Killer Cell Immunoglobulin-Like Receptors Delineate Distinct Phenotypes and Functions in Human γδ T Cells

by

Mahya Razmi

A thesis submitted in partial fulfillment of the requirements for the Master's degree in Biochemistry, Microbiology and Immunology

uOttawa

Department of Biochemistry, Microbiology and Immunology
Faculty of Medicine
University of Ottawa

Abstract

Human circulating γδ T cells are broadly classified into three subsets: Vδ1, Vδ2, and Vδ1/2neg populations, with the Vδ2 subset being the most abundant. In this research, I focus on the contribution and interplay of Natural Killer (NK) receptors to the γδ T cell phenotype and function, with emphasis on Killer-cell Immunoglobulin-like Receptors (KIRs), which are poorly studied in the field of γδ T cell biology. Because cytomegalovirus (CMV) is known to shape the αβ T cell and NK cell repertoires, I studied peripheral blood (PB) γδ T cells from both CMV-seronegative (CMV^-) and CMV-seropositive (CMV^+) healthy adult humans, using spectral flow cytometry. I found that CMV leaves a stable imprint in the γδ T cell repertoire and phenotype. CMV^+ individuals have increased proportions of Vδ1 T cells within the total γδ T cell population. Moreover, γδ T cells from these individuals display increased proportions of cells expressing KIRs, which constitute a highly polymorphic family of receptors for Human Leukocyte Antigen (HLA) class I. These KIRs delineate a dichotomy in the phenotype and function of γδ T cells. KIR^+γδ T cells exhibit characteristics akin to memory like T cells, displaying heightened effector potential, whereas KIR^-γδ T cells resemble naïve T cells with comparatively weaker immune responses. Leveraging the assay for transposase-accessible chromatin with sequencing (ATACseq) technology, I show that KIR^+γδ T cells have more open loci associated with effector functions than KIR^-γδ T cells, which is consistent with the above results. These findings underscore a critical role for KIRs in γδ T cell immunity and hold implications for both our understanding of immune responses influenced by CMV and the potential of γδ T cells in cellular immunotherapy.

Acknowledgement

There were numerous individuals who kindly offered their time, help, and encouragement. While it's impossible to acknowledge everyone individually, their support made this endeavor possible. However, I want to express my heartfelt gratitude to three remarkable women without whom this piece would not have been possible: my mother, my sister, and my supervisor.

I extend my deepest gratitude to my supervisor, who transcended the roles of teacher, mentor, and friend, patiently guiding me from the basics, supporting me through any challenges, and instilling in me a love for science.

To my father, for encouraging me to pursue this path and showing me the profound satisfaction that science can bring. I aim to emulate your dedication and passion.

I express gratitude to the many amazing PIs and scientists I had the opportunity to work with and learn from, listed in alphabetical order: Dr. Alexandre Blais, Dr. Tommy Alain, Dr. Marceline Côté, Dr. Tyson Graber, Dr. Huy-Dung Hoang, Dr. Martin Roffe, Dr. Vera Tang, Dr. Lisheng Wang, and Dr. Behzad Yeganeh. Their never-ending support and guidance have been invaluable.

With hearts intertwined in laughter and shared memories, I want to express profound gratitude to my dearest friends, listed in alphabetical order: Yeganeh Almasi, Mariam Hakoum, Dr. Ali Hamraghani, Marilee Larrivee, Salar Pashangzadeh, and Negar Sharifi. All enriched this journey with their unwavering support and kindness.

I would also like to extend my gratitude to the wonderful teams at CHEO Research Institute and Dr. Angel’s lab for their help to advance this project.

Table of Contents

List of Figures

Chapter 1: Introduction

Chapter 3: Results

Supplementary Materials

List of Tables

Chapter 2: Materials and Methods

List of Abbreviations

Abbreviation	Definition
ADCC	Antibody-dependent cellular cytotoxicity
APC	Antigen-presenting cells
AREG	Amphiregulin
αβ T-depleted	Alpha-beta T cell depleted
β2M	Beta-2 macroglobulin
BTN	Butyrophilin
BTNL	Butyrophilin-like
CMV	Cytomegalovirus
CMV^-	CMV-seronegative
CMV^+	CMV-seropositive
CTLs	Cytotoxic T lymphocytes
DMSO	Dimethyl sulfoxide
EBV	Epstein-Barr virus
EPCR	Endothelial protein C receptor
FBS	Fetal bovine serum
HA	Hemagglutinins
HIV	Human Immunodeficiency Virus
HIV^+	HIV-seropositive
HLA	Human leukocyte antigen
IgG	Immunoglobulin G
ILTs	Immunoglobulin-like transcripts
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
IFN-γ	Interferon-gamma
KIR	Killer cell immunoglobulin-like receptors
KO	Knockout
LILRB1	Leukocyte immunoglobulin-like receptor subfamily B member 1
LIRs	leukocyte immunoglobulin-like receptors
MHC	Major histocompatibility complex
MICA	MHC class I chain-related molecules A

MICB	MHC class I chain-related molecules B
MR1	MHC-related protein 1
M. tuberculosis	Mycobacterium tuberculosis
NCRs	Natural cytotoxicity receptors
NK cell	Natural killer cell
pAg	Phosphoantigen
PB	Peripheral blood
PBMC	Peripheral blood mononuclear cells
P. falciparum	Plasmodium falciparum
SAP	SLAM-associated protein
TCR	T cell receptor
TNF-α	tumor necrosis factor-alpha
ULBP	UL16-binding proteins

Chapter 1: Introduction

1.1 Discovery of γδ T cells

γδ T cells discovery traces back to 40 years ago when Tonegawa *et al.* identified a distinct set of rearranging T cell receptor (TCR) genes in murine cytotoxic T lymphocytes [1]; just two years after James P. Allison's ground-breaking discovery of the TCR in 1982 [2]. In 1985, with further characterization of TCRγ in mice [3-5], separate groups demonstrated the existence of TCRγ in humans. One group pinpointed the presence of TCRγ on the short arm of chromosome 7, previously known for hosting TCRβ genes, and further confirmed the lack of relation between TCRγ and TCRβ through experiments demonstrating no cross-hybridization [6]. Meanwhile, another group isolated the TCRγ gene from humans, revealing a higher degree of gene rearrangements compared to mice in this gene [7].

These investigations led to the identification of the TCRδ chain by two independent research groups. Michael B. Brenner's team observed a subset of peripheral blood (PB) T cells in human blood samples that were $CD3^+$ but unresponsive to antibodies against the α and β TCRs. They further characterized TCRs of the new subset and discovered a novel polypeptide in this TCR protein, naming it TCRδ [8]. Concurrently, another group led by Ilan Bank demonstrated the presence of this structure in immature murine thymocytes and $CD4^-CD8^-$ PB T cells. They identified a novel TCR heterodimer and a polypeptide capable of interacting with major histocompatibility complex (MHC) molecules [9]. However, they were unable to determine which gene expresses TCRδ, the structural arrangement of the TCR γδ complex, the presence of clonal heterogeneity, and whether ligands interacting with this complex could stimulate cellular responses similar to those triggered by the TCR αβ complex.

These questions were answered in following years by separate studies showing the MHC-unrestricted cytotoxicity of γδ T cells [10, 11]. γδ T cells were the first T cell subset found to

respond to *Mycobacterium tuberculosis* (*M. tuberculosis*) or heat shock proteins without requiring MHC [12-14], indicating a unique immune response distinct from that of αβ T cells. The human TCRδ chain was further studied, revealing a smaller repertoire of γδ T cells compared to αβ T cells [15]. Subsequently, research focused on the distribution of γδ T cells in the human lymphatic system, demonstrating their ubiquity [16]. Notably, they were found to be more abundant than αβ T cells in marginal zones and the red pulp of the spleen [16]. This led scientists to further study their antigen recognition patterns and their repertoire.

The lack of dependence on MHC recognition by γδ T cells was surprising because the conventional understanding was that T cell maturation and selection in the thymus and lymph nodes required antigen recognition via MHC molecules. However, research by Bigby *et al.* (1993) demonstrated that γδ T cells in MHC class II knockout (KO) mice proliferate and migrate normally from lymphatic organs to epidermal sites despite the absence of MHC class II molecules [17]. Additionally, whether γδ T cells have MHC class I antigen recognition capabilities is a matter of ongoing debate, as studies have indicated their ability to recognize unprocessed proteins and heat shock proteins. While a few *in vitro* studies suggested γδ T cell recognition of MHC-bound antigenic peptides [18, 19], *in vivo* studies using β-2 macroglobulin (*β2M)* KO mice showed that there was less alteration in γδ T cell development in these mice, compared to αβ T cells. Moderate impairment in the development and maturation of some γδ T cells was observed in the *β2M* KO background suggesting some γδ T cell clones are reactive to MHC or MHC-like molecules [20-22]. These studies provided the unique MHC unrestricted recognition capability of γδ T cells, leading to their classification as unconventional T cells.

1.2 Classification of human γδ T cells

γδ T cells are predominantly found in mucosal tissues, the first line of immune defence, where they play a significant role in immune surveillance [23]. Although they constitute a minor subset in PB, accounting for approximately 5% of peripheral T cells, they are crucial for maintaining homeostasis and responding to pathogens. They do so by producing a wide array of cytokines and chemokines, which lead to the lysis of abnormal cells. γδ T cells can be classified based on the Vδ chain into four main populations: Vδ1, Vδ2, Vδ3, and Vδ5. These TCRδ are primarily paired with Vγ2, Vγ3, Vγ4, Vγ5, Vγ8 and Vγ9 TCRs [24]. It is worth mentioning that four different subsets of γδ T cells were detected (Vδ4, Vδ6, Vδ7, and Vδ8) in PB of patients with B-cell non-Hodgkin lymphoma [25]. γδ T cells are distributed in varied proportions among lymphocytes across different tissues, reflecting their distinct roles in immune surveillance and response (**Fig. 1**).

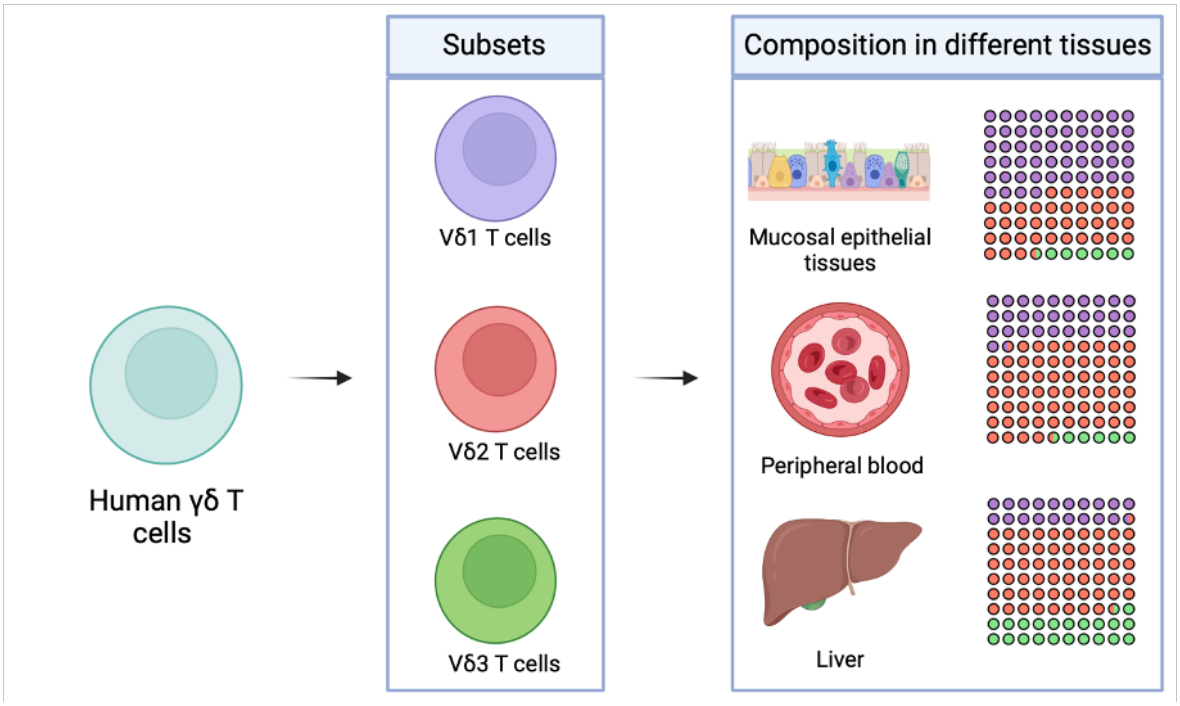


Figure 1: Human γδ T cell distribution in healthy tissues

Vδ1 T cells are predominantly found in epithelial tissues such as the intestinal epithelium [26], breast epithelium [27], lung epithelium [28], spleen [29], and dermis [30]. They possess distinct morphological and biological features, such as abundant cytoskeletal structures, which equip them with the ability to move and migrate to tissues [31]. They constitute approximately 30% of γδ T cells in PB and undergo expansion during viral infections like Human Immunodeficiency Virus (HIV) [32, 33] and Cytomegalovirus (CMV) [34], parasitological diseases such as *Onchocerca volvulus* [35], and autoimmune diseases like Crohn's disease [36]. After CMV infection, Vδ1 T cells undergo clonal expansion in most individuals [37, 38]. Moreover, Vγ4Vδ1 T cells have been shown to be the dominant population in the gut [39]. The ligands recognized by Vδ1 T cells have been shown to include Human Leukocyte Antigen (HLA)∗24:02 [40], MHC-like molecules, including CD1d [41, 42] and CD1c [43], R-phycoerythrin [44] and MHC-related protein 1 (MR1) [45]. Furthermore, the Vγ9Vδ1 T cells have been shown to recognize ephrin receptor A2 on endothelial cells of CMV-seropositive (CMV^+) individuals [44].

Vδ2 T cells are the predominant subset of γδ T cells in PB [46]. Their TCRδ chains mainly pair with Vγ9 TCRs, forming the Vγ9Vδ2 T cells that recognize the conformational changes in Butyrophilin-(BTN)3A and BTN-2A1 molecules induced by the binding of phosphoantigen (pAg) compounds [47]. These compounds can increase in various diseases and conditions, resulting in an elevation of Vγ9Vδ2 T cells to comprise up to 50% of T cells [48]. Vγ9Vδ2 T cells exhibit important immune responses in viral infections like Epstein-Barr virus (EBV) and intracellular pathogens such as *M. tuberculosis, Listeria monocytogenes, Brucella* spp.*, Coxiella burnetii* [49-51] and *Plasmodium falciparum (P. falciparum)* protozoa [52, 53]. Different drugs such as Zoledronate, used in the treatment of osteoporosis and bone metastases, have also been shown to

expand Vγ9Vδ2 T cells by affecting the mevalonate pathway and providing cells with increased pAg content [54]. Vγ9Vδ2 T cells also recognize a cell-surface structure related to the mitochondrial F1-ATPase, which can be expressed on some cancer cells [55]. Furthermore, Vγ9Vδ2 T cells can recognize MHC class I chain-related molecules A and B (MICA and MICB), UL16-binding proteins (ULBP) 1–4, and Retinoic acid early transcript 1, through NKG2D receptor, which is upregulated when Vγ9Vδ2 T cells are activated [56]. Vγ9negVδ2 T cells are another Vδ2 subset that was shown to expand in the blood of CMV^+ individuals [57]. Vδ2 T cells can also be found in tissues, during the development of autoimmune myositis disease, where Vγ3Vδ2 T cells have been demonstrated to react with aminoacyl-tRNA synthetases, key enzymes in protein biosynthesis [58].

