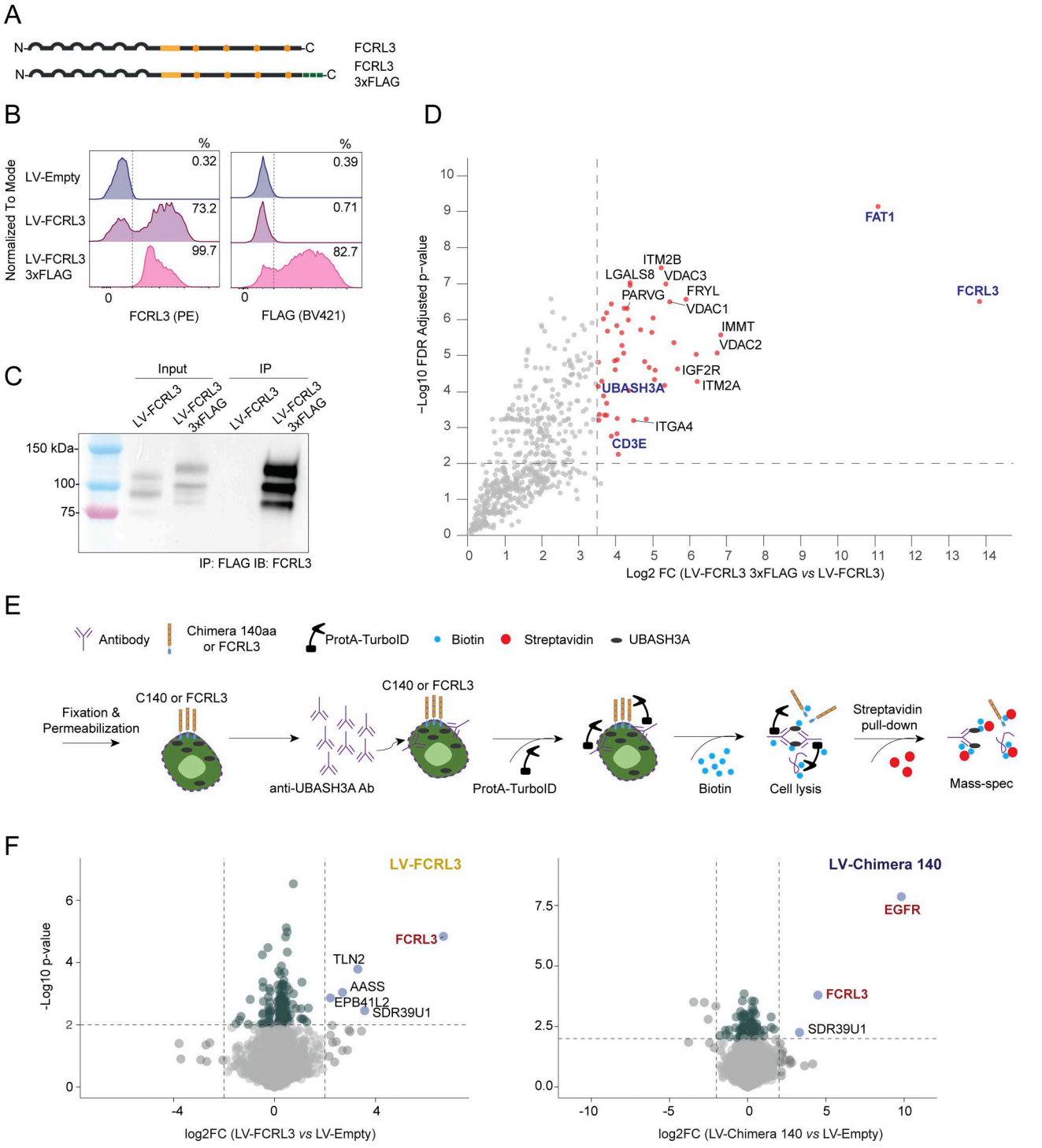

Figure S5. **IP-MS and TurboID-MS confirm the interaction between FCRL3 and UBASH3A. (A)** Schematic representation of FCRL3 and the C-terminally tagged FCRL3-3xFLAG protein. The 3xFLAG tag is shown as green squares. **(B)** Surface (left) FCRL3 staining and intracellular (right) anti-FLAG staining showing surface expression of the FCRL3-3xFLAG construct. **(C)** Western blot showing efficient immunoprecipitation of FCRL3-3xFLAG with anti-FLAG agarose beads in Jurkat cells expressing LV-FCRL3 and LV-FCRL3-3xFLAG. Immunoblot was performed using an anti-FCRL3 antibody. **(D)** Scatter plot showing the enriched proteins in the IP-MS of the FCRL3-3xFLAG compared with untagged FCRL3. In this experiment, to enhance the separation of true interactors from experimental noise, an additional filter was applied by selecting proteins present in at least 3 out of 4 replicates of the FCRL3-3xFLAG condition and in <2 replicates of the untagged control (FCRL3), followed by imputation of the missing values. A two-sided two-samples *t* test (0.01 permutation-based FDR cut-off, 250 randomizations) was employed to identify significant changes, and the results were visualized with a volcano plot generated with R, version 4.4.2. **(E)** Schematic representation of the Protein A-TurboID experimental workflow for UBASH3A. The anti-UBASH3A antibody was added to Jurkat cells ectopically expressing FCRL3 or the EGFR-FCRL3 140 aa chimera (or control cells), followed by the addition of Protein A-TurboID fusion protein and biotin. After streptavidin pull-down, biotinylated proteins were analyzed by mass spectrometry. **(F)** Differentially enriched proteins in the FCRL3-transduced cells versus control (left) and chimera-transduced cells versus control (right). *N* = 4 independent samples. Source data are available for this figure: SourceData FS5.