Vδ3 T cells are predominantly located in the liver and the gut epithelium [59, 60], yet they remain relatively underexplored among γδ T cells. They account for only ~0.2% of PB cells and they exhibit a similar function and phenotype to Vδ1 T cells, as evidenced by their expansion in CMV infection [61] and their responsiveness to CD1d [62]. Additionally, their number have been observed to increase in the gut mucosa of patients with coeliac disease [63]. In a case report involving an HIV/HTLV-negative patient exhibiting CD4 T cell deficiency, a remarkable clonal expansion of Vγ3Vδ3 T cells was observed, reaching more than 25% of total PB lymphocytes [64]. Furthermore, Vγ8Vδ3 T cells have been shown to respond to Annexin A2 in stressed glioblastoma cells [65]. Additionally, Vγ8Vδ3 T cells recognize the antigen-presenting molecule MR1 independently of the presented antigen [66].

Vδ5 T cells are one of the rarest subsets among γδ T cells. Vγ4Vδ5 T cell clone was shown to recognize the BTNL (Butyrophilin-like) molecules [67] and endothelial protein C receptor

(EPCR) upregulated on CMV-infected endothelial cells and epithelial tumor cells [68]. It was shown that when Vγ4Vδ5 T cells sense BTNL molecules, they do not get activated by EPCR [67].

In mice, γδ T cells are classified based on their capacity to produce IL17 or interferon-gamma (IFN-γ) [69]. In humans, IL17-producing γδ T cells are rare and they exhibit a pro-tumor activity [70, 71]. Recently, it has been discovered that amphiregulin (AREG) production serves as a more accurate distinction, equivalent to the classification in mice. γδ T cells expressing *AREG* are comparable to IL17-producing γδ T cells in mice, whereas those lacking *AREG* expression exhibit enhanced cytotoxic function, resembling IFN-γ-producing γδ T cells in mice [72].

γδ T cells comprise a heterogenous group of cells. Like αβ T cells, they can be categorized based on their function and response patterns. Vγ9Vδ2 T cells are often considered as innate lymphocytes and are known for their ability to kill target cells with high pAg-content without prior encounter [73]. Vδ2neg T cells are considered as adaptive-like T cells and demonstrate clonal expansions, resulting in the proliferation of more cytotoxic cells in response to various infections [74, 75].

Of particular interest is the fact that many viral infections associated with γδ T cell expansion coincide with HLA class I downregulation. For instance, Vδ1neg T cells expand in EBV infection while Vδ2neg T cells expand in HIV and CMV infections [32-34, 76, 77]. Furthermore, investigations reveal that even though the clones of Vδ1 T cells in HIV-seropositive (HIV^+) patients are more restricted, there was no difference in Vδ1 T cells' CDR3 of HIV^+ patients and healthy donors, suggesting a different clonal selection and expansion to αβ T cells [78]. This observation hints at the involvement of factors beyond TCR reaggregation within lymph nodes in driving the expansion of specific clones. Consequently, the expansion of Vδ1 T cells in response

to HIV infection may involves mechanisms distinct from the conventional TCR-mediated clonal expansion observed in αβ T cells.

To gain a better understanding of the role of γδ T cells, it is essential to explore their functional and cytotoxic responses, as well as the diversity of receptors that equip these cells.

1.3 NK cell receptors of γδ T cells

While most γδ T cells harbor both activating and inhibitory Natural killer cell (NK cell) receptors, the majority of studies elucidating the functions of these receptors have been focused on NK cells, thus justifying their nomenclature.

1.3.1 Inhibitory receptors

The discrimination between self and non-self-cells is possible by the expression of classical (HLA-A, -B and -C) and non-classical (HLA-E, -G) HLA class I molecules on all healthy cells in the human body. Failure of the antigen presentation due to the HLA class I downregulation disrupts the antigen recognition by αβ T cells, serving as an immune evasion strategy employed by many viruses and cancers [79, 80]. Up to 90% of human tumors have HLA class I downregulation, correlating with a poorer prognosis [80]. NK cell inhibitory receptors can detect the loss of HLA class I, which in turn activates NK cells to kill unhealthy cells. While this role has never been shown in γδ T cell biology, inhibitory receptors for HLA class I are also expressed by most γδ T cells [81, 82]. Of these receptors, Killer cell immunoglobulin-like receptors (KIRs) and leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1) are single-unit type I

glycoproteins, within the immunoglobulin superfamily. On the other hand, CD94/NKG2A receptors are type II glycoproteins with a C-type lectin-like scaffold [83].

KIRs are among the most polymorphic receptors, characterized by 15 genes and numerous allelic types, contributing to extensive diversity within human populations [84]. Thus, both KIRs and HLAs confer individuality to human populations. Predominantly expressed on NK cells and subsets of T cells, KIRs are pivotal in immune regulation. They are mainly found in humans and some non-human primates [85]. Structurally, KIRs are divided into two main groups: KIR2D and KIR3D, differing in the number of extracellular immunoglobulin-like domains. Specifically, KIR2D receptors possess two extracellular domains, while KIR3D receptors have three. Within these groups, the presence of a long cytoplasmic tail designates inhibitory KIRs, denoted as KIR2DL or KIR3DL. The inhibitory functions are mediated by immunoreceptor tyrosine-based inhibition motifs (ITIM). Conversely, KIRs with a short cytoplasmic tail, designated as KIR2DS or KIR3DS, often engage activating pathways via immunoreceptor tyrosine-based activation motifs (ITAM). An exception to this pattern is KIR2DL4, which lacks inhibitory function and instead, through its long cytoplasmic tail, facilitates a unique secretory response from endosomes [86]. Notably, KIR2DL4 does not induce cytotoxicity, but rather recognizes the non-classical HLA-G [87]. While most KIRs recognize epitopes of HLA-A, -B, and -C, KIR3DS1 stands out by recognizing the unclassical HLA-F [88]. The involvement of KIRs extends to various physiological and pathological conditions, including pregnancy, autoimmune diseases, infectious diseases, and cancer [89].

LILRB1, also called ILT2, MIR7, CD85j, or LIR1, belongs to the family of leukocyte immunoglobulin-like receptors (LIRs), also known as immunoglobulin-like transcripts (ILTs). These receptors demonstrate a limited genetic diversity and are found on various immune cells,

including neutrophils, basophils, eosinophils, monocytes, macrophages, dendritic cells, progenitor mast cells, osteoclasts, NK cells, B cells and T cells [90]. LILRB1 specifically recognizes molecules located on β2M and the non-polymorphic α3 domain of the heavy chain within HLA class I. It has also been shown to recognize UL18, a CMV protein homologous to HLA class I expressed by CMV-infected cells [91].

NKG2A, in conjunction with non-signaling CD94, forms a complex that belongs to the NKG2 family of receptors. This complex specifically recognizes the non-classical HLA class I molecule, HLA-E. CD94/NKG2A is predominantly expressed on the surfaces of NK cells and T cells [89].

Similar to NK cells, inhibitory receptors for HLA may regulate the lysosomal compartment in γδ T cells [92], potentially enhancing γδ T cell immune responses to viral infections and cancers. Thus, the expression of these receptors on γδ T cells could make the latter capable of recognizing "missing-self" (the absence of HLA class I on abnormal cells) or "non-self" (*e.g.* HLA on allogeneic cells from transplants). Further investigations are required to validate this hypothesis.

1.3.2 Activating receptors

Activating receptors can also be structurally divided into lectin-like or immunoglobulin-like groups. NKG2D, NKG2C, CD16 and NKp80 belong to the lectin-like group, while NCRs (natural cytotoxicity receptors) are part of the immunoglobulin-like group [93].

NKG2D belongs to the NKG2 family of activating receptors. Through intracellular signaling mediated by the association with adaptor proteins, DAP10 or DAP12, NKG2D triggers cytokine production and the release of perforin and granzyme B, facilitating cytotoxic responses. The ligands for NKG2D include MICA, MICB and ULBPs [94].

NKG2C receptor, also part of the NK2G family, interacts with the non-classical HLA-E molecule [95, 96], similar to its inhibitory counterpart, NKG2A. The interaction of NKG2C with HLA-E, although with a lower affinity compared to NKG2A [97], can activate NK cells, particularly in the presence of antigenic peptides. NKG2C, akin to NKG2A, associates with CD94; however, unlike NKG2A, it transmits activation signals through the DAP-12 adaptor molecule. Variations in the NKG2C gene, such as deletions, impact susceptibility to viral infections like CMV [94].

CD16, also known as FcγRIII, is a transmembrane glycoprotein receptor that recognizes the Fc region of immunoglobulin G (IgG) antibodies [98]. It is expressed on the surface of NK cells, monocytes, macrophages, and some T cells [99]. Upon binding to IgG-coated target cells, CD16 triggers various immune effector functions, including antibody-dependent cellular cytotoxicity (ADCC) and phagocytosis [99]. CD16 has two isoforms; CD16A, contains an ITAM in its cytoplasmic tail, enabling it to transmit activating signals upon ligand binding. In contrast, CD16B primarily serves as a low-affinity receptor for IgG and lacks ITAM motifs [100]. The activation of CD16-mediated signaling cascades leads to lysis of target cells or phagocytosis. Additionally, CD16 engagement stimulates the production of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α) and IFN-γ, further promoting the immune response [101].

The NCRs belong to the family of activating receptors expressed on the surface of NK cells and some T cell subsets, playing a crucial role in recognizing and eliminating infected or transformed cells. The three main members of the NCR family are NKp46 (NCR1, CD335), NKp44 (NCR2, CD336), and NKp30 (NCR3, CD337). NKp46, constitutively expressed on all NK cells, is pivotal in NK cell-mediated cytotoxicity against various targets, including virus-infected cells and tumors. While NKp44 and NKp30 have more restricted expression and are induced upon

NK cell activation, they also contribute to NK cell activation [101]. The ligands for NCRs are diverse, comprising both viral and cellular components. Although not fully characterized for all NCRs, several ligands have been identified. NKp46 recognizes soluble complement factor P (properdin) protein [102], microbial ligands like viral hemagglutinins (HA) [103], Vimentin [104], *Candida glabrata* Epa1, Epa6 and Epa7 adhesion molecules [105], PfEMP1 of *P. falciparum* [106], an unknown ligand from *Fusobacterium nucleatum* [107], as well as cellular ligands like heparan sulfate proteoglycans [108]. NKp44 interacts with viral HAs [109], platelet-derived growth factor receptor [110], proliferating cell nuclear antigen [110], mixed lineage leukemia-5 protein [111], and is activated by the viral protein hemagglutinin-neuraminidase from Newcastle disease virus [112, 113]. Similarly, NKp30 engages with various ligands present on target cells, including B7-H6 [114], BAT3/BAG6 protein complex [115], and PfEMP1 of *P. falciparum* [106].

NKp80 binds to activation-induced C-type lectin, which is expressed on activated T cells and B cells, as well as on some tumor cells [116]. This receptor has previously been introduced as a marker exclusively expressed by mature NK cells [117].

2B4 (CD244) is a complex receptor that exhibits both activating and inhibitory functions in NK cells and T cells. Unlike other immune cell receptors that recognize HLA class I molecules, CD244 interacts with CD48, a surface molecule expressed on various cell types. The dual role of CD244 is hypothesized to be dependent on the presence or absence of the adaptor protein SAP (SLAM-associated protein). In the presence of SAP, CD244 engagement can lead to activating signals. In the absence or dysfunction of SAP, CD244 may predominantly transmit inhibitory signals, attenuating immune cell activity [118]. Dysregulation of CD244 signaling, can lead to impaired immune control of viruses such as EBV [119].

The intricate balance between inhibitory and activating receptors is mainly studied in NK cells, which are capable to recognize the absence of HLA class I on abnormal cells (missing-self) (**Fig. 2**). However, whether γδ T cells are also capable of missing-self recognition is still unknown.

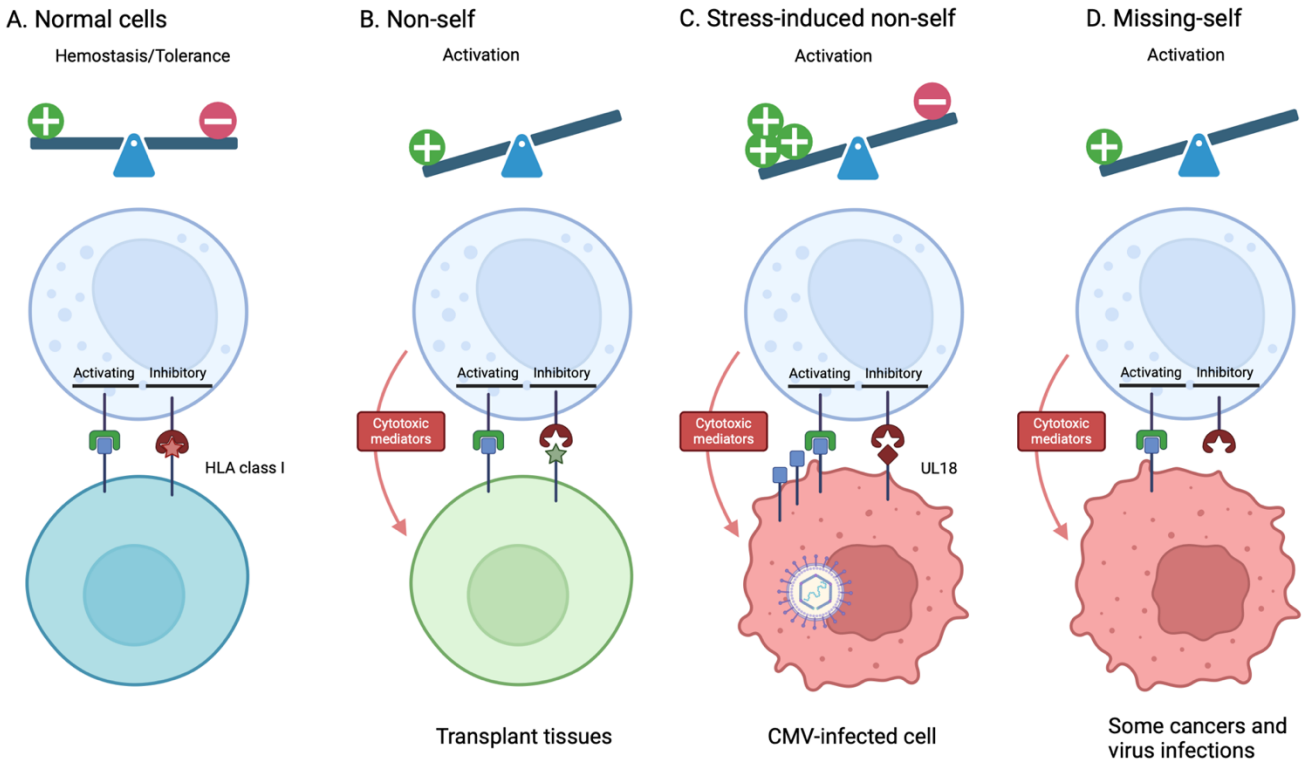


Figure 2: The intricate balance of activating and inhibitory signals involved in the activation of NK cells. (**A**) In healthy autologous cells, the overall level of inhibitory receptor signaling outweighs activating receptor signaling, resulting in tolerance to target cells. When NK cells cannot detect self HLA class I (non-self) (**B**) or detect an upregulation of stimulatory ligands for activating receptors, even in the presence of UL18, a protein structurally similar to HLA produced by CMV (non-self stress-induced) (**C**), the loss of inhibitory signals leads to strong positive signals and cell activation. Similarly, when NK cells cannot recognize HLA class I ligands on transformed cells (missing-self) (**D**), the absence of inhibitory signals leads to strong positive signals and cell activation.