Provided online are Table S1, Data S1, Data S2, Data S3, and Data S4. Table S1 shows materials used in this study. Data S1 shows RNA-seq results from human $CD4^+$, $CD8^+$, and Treg T cells, $FCRL3^+$ versus $FCRL3^-$ cells. Data S2 shows shotgun proteomics of $CD8^+$ T cells, $FCRL3^+$ versus $FCRL3^-$. Data S3 shows TurboID-MS and IP and mass spectrometry datasets. Data S4 shows source data for all figures in this study.

5. Discussion and Open Questions

In this study, we characterized human FCRL3$^+$ T lymphocytes. We found that FCRL3 is highly expressed by CD8$^+$ T lymphocytes, and to a lower extent CD4$^+$ T cells. We observed that these cells display a terminally differentiated effector phenotype. Specifically, they exhibit reduced activation and proliferative capacity, enhanced cytotoxic potential, and upregulation of genes associated with terminal differentiation, including *TOX, EOMES*, and *TIGIT*. *In vitro*, FCRL3 expression was induced by repetitive TCR stimulation, specifically in the absence of co-stimulatory signals. Furthermore, the intracellular domain of FCRL3 alone was sufficient to attenuate T cell activation, a function mediated through the putative interaction with negative regulators of TCR signalling, such as UBASH3A. Overall, we found that FCRL3 functions as an immunoregulatory receptor in human T cells, restraining the activation of conventional T lymphocytes.