1.4 Functional diversity of γδ T cells

γδ T cells can initiate a cell-mediated immune response by orchestrating the activation of other immune cells (indirect response) or acting on target cells (direct response) (**Fig. 3**).

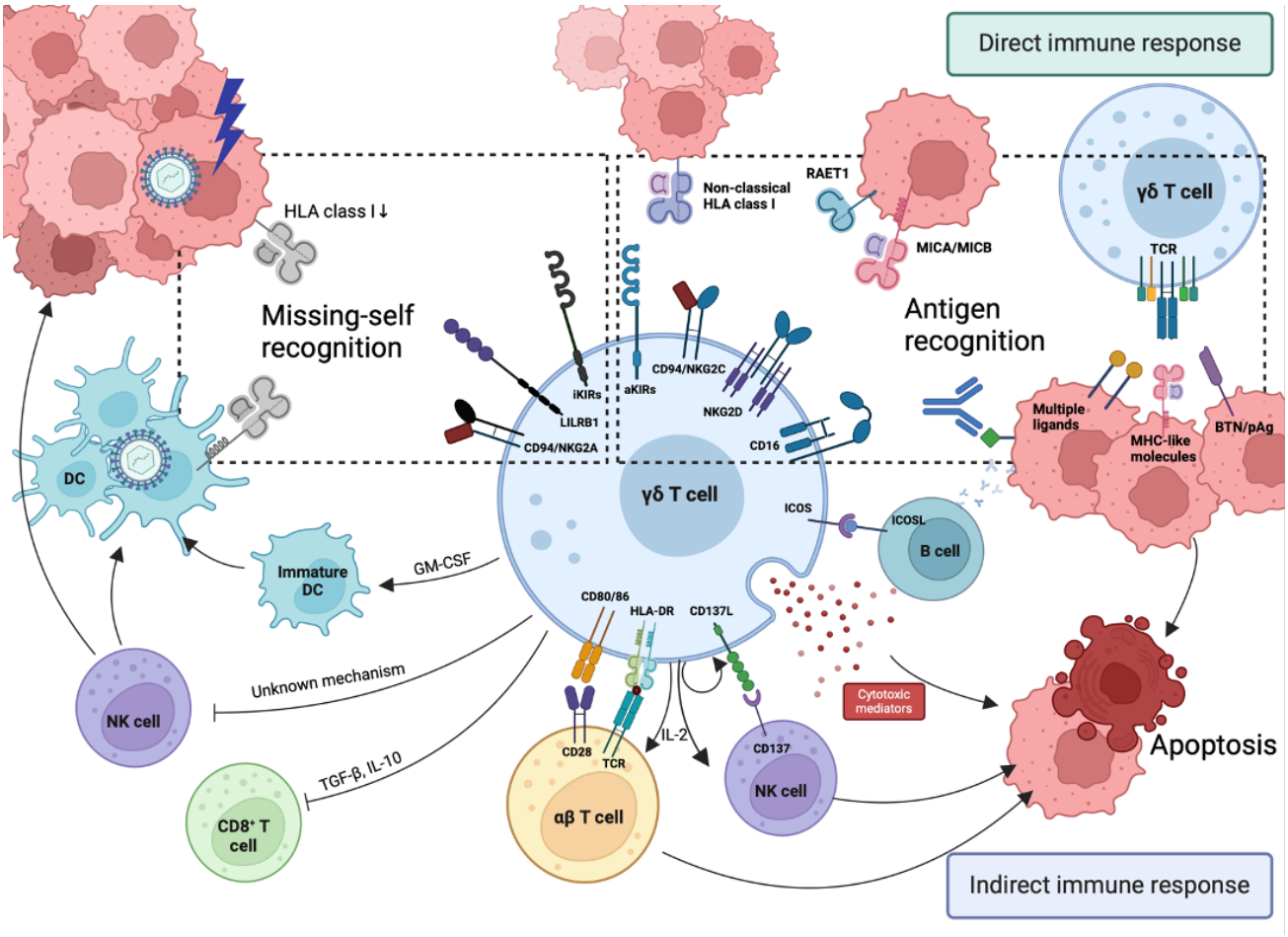


Figure 3: Summary of γδ T cell functions. γδ T cells exhibit remarkable functional plasticity, capable of eliciting indirect immune responses by modulating other immune cells, as well as directly targeting transformed cells.

1.4.1 Indirect immune response

Vγ9Vδ2 T cells have shown features of professional antigen-presenting cells (APC) similar to dendritic cells [120]. Furthermore, Zoledronate-activated Vγ9Vδ2 T cells have the capacity to phagocyte antigens. This was evidenced by the expression of the scavenger receptor CD36, HLA class II, and adhesion molecules CD80, CD86, CD54, and CD40. [121]. These immune cells can induce proliferation and differentiation of naïve $CD4^+$ αβ T cells, into cytotoxic T lymphocytes (CTLs) [122]. Moreover, activated Vγ9Vδ2 T cells have also been shown to promote B cell immunoglobin secretion, by exerting the phenotype and function of follicular B-helper T cells [123]. Like Vγ9Vδ2 T cells, Vδ3 T cells have also been shown to modulate immune responses of other immune cells, as phytohemagglutinin-expanded Vδ3 T cells have been found to promote the release of IgM by B cells [124]. Additionally, several studies have shown that Vγ9Vδ2 T cells can boost DC maturation through cytokine secretion and cell to cell interactions [125, 126]. Activated Vγ9Vδ2 T cells exhibit a dual role in modulating NK cell activity. Walwyn-Brown *et al.* demonstrated that activated Vγ9Vδ2 T cells can inhibit NK cell recognition of missing-self through an unknown mechanism [127]. Conversely, Maniar *et al.* revealed that engagement of CD137 significantly enhances NK cell-mediated antitumor cytotoxicity [128]. Alternatively, tissue-resident Vδ1 subsets exhibit regulatory functions akin to conventional regulatory T cells (Tregs), producing suppressive cytokines such as IL-10 and TGF-β [129].

1.4.2 Direct immune response

γδ T cells, with their HLA-unrestricted recognition, employ a sophisticated mechanism to prevent autoimmune reactions and mount a robust response against abnormal cells. Through their TCR, they are speculated to recognize various changes in their cognate antigens, including the upregulation (e.g., Annexin A2) [130], downregulation (e.g., BTNL molecules recognized by Vγ4Vδ5) [67], conformational changes (e.g., BTN3A1 and BTN2A1 recognized by Vγ9Vδ2)[131] and multimerization (e.g., HLA*24:02 recognized by Vγ5Vδ1) of antigens [22, 67]. Furthermore, the binding site of the γδ TCR to its ligand could influence the degree of activation. For instance, the interaction of Vδ1 TCR with the α3-domain on the side of MR-1 leads to ERK phosphorylation with no upregulation of the activation marker CD69, while binding from the top to the actual antigen-presenting cleft of MR-1 induces full activation of the employed reporter cells [45, 67].

1.5 γδ T cell immune response to infections

γδ T cells play a crucial role in various infections, many of which pose a significant health burden worldwide, leading to acute, chronic, or even lethal outcomes. Such infections include bacteria like *M. tuberculosis* [132], protozoa like *P. falciparum*[133], and numerous viruses like influenza viruses [134], HIV [135], EBV [136], and CMV [137].

1.5.1 γδ T cell immune responses to bacteria and protozoa

M. tuberculosis: The intracellular bacterium responsible for tuberculosis, is a protozoan that primarily infects the lungs but also other organs like kidneys, spine, and brain. Tuberculosis is a leading cause of death worldwide, surpassing HIV/AIDS as the deadliest single infectious

agent. [138]. Vγ9Vδ2 T cells respond to the primary infection through recognition of pAgs in infected cells. In persistent *M. tuberculosis* infection, there is an expansion of KIR^+ $CD8^+$ γδ T cells with enhanced effector potential [132].

P. falciparum: The causative agent of malaria, poses a significant threat to public health, particularly in endemic regions such as Africa. It evades the immune system, especially T cell recognition, by infecting red blood cells, which do not express HLA class I molecules. Infections are particularly prevalent in children repeatedly exposed to the parasite, as they exhibit significant decrease in Vγ9Vδ2 T cells, which are crucial for immune defense [139]. The remaining Vγ9Vδ2 T cells display signs of exhaustion, including the expression of inhibitory coreceptors and reduced cytokine production [133]. Interestingly, repeated *P. falciparum* infections lead to a clonal expansion of $Vδ1^+$ γδ T cells with a heightened effector potential[140], as described previously for *M. tuberculosis*. Notably, the Vδ1 and Vδ3 T cells, which are predominant in the liver where *P. falciparum* resides during its lifecycle, display an activated phenotype, produce pro-inflammatory cytokines, and exhibit clonal focusing [141].

1.5.2 γδ T cells immune responses to viruses

Influenza virus: Despite global vaccination efforts, influenza viruses continue to present a significant burden to human and animal health. γδ T cells play a crucial role in controlling the infection [142]. They are shown to play a protective role against the H5N1 strain [143]. Recent research suggests that natural HA trimers of H5N1 can directly activate γδ T cells through sialic acid receptors on the surface of γδ T cells [143].

HIV: Vδ1 T cells are clonally expanded in the PB of viremic HIV^+ patients [82], which suggests a potential role of these cells in the immune response to the infection. These cells express

NKG2C and effectively recognize and kill HIV-infected CD4 T cells, which upregulate HLA-E, the ligand of NKG2C.

EBV: EBV is one of the most common viruses in humans. It infects B cells and epithelial cells, leading to a lifelong latent infection. EBV infection is associated with various diseases, including infectious mononucleosis [144], certain cancers [145, 146], such as Hodgkin's lymphoma, Burkitt's lymphoma [147], and nasopharyngeal carcinoma [148, 149], and autoimmune disorders, such as multiple sclerosis [150]. Vγ9Vδ2 T cells are shown to proliferate in response to B cells that maintain an EBV latency type I, such as Burkitt Lymphoma cells, but not to cells that maintain type III EBV latency [136].

CMV: CMV is one of the most prevalent viral infections worldwide, causing a lifelong infection [151]. This virus shows a sophisticated interplay with the host immune system, which has evolved with the first vertebrates [152]. It employs various strategies to evade immunosurveillance. One of these strategies is the downregulation of HLA class I molecules, which disrupts antigen presentation. Additionally, it stimulates inhibitory pathways by increasing the expression of non-classical HLA class I molecules, such as HLA-E, which serves as a ligand for inhibitory receptors like CD94/NKG2A. Also, CMV downregulates activating ligands, such as ULBP 1-4. This event prevents the interaction of the NKG2D activating receptor with these ligands [153, 154]. However, most people with CMV remain asymptomatic during their life suggesting a robust immune response to CMV infection.

Indeed, in these viral infections, both NK cells and γδ T cells undergo clonal expansions, leading to the formation of memory-like subsets with enhanced cytotoxicity. These NK cell subsets in CMV infection are distinguished by the co-expression of NKG2C and KIRs [155]. Harnessing the potential of these NK cells has shown promising results in preclinical studies for

immunotherapy [156]. One proposed mechanism for this increased cytotoxicity can be the unique accumulation of granzyme B in dense-core secretory lysosomes, near the centrosome, in KIR^+ cells, facilitating a rapid and stronger response [157]. However, the specific characteristics of memory-like subsets within γδ T cells, including the expression patterns of receptors such as KIRs are still not fully understood.

1.6 γδ T cell-based clinical studies

γδ T cells hold substantial promise for cancer immunotherapy. In a published extensive transcriptomic analysis spanning 18,000 human tumors across 39 cancer types, γδ T cells emerged as a prominent immune cell associated with favorable prognosis[158]. Despite this potential, clinical trials involving γδ T cells have thus far demonstrated only moderate efficacy.

Over 48 γδ T cell-based clinical trials have been registered on *ClinicalTrials.gov*, with the majority focusing on Vγ9Vδ2 T cells. Notably, only two trials have initiated treatments using allogeneic Vδ1 T cells for hematological malignancies. A significant portion of γδ T cell-based treatments is being conducted in China and the United States. The main focus on Vγ9Vδ2 T cell subset only could be a limiting factor for the efficacy of these clinical trials. A recent meta-analysis conducted by Ma Ling et al. (2023), revealed an objective response rate (comprising complete response and partial response) of 18% [159]. The analysis involved a total of 307 patients across 27 cohorts, irrespective of the treatment strategy employed [159].

This current landscape of γδ T cell-based immunotherapy highlights the need for further research and optimization to enhance its clinical efficacy.

1.7 Rationale, hypothesis and objectives

Most of the existing research on γδ T cells has been centered around the Vγ9Vδ2 T cell subset, the TCR recognition mechanisms and the ongoing quest to identify ligands for γδ TCRs. Additional work on other γδ T cell subsets is needed. Furthermore, while CMV is recognized as an immunodominant virus shaping the human immune system, its specific influence on γδ T cells in healthy individuals requires further investigations. My hypothesis proposes that, similar to NK cells, memory-like subsets of γδ T cells expressing KIRs could serve as a hallmark of γδ T cell functional maturation. CMV is expected to play a contributory role in this maturation process. I tested this hypothesis through the following objectives:

Objective 1. Defining the effect of CMV on the γδ T cell repertoire

Objective 2. Investigating the effector potential of KIR^{+}γδ T cells.

Objective 3. Characterizing and comparing the epigenetics of KIR^{-} and KIR^{+}γδ T cell subsets.

Objective 4. Expansion and subsequent characterization of KIR-enriched polyclonal γδ T Cells.

Chapter 2: Materials and Methods

2.1 PBMC isolation

Blood samples were taken from consenting healthy donors recruited by Dr. Jonathan Angel's laboratory at the Ottawa Hospital Research Institute (Protocol approved by Children's Hospital of Eastern Ontario Research Ethics Board; REB Protocol No: 22/98X). Heparinized blood samples were spun down in a centrifuge (Beckman Avanti J20) at 750g for 10 minutes. After the formation of three layers comprised of plasma, a ring of leukocytes, and erythrocyte, the plasma was collected and kept at -20ºC for further immunological assays. The leukocytes were harvested and then resuspended to one-third in PBS. In a separate tube, one volume unit of Ficoll solution (Sigma-Aldrich) was added. Then, two volume units of diluted leukocytes were added through the sides of the tube such that the leukocytes overlaid the Ficoll solution. The tubes were then centrifuged for 30 minutes at 1250g with minimum acceleration and deceleration speed at room temperature. The Peripheral blood mononuclear cells (PBMC) were resuspended in PBS and washed twice. Isolated PBMCs were stored in cryogenic vials overnight at -80°C, in fetal bovine serum (FBS) (Millipore Sigma) supplemented with 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich). Within 24-48 hours, the cells were transferred into liquid nitrogen.

2.2 Detection of serological CMV status

CMV serological status was determined using the CMV IgG ELISA kit from Calbiotech following the manufacturer's instructions on thawed plasma. A BioTek Synergy HTX multimode reader was used to read absorbance at 450 nm with a reference filter of 650 nm.

2.3 Depletion of αβ T cells from PBMCs

PBMCs were thawed and incubated at 37°C with 5% CO_2 overnight. Given that γδ T cells account for only 5% of T cells, αβ T cells were depleted from the PBMCs, using the EasySepTM Human Alpha/Beta Depletion kit (STEMCELL Technologies) according to the manufacture's protocol. Not only was the PBMC culture enriched with γδ T cells by this approach, but it also allowed a comparison between γδ T cells and NK cells. The αβ T cell depleted (αβ T-depleted) PBMCs were obtained and used for subsequent experiments.