Our study raises several open questions. In particular, the overall understanding of the role of this receptor *in vivo* remains limited, as there is no murine ortholog of *FCRL3*, which constrains the possibility of investigating its function in the context of autoimmune disease using mouse models. Since the -169C SNP in the *FCRL3* promoter region has been associated to several autoimmune diseases (Kochi, Yamada et al. 2005, Bajpai, Swainson et al. 2012), an interesting approach would be to examine how this variant shapes the functionality and transcriptome of different cell types expressing FCRL3, including T cells, B cells, and NK cells. Specifically, it is important to underline that within the T cell compartment, the -169C SNP could impact CD4$^+$ and CD8$^+$ T lymphocytes differently compared to Tregs. Building on this, the contexts in which this modulation becomes pathogenic or protective remain unclear. Tregs represent a particularly relevant subset to investigate: as illustrated in Figure 8 of this thesis, they play a central role in restraining immune responses, and loss of their regulatory function can lead to autoimmunity. Notably, Tregs typically express high levels of FCRL3, and stimulation with a FCRL3-specific monoclonal antibody has been shown to inhibit the suppressive activity of Tregs (Agarwal, Kraus et al. 2020). Another group reported that FCRL3$^+$ Tregs are hypoproliferative in response to IL-2 compared to FCRL3$^-$ Tregs (Nagata, Ise et al. 2009). In our work, we show that FCRL3 receptor exerts inhibitory activity in conventional T lymphocytes, particularly by downregulating CD25 expression. If this mechanism applies to Tregs, defined by the surface co-expression of CD4 and CD25 (Sakaguchi, Sakaguchi et al. 1995), an hypothesis is that increased expression of FCRL3 due to the -169T/C SNP in *FCRL3* promoter could lead to reduced IL-2 responsiveness, critical for Tregs function, and weaken their suppressive phenotype. A compelling direction is to assess *in vitro* whether the −169T/C SNP modulates Treg suppressive function, and to determine whether FCRL3 pharmacological blockade could restore it.

For what refers to conventional T lymphocytes, in our study we showed that FCRL3 expression in the peripheral blood of healthy human donors is highly variable, ranging from 0% to over 80%, with no correlation to biological sex or age. Donors carrying the -169C SNP exhibited higher levels of receptor expression. However, it remains unclear whether FCRL3 expression is stable or transient and longitudinal studies following the same donors will be required to resolve this question. Notably, we observed that FCRL3 is lowly expressed by naïve T cells, enriched within the T_{EMRA} compartment, and upregulated *in vitro* following repetitive TCR engagement. Moreover, FCRL3 was observed to be upregulated in B cells and T cells in the setting of chronic viral infection (Portugal, Obeng-Adjei et al. 2017, Poonia, Ayithan et al. 2018). Whether FCRL3 expression increases following natural antigen exposure *in vivo* remains unknown and could be investigated by monitoring expression before and after infection or vaccination. Moreover, ATAC-Seq data showed that the *FCRL3* locus in effector memory $CD4^+$ T cells was selectively accessible in cells that did not produce the inflammatory cytokine GM-CSF (Emming, Bianchi et al. 2020); therefore, *FCRL3* locus accessibility may be altered during inflammation or following infections. Under these conditions, the presence of the -169C SNP could facilitate NFκB binding and thereby enhance *FCRL3* transcription, potentially disrupting signalling balance.

More specific to the $CD4^+$ T lymphocyte compartment, we observed that $CD4^+$ T cells expressing FCRL3 show features that are typical of CD4 CTLs. At the transcriptomic level, they upregulate hallmark CD4 CTL-associated genes, including *CRTAM* and *EOMES*. Notably, protein analysis of granzyme B, a canonical cytotoxic effector molecule, revealed that within circulating $CD4^+$ T cells, its expression is significantly higher in the $FCRL3^+$ subset. CD4 CTLs are known to play a critical role in antiviral immunity (Juno, van Bockel et al. 2017) and are enriched in centenarians (Hashimoto, Kouno et al. 2019), consistent with our hypothesis that FCRL3 expression may be upregulated following natural antigen exposure. Further investigation into the relationship between FCRL3 and CD4 CTLs could clarify whether in this subset FCRL3 expression serves primarily to dampen TCR signaling and prevent excessive T cell activation, or whether it serves additional, unidentified, functions.