2.4 Assessing the ADCC capability of γδ T cells

Raji cells (ATCC CCL86) were coated with 20 μg/ml Rituximab (kindly provided by Dr. Seung-Hwan Lee), an FDA-approved antibody against CD20. Raji cells were then incubated for 30 minutes at 4°C. After the incubation period, Raji cells were washed by removing the supernatant following centrifugation at 530g for 5 minutes. Rituximab-coated Raji cells were then co-cultured for 6 hours with αβ T-depleted PBMC at the ratio of 10:1 effector-to-target cells (E: T), in RPMI supplemented with 10% FBS and 200 IU/ml IL2 (R&D Systems), in round-bottomed 96-well plate. For assessing the degranulation level, 96 well-plates were supplemented with anti-CD107a-PE (REA792, Miltenyi). Brefeldin A (BioLegend) and Monensin (BioLegend) were added to each well after a one-hour incubation, followed by remaining incubation time. After the incubation period, cells were processed for antibody staining and flow cytometry.

2.5 PMA/Ionomycin stimulation assay

αβ T-depleted PBMCs were treated for 4 hours with PMA/Ionomycin (BioLegend). Since γδ T cells were too sensitive to the stimulation with the 2μMof PMA/Ionomycin recommended by the manufacturer, the reagent's concentration was reduced to 0.05uM (**Fig. S1**). Degranulation levels were assessed as described in section 2.4. After the incubation period, cells were processed for antibody staining and subsequent analysis via flow cytometry.

2.6 Antibody staining and flow cytometry

Antibody staining was carried out in v-bottom 96-well plates, with washes between each step performed using 1X PBS. Freshly thawed αβ T-depleted PBMCs or cultured cells were labeled with Ghost dye red 710 (Sigma-Aldrich) or Zombie Aqua Fixable Viability Kit (BioLegend) to assess cell viability, following the manufacturer's protocol, for 20 minutes at room temperature in the dark. Subsequently, FC block (BioLegend) was added according to the manufacturer's instructions to minimize nonspecific binding, and the cells were incubated for 10 minutes at room temperature in the dark. Surface marker staining was then performed using specific antibodies in FACS buffer containing 1% bovine serum albumin (Avantor), as detailed in Table 1. Additionally, a monocyte blocker (BioLegend) was included in the staining cocktail. The cells were incubated with the antibody mixture for 30 minutes at 4°C in the dark. Following surface staining, excess antibodies were removed by washing the cells with 1X PBS.

For intracellular staining, cells were fixed overnight using the fixation buffer (Life Technologies eBioscience™ Intracellular Fixation & Permeabilization Buffer Set) at 4°C,

protected from light. The next day, cells were permeabilized and stained according to the manufacturer's protocol.

After completion of all staining steps, cells were resuspended and fixed in PBS containing 1% PFA. Subsequently, cells were analyzed using spectral flow cytometry (Flow Cytometry and Virometry Core Facility of the University of Ottawa). Data (density plots, histograms and tSNE plots) were generated with FlowJo 10.8.1 software (TreeStar).

Table 1: List of monoclonal antibodies used in flow cytometry.

	Marker	Clone	Supplier
1	CD3	OKT3	BioLegend
2	CD56	5.1H11	BioLegend
3	TCR V delta 1	REA173	Miltenyi
4	TCR V delta 2	123R3	Miltenyi
5	TCR V gamma 9	B3	BioLegend
6	KIR2D	REA1042	Miltenyi
7	CD158e1/e2(KIR3D)	REA168	Miltenyi
8	CD19	HIB19	BioLegend
9	CD57	REA769	Miltenyi
10	CD16	REA423	Miltenyi
11	CD45RA	HI100	BioLegend
12	CD27	L128	BD Biosciences
13	LILRB1 (CD85j)	HP-F1	Thermo Fisher Scientific
14	CD244	C1.7	BioLegend
15	CD14	M5E2	BioLegend
16	PD-1	A17188B	BioLegend
17	Granzyme B	REA226	Miltenyi
18	Perforin	dG9	BioLegend
19	IFN-γ	REA600	Miltenyi
20	TNF-α	REA656	Miltenyi
21	CD107a	REA792	Miltenyi

2.7 Preparation of ATAC sequencing libraries

To assess the epigenetic features of γδ T cells, based on KIR expression, and compare these features to the well published epigenetic signatures of naïve and adaptive αβ T cells [160], ATAC (Assay for Transposase-Accessible Chromatin) libraries were prepared in collaboration with Dr. Alexander Blais. For this purpose, PBMCs were thawed a day prior to experimentation to ensure optimal viability. Samples were selected from CMV^+ blood donors as these individuals have sufficient numbers of KIR^+ γδ T cells. Following αβ T cell depletion, PBMCs were stained with anti-CD3 (OKT3; BioLegnd), anti-KIR2D (REA168; Miltenyi), anti-KIR3D (REA1042; Miltenyi), and Zombie Aqua viability dye, as previously described. 25,000 KIR^+ and 25,000 KIR^- γδ T cells were sorted into FBS using a Sony SH800 instrument (Flow Cytometry & Virometry Core Facility).

ATAC sequencing libraries were prepared according to the protocol by Corces *et al.*'s (2017) [161], involving cell lysis, transposition, purification, amplification, size selection and DNA quality assessment. DNA quality assessment consisted of evaluating amplification efficiency and fragment size distribution using gene-specific qPCR primer sets targeting closed and open chromatin loci. These primers were designed based on the result of McMurray *et al.*'s (2022) study [162]. Primers were designed for specific target genes using Primer-BLAST designing tool. The primer sequences used in this study, ordered from ThermoFisher scientific, are as detailed in the following table (**Table 2**).

Table 2: List of primer sequences used for qPCR

	Target	Forward primer	Reverse primer
1	Granzyme B	CCCAAGCCTATGAAGTGAATTG	GTTCTATCACTGGGAACTCAGG
2	EOMES	CTAGGCCTCAGAATGGAAAG	GGCGGGAAGAAATCGTAACC
3	STAT4	ACAAAATTACCTAACACCTCAGTC	GCAGTTTCTCCAGTTTGCTC
4	Granzyme H	TGTCCCCTTCTGGTTTTGATGG	ACAGTGCCATGTCAACTTAAACC
5	CX3CR1	CTCATTTCAACCATACCTCTCAC	AGACAGAGCTGCAATTACCC
6	Close loci 1	TGTTTGTGATTGGAATACGCCTG	GTGGATAGACTCTGGGGTTGAC
7	Close loci 2	TTGTCATTGGAACAAGGCTGTG	TGTGGCAGCGTAGCTCTC
8	Close loci 3	TTGGCCTATTGGAATTGCATCC	TGGCAATAGGGGTAAGCAGTC
9	Open loci 1	CAAGTGTGTTACTCACCGGG	ACTCCTCAACTCCCCACTTG
10	Open loci 2	TTTGGGCCTGTGTGGGTTAG	CAGACGTCGGAGTCCTAGTG
11	Open loci 3	AACGTGACTCTCTGTGGACG	GCTTTGACTCAGATGACGCAC

The qPCRs were done on all libraries. Genomic DNA and water were used as positive and negative controls, respectively. Phusion™ High-Fidelity DNA Polymerase and SYTO-13 DNA-intercalating dye (provided by Dr. Alexander Blais, ThermoFisher Scientific) were used according to the manufacturer's instructions. Amplification, for each primer, was conducted in 10 µL reaction and was performed in duplicates, using 96-well PCR plates. The amplification process was carried out using a CFX Opus 96 thermal cycler under the following cycling conditions: The temperature was held at 98.0°C for 30 seconds, at 98.0°C for 5 seconds, at 66.5°C for 15 seconds, and finally at 72.0°C for 10 seconds. The plates were read at the end of the 72.0°C period, and these steps were repeated for a total of 49 cycles. Subsequently, melting curve analysis was conducted over a temperature range from 65°C to 90°C. The 2^{-Ct} method was employed to estimate DNA amount, and the ratio of average open loci to closed loci was calculated for the validation of library preparation. Ratios exceeding 50 were indicative of a successful library preparation. Additionally,

Lib-Quant primer set (kindly provided by Dr. Alexander Blais) was used to estimate library concentration.

2.8 γδ T cell expansion

γδ T cells were negatively sorted from PBMCs using the STEMCELL Technologies γδ T cell sorting kit. A mixture of irradiated allogenic-PBMCs and K562 cells (ATCC CCL-243) was added to sorted γδ T cells and centrifuged at 530g for 10 minutes. The pellet was resuspended in OpTmizer containing 50 mg/mL penicillin/streptomycin, 10% FBS, 200 IU/mL IL-2, 50 ng/mL IL-15 (R&D Systems), and 150 ng/ml OKT3 (BioLegend). The cell suspension was transferred to 96-well round-bottom plate (400,000 cells/well) and incubated at 37°C with 5% CO2 for 2 weeks. Daily monitoring of the plates was performed by counting the cells with hemocytometer and Trypan blue solution (Sigma) and adding fresh OpTmizer medium containing 50 mg/mL penicillin/streptomycin, 10% FBS and 200 IU/ml IL2.

2.9 Statistical analyses

Comparisons of different values were conducted using the paired t-test within the same individual and the unpaired t-test between different individuals, with Prism software version 10.2.1s. Additionally, one-way and two-way ANOVA analyses were performed to further analyze the data, in order to assess the influence of multiple variables on the observed outcomes. Statistical significance was denoted by * for $p < 0.05$, ** for $p < 0.01$, *** for $p < 0.001$, and **** for $p < 0.0001$.

Chapter 3: Results

3.1 Objective One. Defining the effect of CMV on the γδ T cell repertoire.

3.1.1 Description of the study cohort

The sera of blood donors were immunoassayed for the presence of anti-CMV IgG, while demographic information including sex, age, and ethnicity was collected. Samples from a total of 20 donors were immunoprofiled using spectral flow cytometry and a panel of 16 antibodies. The cohort includes 10 CMV^- and 10 CMV^+ individuals, 15 females and 5 males, and different ages and ethnic groups (**Fig. 4**).

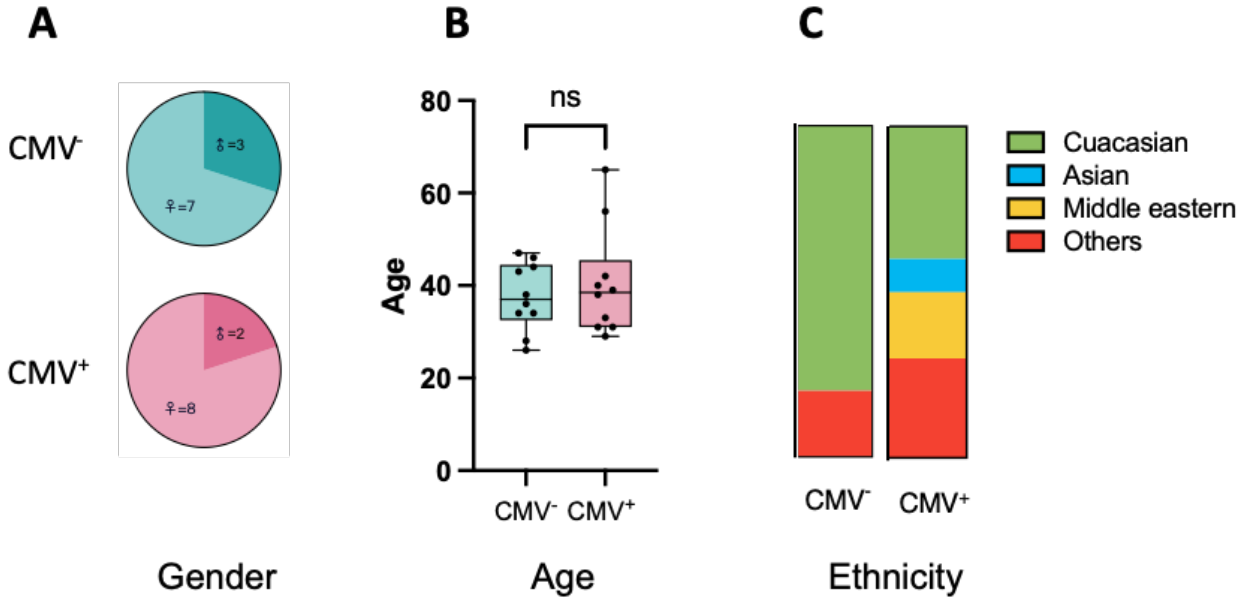


Figure 4: Cohort of healthy adult blood donors: Analysis of the demographic distribution within the study cohort of 20 individuals, including sex, age, ethnicity and CMV-status.

3.1.2 Enrichment in γδ T cells through αβ T cell depletion of PBMCs

γδ T cells constitute a minor subset of lymphocytes, comprising only ~0.5-5% of total PBMCs, which makes it challenging to obtain a sufficient number of cells for subsequent immunoprofiling assays. Thus, αβ T cells were depleted from PBMC samples. Through this targeted depletion, the proportion of γδ T cells is enhanced up to ~10-45%. This enrichment facilitates more robust flow cytometry analyses of all Vδ1, Vδ2, and Vδ1/2neg populations which is difficult to achieve in undepleted samples (**Fig. 5A, B**). It also facilitates a comparison of the findings to those pertaining to NK cells, since NK cells are still present in αβ T-depleted PBMCs and can serve as internal controls (**Fig. 5C**).

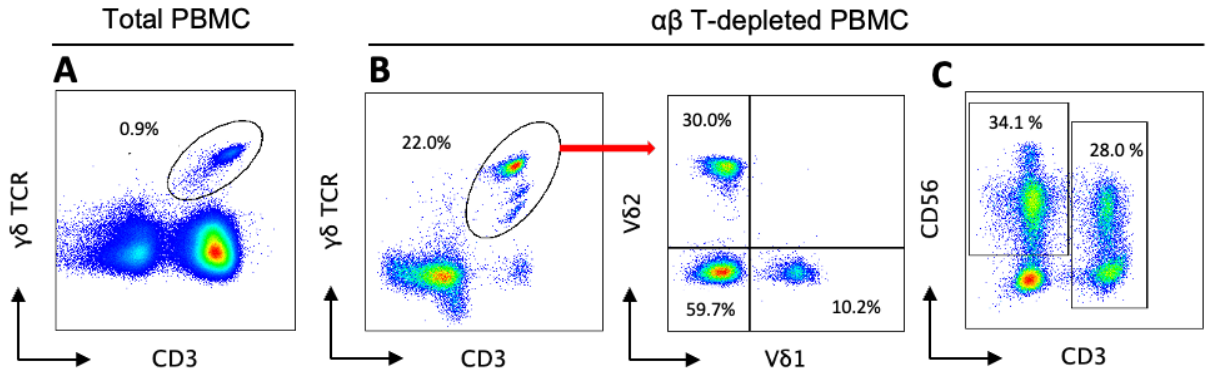


Figure 5: Enrichment of γδ T cells in αβ T-depleted PBMCs: Representative plots showing (**A**) γδ T cells in bulk PBMCs, (**B**) γδ T cells in αβ T-depleted PBMCs (left panel) and the three main subsets within total γδ T cells (right panel), (**C**) NK cells ($CD3^{-}CD56^{+}$) in αβ T-depleted PBMCs.