It will be important to further investigate the gene network that, according to our hypothesis, drives FCRL3 upregulation following natural antigen encounters. $FCRL3^+$ $CD8^+$ T lymphocytes display significantly higher TOX and EOMES protein expression, and knocking-out FCRL3 leads to reduced *TOX* and *EOMES* transcripts, indicating a strong association between these transcription factors and FCRL3. To determine the directionality of this regulatory relationship, and to assess the potential existence of feedback loops, one approach would be to enforce TOX and EOMES expression, using systems such as mRNA *in vitro* transcription or CRISPR activation, and assess chromatin accessibility at the FCRL3 locus by ATAC-seq, together with the evaluation of FCRL3 protein expression across T lymphocytes. In fact, as discussed in Chapter 2.1, TOX and EOMES play a crucial role in establishing transcriptional and epigenetic programs

during T cell exhaustion, including the induction of the expression of some genes encoding for inhibitory receptors, such as *PDCD1* and *TIGIT* (Buggert, Tauriainen et al. 2014, Khan, Giles et al. 2019, Scott, Dündar et al. 2019, Kim, Park et al. 2020).

Interestingly, we found that in peripheral blood $CD8^+$ T lymphocytes CXCR5 expression is restricted to the $FCRL3^+$ subset (Bianchi, Foli et al. 2025). CXCR5 is a chemokine receptor that directs T cells to the B-cell zones of SLOs. Its canonical expression is found on T_{FH} cells (Hardtke, Ohl et al. 2005, Junt, Fink et al. 2005). More recently, attention has turned to a less common $CD8^+CXCR5^+$ population, characterized by potent cytotoxic activity and by the ability to home to lymph nodes, where it contributes to both antitumor immunity and to the control of viral infections (Yu and Ye 2018). Specifically, the pivotal role of $CD8^+$ $CXCR5^+$ T cells in controlling viral replication has been demonstrated in chronic infections (He, Hou et al. 2016), including their contribution to the eradication of simian immunodeficiency virus reservoirs in macaques, which persist in lymph node germinal centers (Mylvaganam, Rios et al. 2017). Moreover, their presence has been associated with favourable prognosis in patients with follicular lymphoma (Chu, Li et al. 2019). In our RNA-seq analysis, *CXCR5* emerged as one of the most differentially expressed genes in $FCRL3^+$ cells. This was corroborated at the protein level, as surface staining in peripheral blood revealed that CXCR5 expression is restricted to the $FCRL3^+$ subset (Bianchi, Foli et al. 2025). Together, these data demonstrate a strong association between FCRL3 and CXCR5 at both the transcript and protein levels. The biological significance of this relationship remains unclear, but it suggests the existence of a $CD8^+FCRL3^+$ subset with potential for follicular homing and effector activity.

In the context of the FCRL3 interactome, we identified interactions between the intracellular tail of FCRL3 and negative regulators of TCR signaling, such as UBASH3A. From a functional perspective, definitive evidence for the role of this interaction in T cells would come from CRISPR-mediated knockout of *UBASH3A* in both $FCRL3^+$ and $FCRL3^-$ T lymphocytes, followed by transcriptomic profiling after TCR activation to determine whether alterations in propagating TCR signalling are restricted to a specific subset. Another approach would involve complementing *UBASH3A*-CRISPR KO with ectopic expression of FCRL3, followed by analysis of T lymphocyte activation patterns, to determine whether FCRL3 can exert its inhibitory function also in the absence of UBASH3A. Moreover, variants of *UBASH3A* gene carrying SNPs have been correlated with several autoimmune diseases (Diaz-Gallo, Sánchez et al. 2013, Howarth, Sneddon et al. 2023), strengthening the rationale for exploring its potential role in a molecular network with FCRL3.

An important open question regards the identification of a physiological ligand for FCRL3. High-throughput receptor–ligand interaction screens using comprehensive libraries of most surface proteins detectable on human leukocytes have been conducted in recent years. However, even across these large, unbiased extracellular interactome studies, FCRL3 has remained an orphan receptor (Verschueren, Husain

et al. 2020, Wojtowicz, Vielmetter et al. 2020, Shilts, Severin et al. 2022). Further investigation will be required to elucidate whether FCRL3 functions through engagement of a physiological ligand, via ligand-independent mechanisms, or both, before definitive conclusions can be drawn.

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