3.1.3 The Vδ2/Vδ1 γδ T cell ratio is low in CMV$^+$ individuals.

While Vδ2 T cells are typically considered as a dominant γδ T cell subset in PB of healthy adults [46], in CMV$^+$ individuals, a significant increase in the frequency of Vδ1 T cells was observed, resulting in Vδ2/Vδ1 ratio approaching 1 (**Fig. 6A-D**). The proportions of Vδ1/2neg populations are similar between CMV$^-$ and CMV$^+$ individuals. (**Fig. 6E**). Willcox *et al.* have reported a discrete Vγ9negVδ2 T cell population that expands during active CMV infection [57]. Although the difference is statistically not significant, a slight increase in the size of this cell subset is observed in CMV$^+$ individuals, compared to CMV$^-$ individuals (**Fig. 6F**). The gating strategy used to assess different populations is shown in **Fig. S2**. These findings challenge the prevailing belief regarding the predominance of Vδ2 T cells in the PB of healthy adults and demonstrate that Vδ1 T cells could be highly abundant in CMV$^+$ individuals.

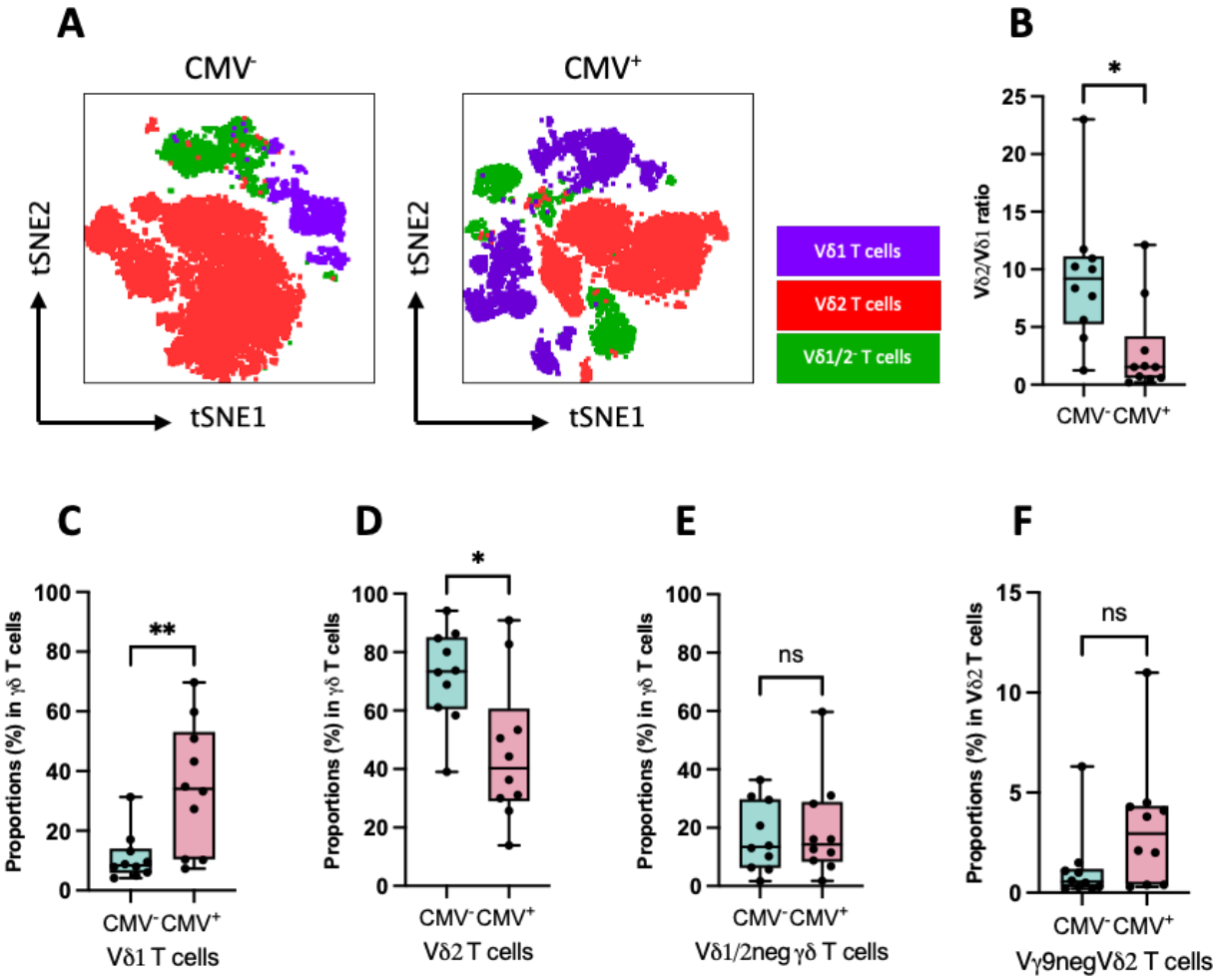


Figure 6: Vδ2/Vδ1 γδ T cell ratio decreases in CMV⁺ individuals. (**A**) tSNE plots from concatenated data showing γδ T cell subsets from CMV⁻ (*n=10*) and CMV⁺ (*n=10*) individuals within total γδ T cells. Graphs displaying (**B**) cell ratio of Vδ2 over Vδ1 γδ T cells, (**C**) proportions of Vδ1 γδ T cells, (**D**) proportions of Vδ2 γδ T cells, (**F**) proportions of Vδ1/2neg T cells in CMV⁻ and CMV⁺ individuals. Unpaired t-tests were performed to compare variables. Statistical significance denoted by asterisks: *= p<0.05, ** =p<0.01, ns = non-significant.

3.1.4 CMV$^+$ individuals have increased proportions of γδ T cells expressing KIRs.

There is a significantly higher proportion of KIR$^+$γδ T cells in CMV$^+$ individuals, compared to CMV$^-$ individuals (**Fig. 7A-D**). Notably, KIR$^+$Vδ1 T cells exhibit the highest proportions in CMV$^+$ individuals, with up to 70% of Vδ1 T cells expressing KIRs (**Fig. 7C, E**). There was no significant difference between the proportions of KIR$^+$Vδ2 T cells between CMV$^-$ and CMV$^+$ individuals (**Fig. 7F**). Finally, although the difference is not statistically significant, Vδ1/2neg T cells tend to express more KIRs in CMV$^+$ individuals (**Fig. 7G**). These findings suggest a role for KIRs in γδ T cell immunity to CMV, especially within the Vδ2neg compartment. This is consistent with previous findings showing that Vδ2neg γδ T cells play a more substantial role in the immunity against active CMV infection, compared to the Vδ2 population [163].

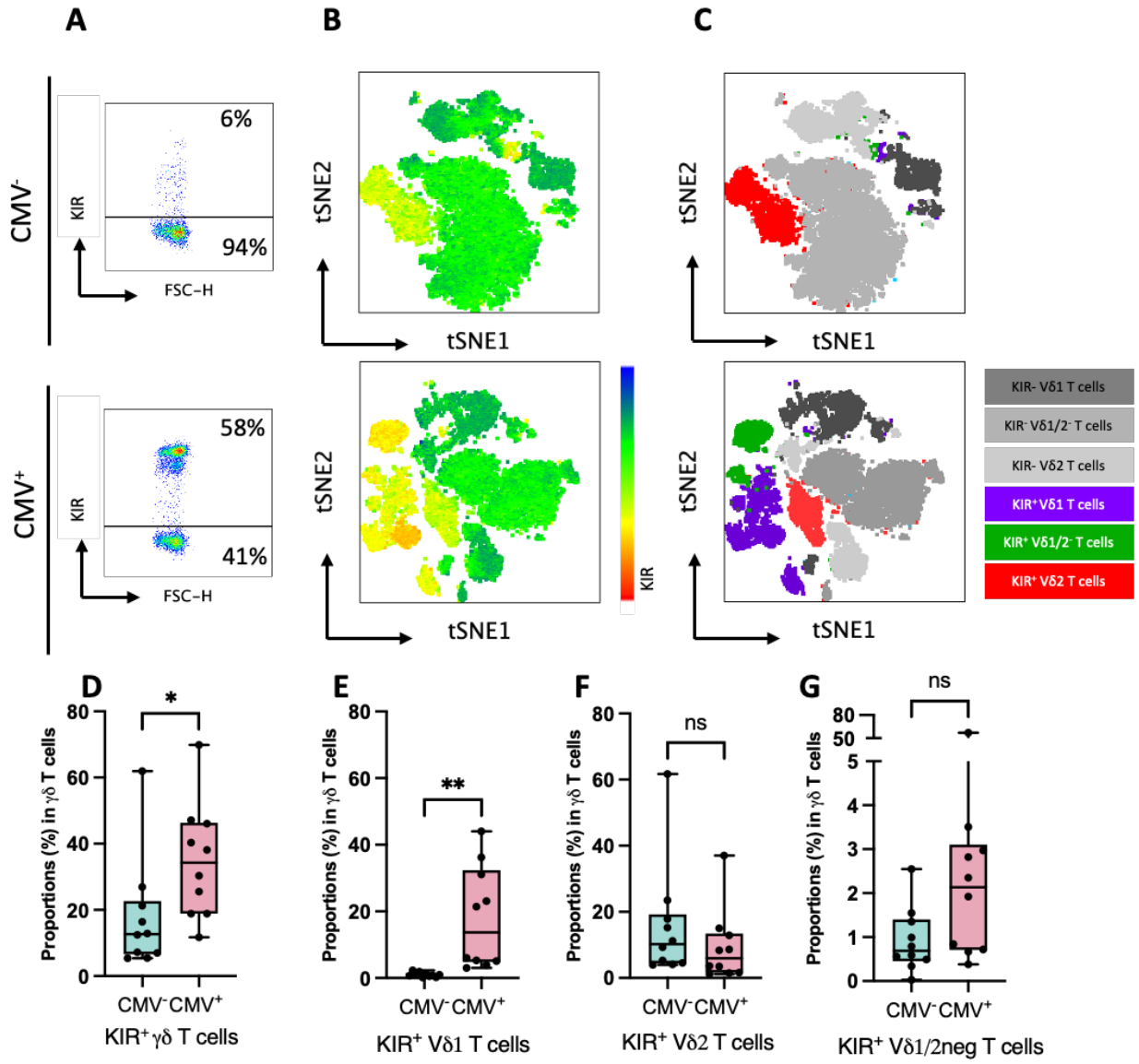


Figure 7: CMV⁺ individuals have higher proportions of KIR⁺γδ T cells in their blood. (**A**) Representative plots showing the proportion of KIR⁻ and KIR⁺ γδ T cell subsets within γδ T cells in CMV⁻ (top panel) and CMV⁺ (bottom panel). (**B**) tSNE plots from concatenated data showing KIR expression intensity on γδ T cells from CMV⁻ (*n=10*, top panel) and CMV⁺ (*n=10*, bottom panel) individuals. (**C**) tSNE plots from concatenated data showing KIR⁻ and KIR⁺ γδ T cell subsets within total γδ T cells. (**D-G**) Graphs showing proportions of KIR⁺γδ T cells (**D**), KIR⁺ Vδ1 T cells (**E**), KIR⁺ Vδ2 T cells (**F**), and KIR⁺ Vδ1/2neg T cells (**G**) in samples from CMV⁻ and CMV⁺ individuals. Unpaired t-tests were performed to compare variables. Statistical significance denoted by asterisks: *= p<0.05, ** =p<0.01, ns = non-significant.

3.1.5 KIRs delineate a dichotomy in the phenotype and function of γδ T cells.

A dichotomy in the γδ T cell phenotype associated with KIRs was observed, as most KIR^+γδ T cells exhibit a $CD27^-CD57^+CD45RA^{high}$ phenotype, whereas most KIR^- subsets are $CD27^+CD57^-CD45RA^{low}$ (**Fig. 8A-C**). This suggests an advanced stage of maturation in KIR^+γδ T cells and the establishment of a memory-like phenotype [164]. Although KIR^+γδ T cells are more prevalent in CMV^+ individuals, this dichotomy between KIR^+ and KIR^-γδ T cells persists across all samples, regardless of CMV status. Notably, only CD27 exhibits a more pronounced decrease in KIR^+γδ T cells from CMV^+ individuals compared to CMV^- individuals (**Fig. 8D-F**). These findings suggest that similar to NK cells [165], KIR acquisition marks an advanced stage of maturation in γδ T cells.

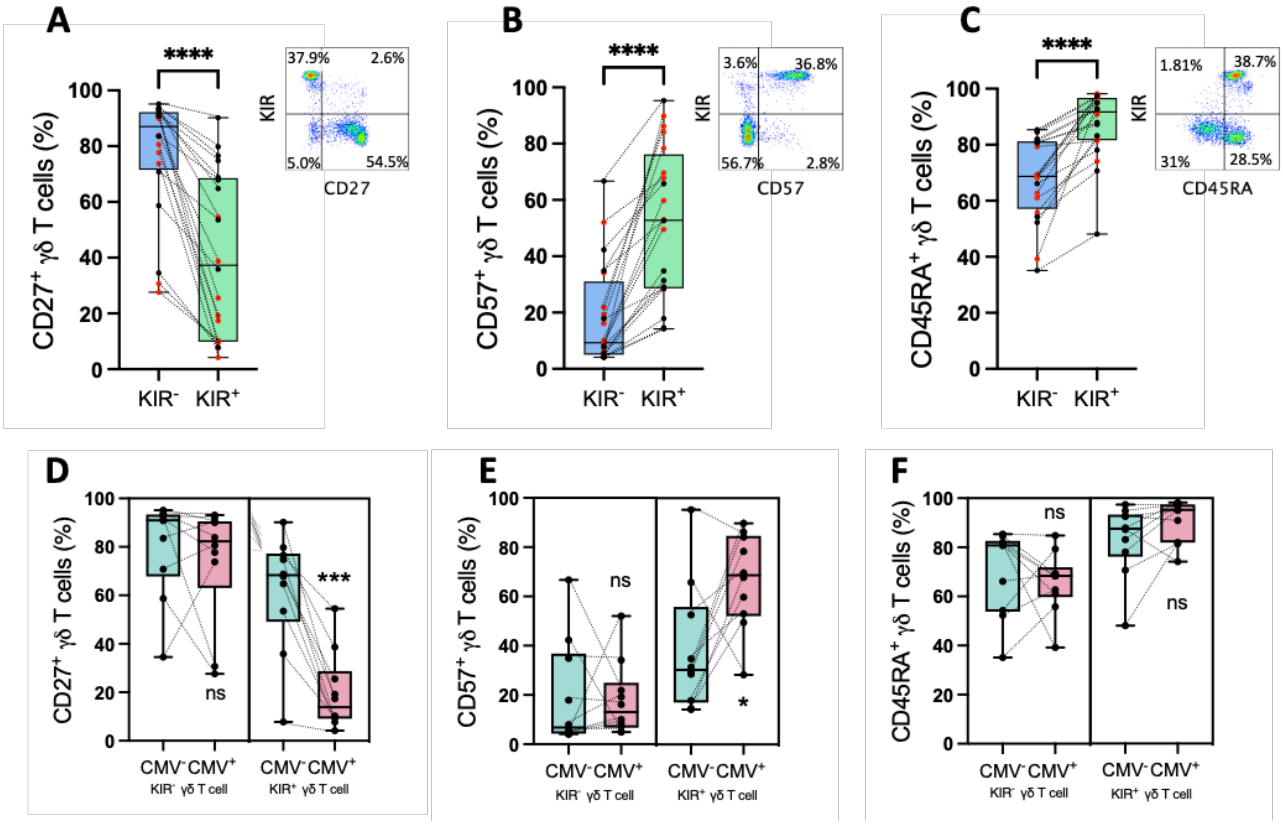


Figure 8: KIR$^+$γδ T cells exhibit a more mature phenotype compared to KIR$^-$ γδ T cells. (A-C) Graphs and representative plots showing the proportions of CD27$^+$(**A**), CD57$^+$(**B**) and CD45RA$^+$ (**C**) within KIR$^-$ and KIR$^+$γδ T cells (*n=20*; CMV$^-$= black dots, CMV$^+$= red dots). (**D-F**) Graphs comparing the proportions of CD27$^+$(**D**), CD57$^+$(**E**), CD45RA$^+$(**F**) in KIR$^-$ and KIR$^+$γδ T cells between CMV$^-$ (*n=10*) and CMV$^+$ (*n=10*) individuals. Paired t-tests were performed to compare variables. Statistical significance denoted by asterisks: *= p<0.05, **= p<0.01, ***= p<0.001, ****= p<0.0001, ns = non-significant.

3.1.6 KIR$^+$γδ T cells exhibit increased levels of granzyme B and perforin.

KIR$^+$γδ T cells exhibit higher expression levels of granzyme B and perforin across all individuals (**Fig. 9A-C**), consistent with their more differentiated phenotype (**Fig. 8**). While not statistically significant, the expression levels of granzyme B and perforin in KIR$^+$γδ T cells tend to be more pronounced in CMV$^+$ individuals (**Fig. 9D-F**). These findings suggest that KIR$^+$γδ T cells have more cytotoxic potential than their KIR$^-$ counterparts.

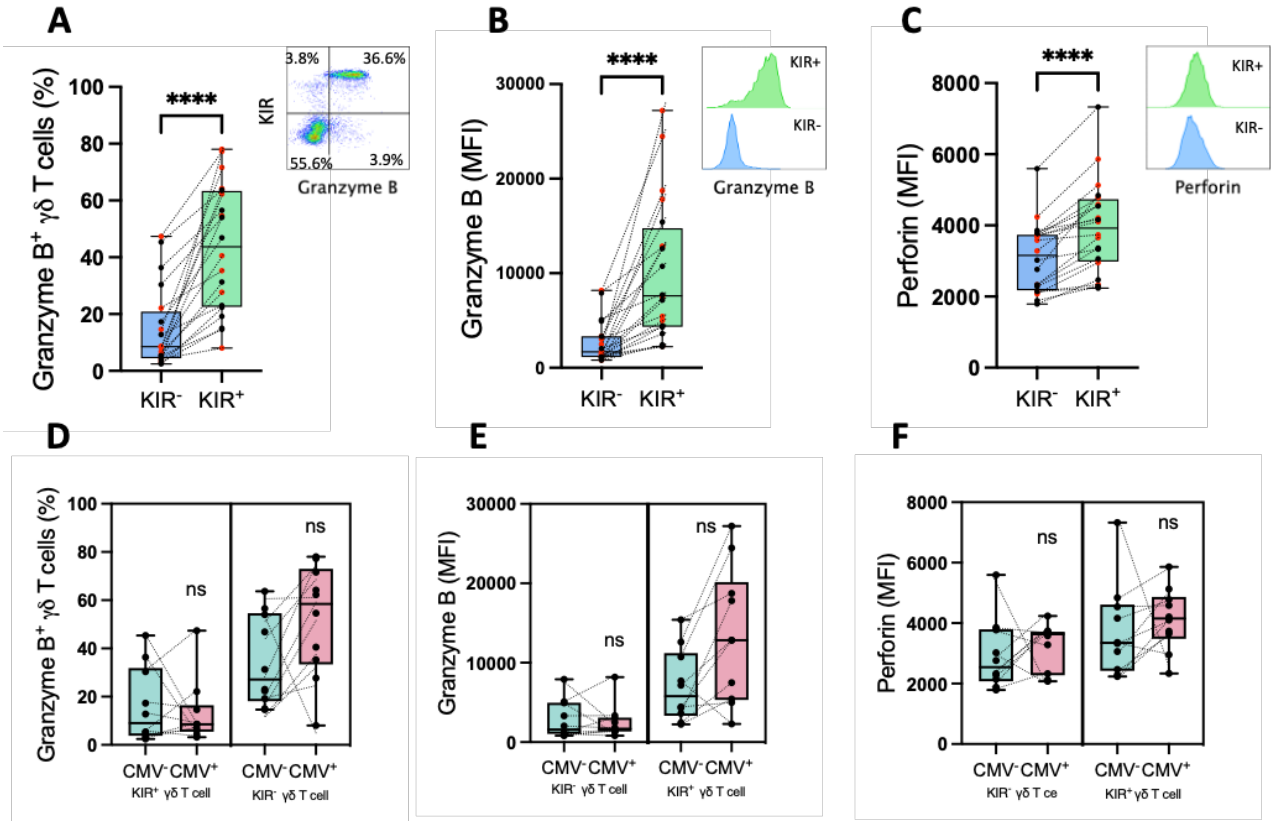


Figure 9: KIR$^+$γδ T cells have more stored cytotoxic mediators compared to KIR$^-$γδ T cells. (**A-C**) Graphs and representative displaying the proportion of granzyme B$^+$(**A**) and the mean fluorescence intensity (MFI) of granzyme B(**B**) and perforin(**C**) within KIR$^-$ and KIR$^+$γδ T cells (*n=20*; CMV$^-$= black dots, CMV$^+$= red dots). (**D-F**) Graphs comparing the proportion the proportion of granzyme B$^+$ (**D**) and the MFI of granzyme B(**E**) and perforin(**F**) in KIR$^-$ and KIR$^+$γδ T cells between CMV$^-$ (*n=10*) and CMV$^+$ (*n=10*) individuals. Paired t-tests were performed to compare variables. Statistical significance denoted by asterisks: *= p<0.05, **= p<0.01, ***= p<0.001, ****= p<0.0001, ns = non-significant

3.1.7 KIR$^+$γδ T cells express more NK cell receptors and exhibit a phenotype similar to that of NK cells.

KIR$^+$γδ T cells also express higher levels of other NK cell receptors, including CD244 (2B4), LILRB1, and CD16 (**Fig. 10A-C**). Notably, LILRB1 and CD16 expression is significantly more pronounced in CMV$^+$ individuals (**Fig. 10D-F**), suggesting a role for CMV in the observed phenotypic and functional differentiation of γδ T cells. Furthermore, my data shows that KIR$^+$ γδ T cells, especially KIR$^+$Vδ2neg γδ T cells, exhibit characteristics akin to NK cells. Notably, both populations express high levels of CD57 (**Fig. 11A**), granzyme B (**Fig. 11B**), CD244 (**Fig. 11C**), LILRB1 (**Fig. 11D**) and CD16 (**Fig. 11E**), while KIR$^-$γδ T cells do not exhibit this phenotype. Together, these findings suggest that CMV is associated with a differentiation of γδ T cells into KIR$^+$ cells, with enhanced capacity for natural cytotoxicity.

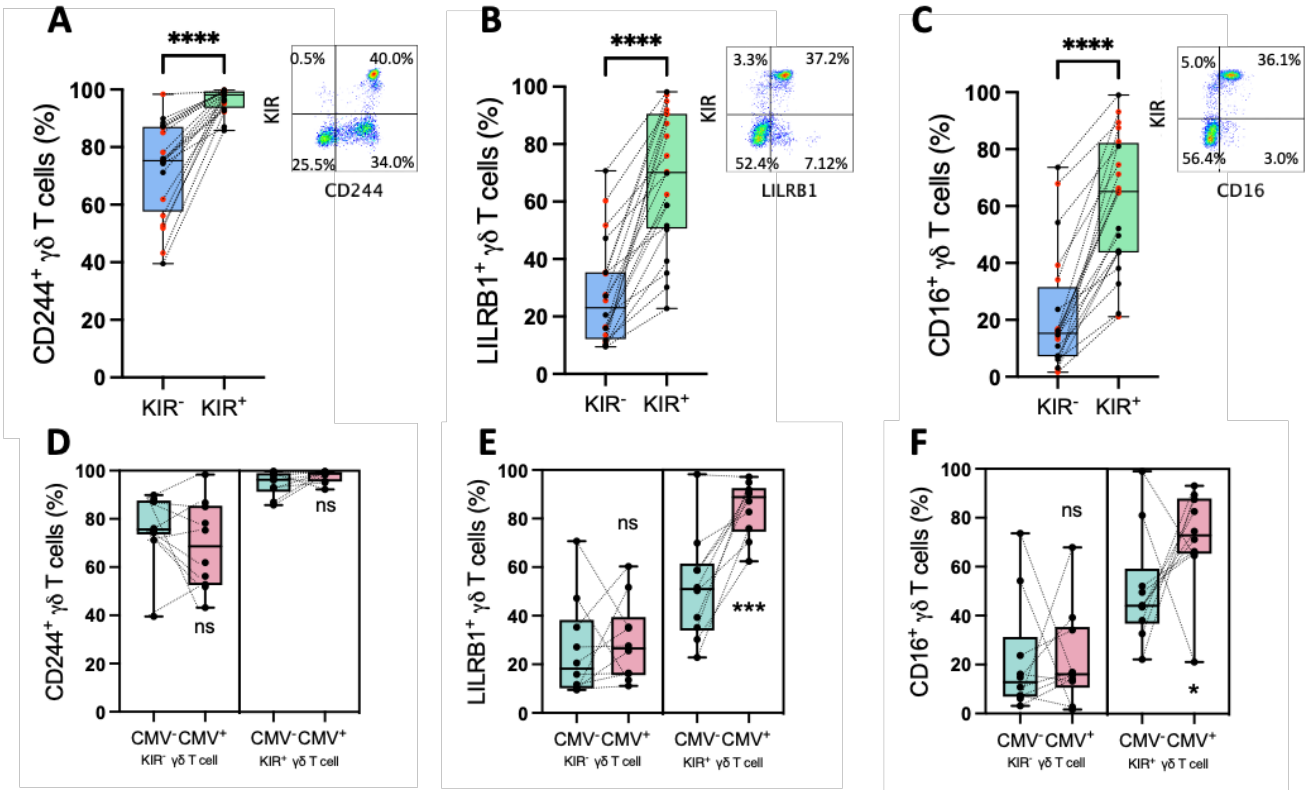


Figure 10: KIR$^+$γδ T cells are equipped with more innate receptors compared to KIR$^-$γδ T cells, especially in CMV$^+$ individuals. (A-C) Graphs and representative plots showing the proportions of CD244$^+$(**A**), LILRB1$^+$(**B**) and CD16$^+$ (**C**) within KIR$^-$ and KIR$^+$γδ T cells (*n=20*; CMV$^-$= black dots, CMV$^+$= red dots). (**D-F**) Graphs comparing the proportions of CD244$^+$(**D**), LILRB1$^+$(**E**), CD16$^+$**(F)** in KIR$^-$ and KIR$^+$γδ T cells between CMV$^-$ (*n=10*) and CMV$^+$ (*n=10*) individuals. Paired t-tests were performed to compare variables Statistical significance denoted by asterisks: *= p<0.05, **= p<0.01, ***= p<0.001, ****= p<0.0001, ns = non-significant.

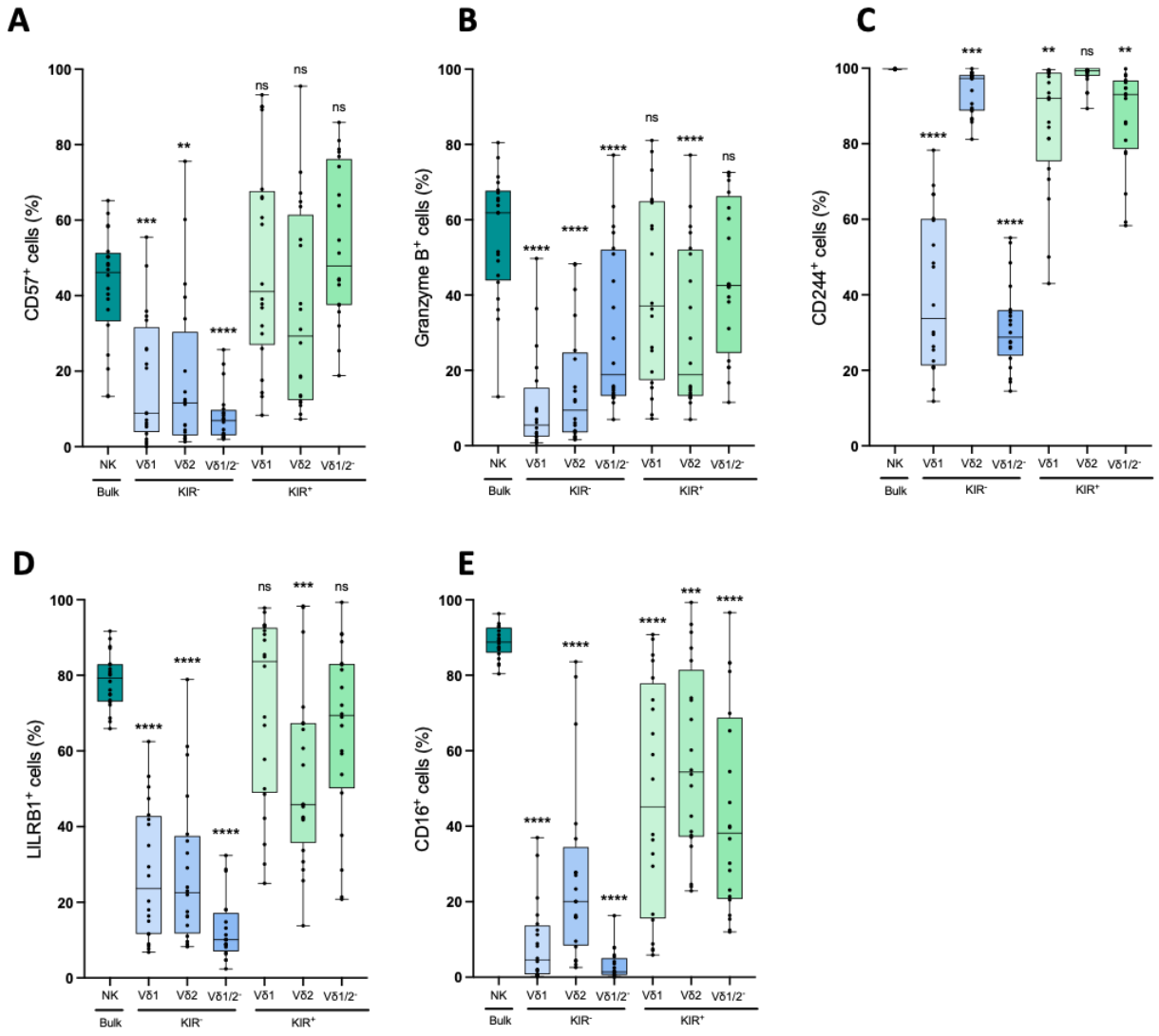


Figure 11: KIR$^+$γδ T cells resemble NK cells. Graphs displaying the proportions of CD57 (**A**), granzyme B (**B**), CD244 (**C**), LILRB1 (**D**) and CD16 (**E**) in different KIR$^-$ and KIR$^+$ γδ T cell subsets compared to bulk NK cells (*n=20*). One-way ANOVA were performed to compare variables. Statistical significance denoted by asterisks: *= p<0.05, **= p<0.01, ***= p<0.001, ****= p<0.0001, ns = non-significant.

3.2. Objective Two. Investigating the effector potential of KIR$^+$γδ T cells.

3.2.1 KIR$^+$ Vδ1neg T cells have stronger response to ADCC compared to other subsets.

αβ T-depleted PBMCs were exposed to Rituximab-coated Raji cells for a duration of six hours to assess their capacity for ADCC. KIR$^+$Vδ2 T cells exhibited a greater degranulation compared to KIR$^-$Vδ2 T cells and other γδ T cell subsets (**Fig. 12A**). Moreover, KIR$^+$ Vδ2 T cells exhibited a significantly higher production of IFN-γ compared to KIR$^-$Vδ2 T cells. This distinction related to KIR expression was also evident in Vδ1/2neg γδ T cells, whereas the IFN-γ production of Vδ1 T cells remained comparable between the two subsets (**Fig. 12B**). The disparity observed between KIR$^-$ and KIR$^+$ γδ T cell subsets was consistent across both CMV$^-$ and CMV$^+$ individuals (**Fig. 12A, B**).

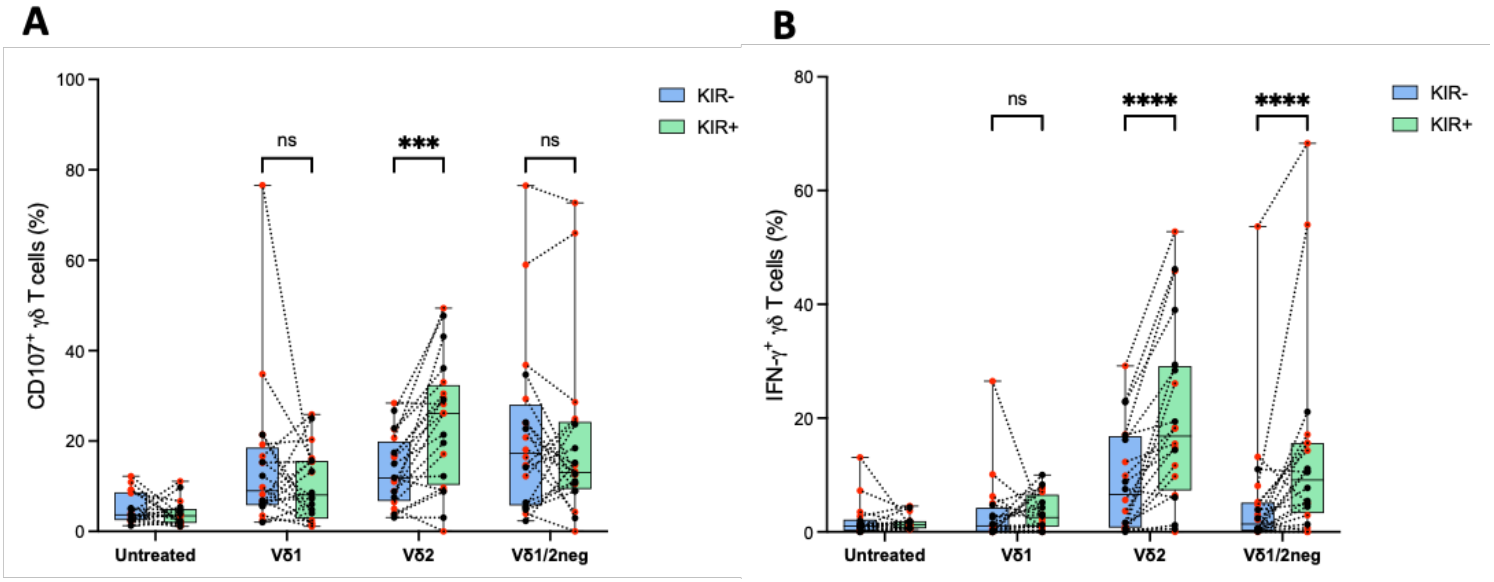


Figure 12: ADCC response is more pronounced in KIR$^+$γδ T cells compared to KIR$^-$ γδ T cells. Graphs comparing the proportions of CD107a$^+$(**A**) and IFN-γ$^+$(**B**) γδ T cells in response to Rituximab coated Raji cells between KIR$^-$ and KIR$^+$ γδ T cell subsets (*n=20*; CMV$^-$= black dots, CMV$^+$= red dots). Two-way ANOVA were performed to compare variables. Statistical significance denoted by asterisks: *= p<0.05, **= p<0.01, ***= p<0.001, ****= p<0.0001.

3.2.2 KIR^{+}γδ T cells have higher cytokine production and degranulation level when activated with PMA/Ionomycin.

To compare the effector potential of KIR^{-}γδ T cells to that of KIR^{+} γδ T cells, αβ T-depleted PBMCs were treated with PMA/Ionomycin for four hours. In Vδ2neg subsets, KIR^{+}γδ T cells exhibited greater degranulation than KIR^{-}γδ T cells (**Fig. 13A**). IFN-γ production was, however, higher in KIR^{+} cells, compared to KIR^{-} cells, in all γδ T cell subsets (**Fig. 13B**). Similarly, all KIR^{+}γδ T cells showed more elevated TNF-α, compared to KIR^{-} cells (**Fig. 13C**). This data show that KIR^{+}γδ T cells exhibit a greater potential for cytotoxicity and cytokine production compared to KIR^{-}γδ T cells, aligning with their mature phenotype.

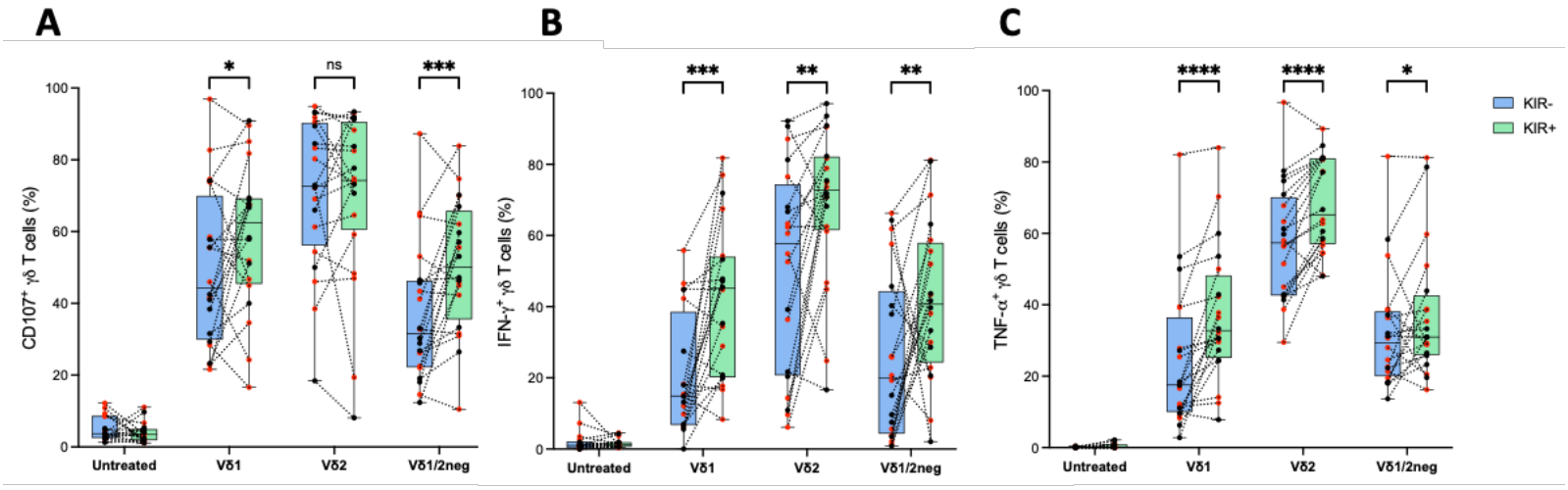


Figure 13: KIR^{+}γδ T cells are more cytotoxic compared to KIR^{-} γδ T cells. (**A-C**) Graphs comparing the proportions of CD107a^{+} (**A**), IFN-γ^{+} (**B**) and TNF-α^{+} (**C**) cells in response to 0.05uM PMA/Ionomycin, between KIR^{-} and KIR^{+} γδ T cell subsets (*n=20*; CMV^{-}= black dots, CMV^{+}= red dots). Two-way ANOVA were performed to compare variables. Statistical significance denoted by asterisks: *= p<0.05, **= p<0.01, ***= p<0.001, ****= p<0.0001.

3.3 Objective Three. Characterizing and comparing the epigenetics of KIR$^-$ and KIR$^+$ γδ T cell subsets.

3.3.1 KIR$^+$γδ T cells have more accessible genes with effector functions than KIR$^-$ γδ T cells.

I sorted KIR$^-$ and KIR$^+$γδ T cells from three CMV$^+$ individuals and prepared six OMNI-ATAC libraries as previously described [161]. qPCRs indicated an enrichment of known high-accessibility genomic regions compared to heterochromatic loci in all libraries, effectively validating the ATAC procedure (typically, libraries with ratios above 50 give excellent results in sequencing). (**Fig. 14A**). qPCRs were performed on the six library samples, targeting genomic loci known to be associated with effector functions. These included granzymes B and H (cytotoxicity), STAT4, CX3CR1 and EOMES (all higher in effector memory αβ T cells [166-171]. As expected, a higher abundance of all five loci was seen in the libraries prepared from KIR$^+$γδ T cells compared to those prepared from KIR$^-$γδ T cells (**Fig. 14B**). While some interindividual variation was noticed, this was anticipated, aligning with granzyme B expression, which similarly exhibited differences among individuals, but was consistently higher in KIR$^+$γδ T cells (**Fig. 14C**). These findings demonstrate that effector genes are more open in KIR$^+$γδ T cells, aligning with the phenotypic and functional characterization of these cells.

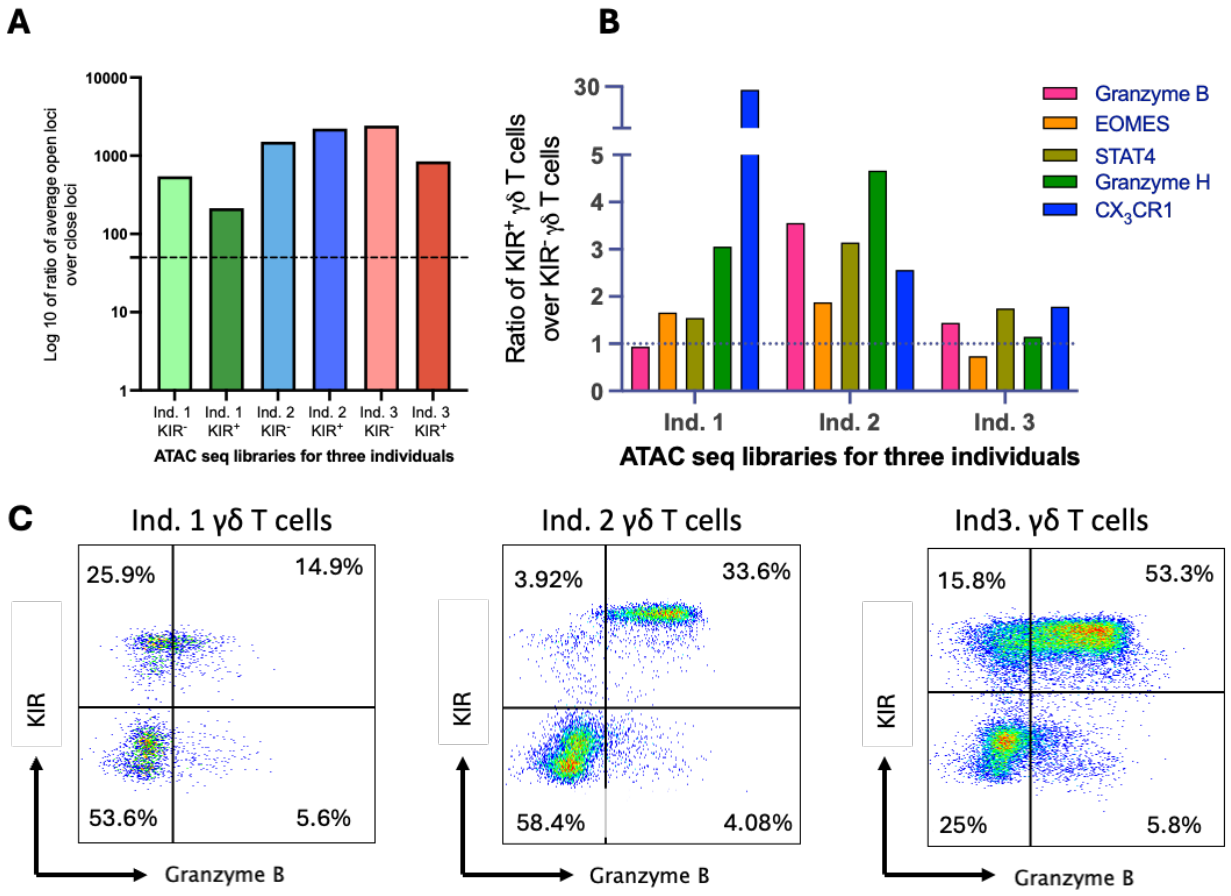


Figure 14: Epigenetic signature of γδ T cells. (A) Histogram displays the ratio of average open loci over closed loci in KIR$^-$ and KIR$^+$ samples of three individuals (ind.). (**B**) Histogram shows the ratio of KIR$^+$γδ T cells over KIR$^-$γδ T cells in three individuals across five genes. (**C**) Representative plots illustrate the proportions of granzyme B in KIR$^-$ and KIR$^+$γδ T cells.

3.4 Objective Four. Expansion and subsequent characterization of KIR-enriched polyclonal γδ T cells.

3.4.1 Efficient large-scale expansion of KIR-enriched polyclonal γδ T cells.

Given that polyclonal KIR^+ γδ T cell subsets from CMV^+ individuals all show mature effector phenotypes and functions, these cells were expanded *in vitro* to explore their anti-cancer effector potential. After numerous attempts, γδ T cells were successfully expanded by using a combination of feeder cells, including PBMCs and HLA-deficient K562 cells, supplemented by a soluble monoclonal antibody anti-CD3 and either IL2 alone or IL2 and IL15 together. In some cultures, the expansion rate was up to ~350 fold by day 17 post-stimulation (**Fig. 15A**). Remarkably, in all cultures, the expanded cells encompass all γδ T cell subsets (**Fig. 15B**) while preserving elevated KIR expression (**Fig. 15C**). These polyclonal γδ T cells maintain their cytotoxicity, evidenced by a high content of granzyme B and perforin, which is particularly notable in KIR^+ cells (**Fig. 15D, E**) and express low levels of PD1, especially within the KIR^+ cells (**Fig. 15F**). The expression of CD16 and CD244 was maintained in the expanded cells, compared to unstimulated cells. Only, LILRB1 expression was slightly decreased (**Fig. S3**).

In conclusion, this protocol successfully expanded KIR-enriched polyclonal γδ T cells with potent cytotoxic potential. This cell product will undergo testing in our laboratory to assess its *in vitro* and *in vivo* efficacy against cancer cells. Furthermore, it will serve as a foundation for another project in our laboratory aiming to develop γδ T cells equipped with a chimeric antigen receptor.

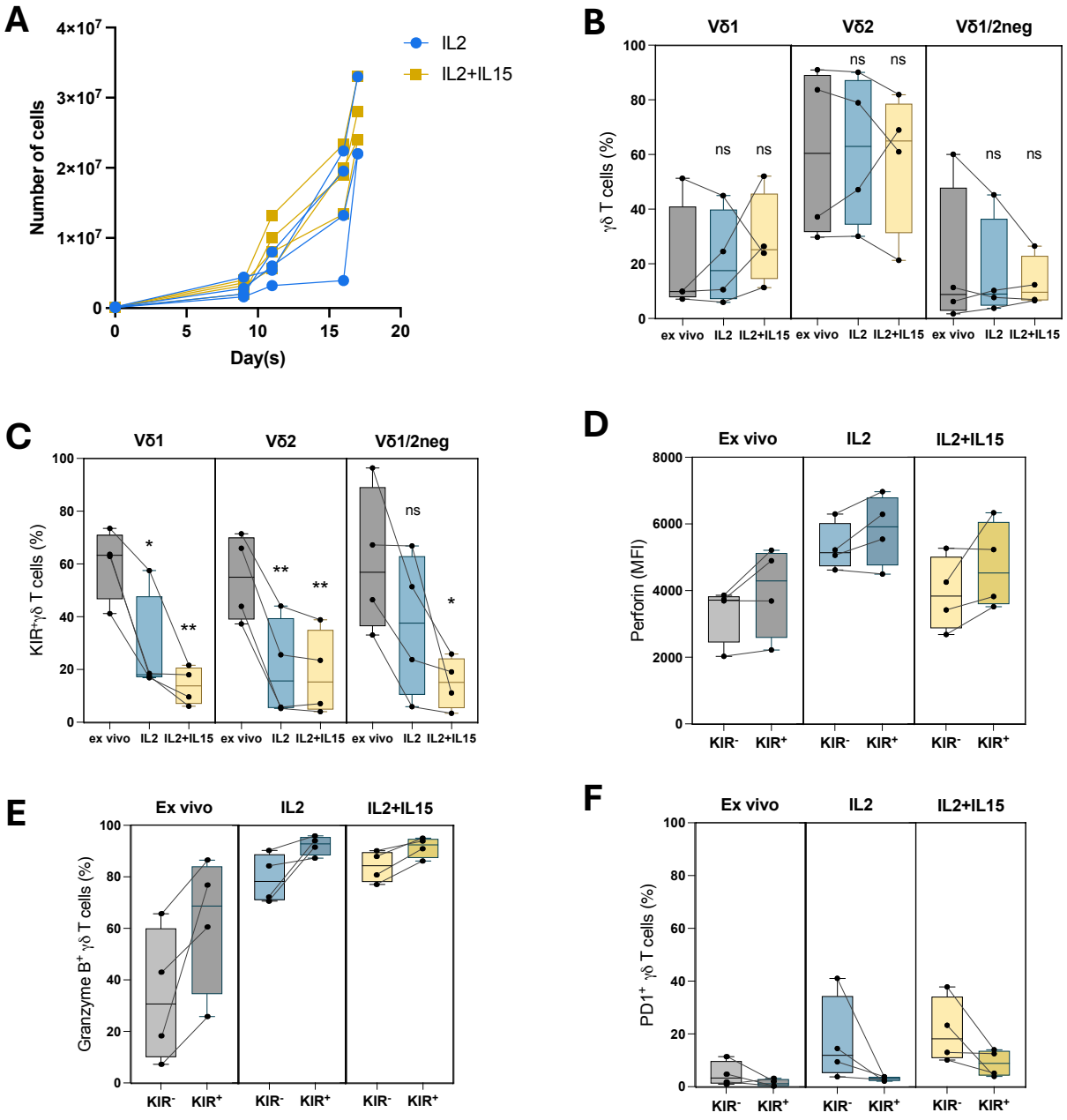


Figure 15: KIR-enriched polyclonal γδ T cells have high storage of cytotoxic mediators. (**A**) Cell numbers of γδ T cells under two conditions of IL2 alone and IL2+IL15. Graphs displaying the proportions of γδ T cell subsets(**B**) and KIR$^+$ γδ T cells(**C**) in different conditions. (**D-F**) Graphs comparing the MFI of perforin(**D**) and the proportions of granzyme B$^+$(**E**) and PD1$^+$(**F**) between KIR$^-$ and KIR$^+$γδ T cells in different condition of *ex vivo*, IL2, and IL2+IL15 conditions at day 17. Paired t-tests were performed to compare variables. Statistical significance was denoted by asterisks: * = p<0.05, **= p<0.01, ns= non-significant.

Chapter 4: Discussion

This study shows a significant increase in $KIR^+\gamma\delta$ T cells in CMV^+ individuals. These KIRs are associated with a more mature phenotype and a more potent effector potential of γδ T cells. Specifically, flow cytometry analyses show that $KIR^+\gamma\delta$ T cells exhibit a terminally differentiated profile, characterized predominantly by a $CD27^-CD57^+CD45RA^{high}$ phenotype and a high expression of NK cell receptors. Moreover, $KIR^+\gamma\delta$ T cells express more cytotoxic granules, such as granzyme B and perforin. qPCRs performed from ATAC libraries support these findings and show more open genes associated with effector potential. These genes include granzyme B, granzyme H, STAT4, EOMES, and CX_3CR1. Additionally, upon *in vitro* stimulation with PMA/Ionomycin, $KIR^+\gamma\delta$ T cells exhibit enhanced degranulation and cytokine production, compared to $KIR^-\gamma\delta$ T cells. These results were also observed when the cells were tested for their capacity to mediate ADCC. Recognizing the potential of adaptive-like γδ T cells, the expansion of cytotoxic, polyclonal populations, enriched with a substantial proportion of $KIR^+\gamma\delta$ T cells represents an important step in the field of immunotherapy.

The increase in the proportions of Vδ2neg subsets, predominantly composed of Vδ1 T cells, and the decrease in the ratio of Vδ2/Vδ1 T cells has been consistently observed in numerous studies of active CMV infection [172-176]. Moreover, expansion of adaptive-like Vγ9negVδ2 T cells was observed by Willcox's group in patients with active CMV infection [177]. They showed that Vγ9negVδ2 T cells are only detected with the anti-Vδ2 TCR antibody clone 123R3 (Miltenyi). Despite the same antibody being used, similar proportions of these cells were observed between CMV^- and CMV^+ individuals in this study, suggesting that this population expands only during active CMV infection. Furthermore, no difference was observed in the proportions of KIR^+Vδ2 T cells between CMV^- and CMV^+ individuals. Two CMV^- individuals displayed high expression of KIRs by their Vδ2 T cells. While this study focused exclusively on CMV serostatus, existing

literature suggests that other herpesvirus infections may also induce the expansion of adaptive-like γδ T cells [160].

The $CD27^{-}CD57^{+}CD45RA^{high}$ phenotype of KIR^{+}γδ T cells is indicative of terminal differentiation, similar to that of memory effector T cells (TEMRA). These cells respond better to PMA/Ionomycin stimulation and can mediate ADCC indicates that they are not exhausted, despite expressing high levels of CD57. According to several studies [37, 162, 163], $CD27^{-}$ Vδ1 T cells bear a striking resemblance to $CD8^{+}$ αβ TEMRA cells, which was also shown by ATAC-sequencing [162]. It is shown by this study that all KIR^{+}γδ T cell subsets display TEMRA characteristics.

These findings and evidence from other studies, suggest that KIR^{+}γδ T cells play a role in both infection and cancer, particularly in instances where there are defects in HLA class I expression [178]. Downregulation of HLA class I expression by unhealthy cells is a common immune evasion strategy employed by many cancers and viruses [80, 179]. It was shown that KIR^{+}γδ T cells expressing PD1 infiltrate MMR-d deficient colorectal cancers lacking HLA class I. Patients bearing these cancers have better response to anti-PD1 and a better prognosis [178]. Another study shows that Vδ1 T cells infiltrating non-small-cell lung cancer and exhibiting a $CD27^{-}$ $CD45RA^{high/low}$ phenotype contain high levels of granzyme B and are associated with a more favorable prognosis [28].

γδ T cells have significantly less perforin compared to granzyme B. This intriguing discrepancy in expression was also observed in other studies focusing on Vγ9Vδ2 T cells' expression of granzyme B and perforin [180]. It has been suggested that modulation of perforin serves to safeguard γδ T cells and adjacent healthy tissues from inadvertent damage caused by untargeted degranulation or dying cells. Furthermore, the substantial amounts of granzyme B can also be used by various processes such as extracellular matrix remodeling, immune cell

transmigration facilitation, and modulation of receptor and cytokine maturation, all relevant in multiple diseases [180]. Higher levels of both perforin and granzyme B were seen in $KIR^+V\delta1/2neg$ T cells compared to other subsets, indicating the potential of this subset to serve as potent cytotoxic lymphocytes in immunity.

Expression of inhibitory receptors by immune cells is vital for self-tolerance and prevention from autoimmune diseases. These receptors help regulate cell responsiveness, preventing overactivation and tissue harm. Their presence is tied to immune cell maturity and effectiveness, especially in chronic viral infections. The elevated expression of LILRB1 and CD244 observed in $KIR^+\gamma\delta$ T cells mirrors that of effector memory $CD8^+$ $\alpha\beta$ T cells and NK cells during infections such as HCV, HTLV1, CMV and EBV[181-185].

The heightened expression of CD16 and increased capacity for ADCC in $KIR^+\gamma\delta$ T cells, particularly within the $KIR^+V\delta1neg$ subset, suggests that activation via activating receptors such as CD16 can stimulate immune responses in γδ T cells beyond TCR engagement. This observation aligns with findings from other studies, indicating a potentially significant role for CD16-mediated activation in γδ T cell immune responses [186, 187]. It is noteworthy that despite the high expression of CD16 in $KIR^+V\delta1$ T cells, they did not show an ADCC response [188]. While the mechanism underlying this phenomenon is unknown, one possible explanation could be linked to the CD16 isoform expressed by these cells. CD16 exists in two isoforms, CD16A and CD16B [100]. CD16B is believed to be predominantly expressed on neutrophils and exhibits lower affinity to IgG, thus resulting in a diminished ability to mediate ADCC responses. However, it has been observed that it is also expressed on $CD56^-CD16^+$ NK cells, which are expanded during HIV infection [189]. It is thus possible that $V\delta1^+\gamma\delta$ T cells express the CD16B isoform.

KIR^{+}γδ T cells have more open genes associated with maturity, cytotoxicity, and migration compared to KIR^{-} subsets. EOMES plays a key role in the differentiation and maintenance of effector and memory T cells [170, 171]. STAT4 is involved in transmitting signals and enhanced immune response activation [190]. CX_3CR1, a memory marker of αβ T cells [168, 191], is responsible for cell migration, adhesion, and activation [168].

In recent years, *in vitro* expansion of γδ T cells has been a focus in immunotherapy, using either pAg compounds to target Vγ9Vδ2 T cells, or a combination of OKT3 and cytokines, including IL-1β, IL-4, IFN-γ, IL-21, to target Vδ1 T cells [159, 192]. The limitation of these methods is that they expand only one subset of γδ T cells, and these cells are naïve-like T cells and do not express KIRs. Recent findings from Guo *et al.* (2024) highlight the important anti-cancer role of diverse T cell clonotypes within the tumor microenvironment [193]. Moreover, the efficient response of polyclonal γδ T cells, as shown by Cooper's group, showcased a robust ability to target acute and chronic leukemia, colon, pancreatic, and ovarian cancer cell lines. Importantly, this response was observed without attacking healthy autologous or allogeneic normal B cells. Remarkably, the study highlighted that polyclonal γδ T cells exhibited superior efficacy compared to single subsets of γδ T cells *in vivo* [194]. However, the results of my study suggest that incorporating KIR^{+}γδ T cells abundant in CMV^{+} individuals could enhance the anti-tumor response leading to improved therapeutic outcomes in cancer immunotherapy. Here, we developed a method that efficiently expands polyclonal γδ T cells from a mere few thousands cells to million cells. These expanded cells are fully functional and show no signs of exhaustion. Expansion of a pure KIR^{+}γδ T cell population is challenging due to its scarcity in the PB. Nevertheless, our approach successfully maintains KIR expression by the expanded cells. Prior to *in vitro* expansion, blood donors could be selected based on their KIR expression levels to target high proportions of KIR^{+}γδ

T cells post-expansion. This new protocol marks a significant milestone for future research in our group enabling investigations into its efficacy both *in vitro* and *in vivo* against cancer cells and virus-infected cells.

4.1 Conclusion

CMV infection leaves a stable imprint on γδ T cells, resulting in the expansion of $CD27^{+}CD57^{-}CD45RA^{low}$ mature γδ T cells with high cytotoxic potential. These γδ T cells are predominantly equipped with KIRs and other innate receptors. Recognizing the potential of this subset and the necessity for expansion for successful immunotherapy, I propose the initial steps of expanding a cytotoxic, polyclonal γδ T cell population enriched with a substantial proportion of KIR^{+} cells.

4.2 Future Direction

This work sets the stage for exploring the potential benefits of KIR-enriched polyclonal γδ T cells in immunotherapy. As stated above, these cells will undergo testing both *in vitro* and *in vivo* against cancer cells. Furthermore, the expansion protocol developed in this study will serve as a foundational framework for the generation of CAR polyclonal γδ T cells. Notably, γδ T cell education by KIRs was not studied in this work. It is crucial to explore the effects of individual KIRs on γδ T cell responses to HLA deficiency and HLA allotypes. Such investigations will help in the selection of donors for individual patients when it comes to immunotherapy of cancer.

4.3 Limitation

While this study provides valuable insights into the characteristics and functions of $KIR^{+}\gamma\delta$ T cells, it is essential to acknowledge several limitations that may impact the interpretation and generalization of findings. Firstly, the majority of donors were Caucasian females, potentially limiting the generalizability of this study findings to other demographic groups. Additionally, while CMV status was assessed, other viral or infectious statuses were not evaluated, which could have provided a more comprehensive understanding of immune responses. Moreover, some donors did not return for follow-up assessments, resulting in a mismatch between phenotypic analysis and functional assays in certain cases. Functional assays were conducted using two different instruments of three-laser and five-laser Cytec Aurora, which may introduce variability in the results. Also, this study did not comprehensively investigate all possible receptors due to the vast array of receptors present on γδ T cells, such as NCRs. Furthermore, in the transcriptional signature analysis, sorting based on KIR expression resulted in a dominance of Vδ2Vγ9 cells in the KIR^{-} subset, potentially biasing the results toward limited difference between KIR^{-} and KIR^{+} cells. This imbalance is attributed to the lower cell numbers within each subset. Lastly, the expansion assay relied on activation-induced markers such as CD107a, IFN-γ, and CD69, which can be expressed even in the absence of target cells. Incorporating live-cell imaging techniques could provide a more accurate assessment of γδ T cell responses under stimulation conditions.

References

1. Saito, H., et al., *Complete primary structure of a heterodimeric T-cell receptor deduced from cDNA sequences.* Nature, 1984. **309**(5971): p. 757-62.
2. Allison, J.P., B.W. McIntyre, and D. Bloch, *Tumor-specific antigen of murine T-lymphoma defined with monoclonal antibody.* The Journal of Immunology, 1982. **129**(5): p. 2293-2300.
3. Chien, Y.-h., et al., *A third type of murine T-cell receptor gene.* Nature, 1984. **312**(5989): p. 31-35.
4. Kranz, D.M., et al., *Limited diversity of the rearranged T-cell γ gene.* Nature, 1985. **313**(6005): p. 752-755.
5. Hayday, A.C., et al., *Structure, organization, and somatic rearrangement of T cell gamma genes.* Cell, 1985. **40**(2): p. 259-269.
6. Murre, C., et al., *Human γ-chain genes are rearranged in leukaemic T cells and map to the short arm of chromosome 7.* Nature, 1985. **316**(6028): p. 549-552.
7. Lefranc, M.-P. and T.H. Rabbitts, *Two tandemly organized human genes encoding the T-cell γ constant-region sequences show multiple rearrangement in different T-cell types.* Nature, 1985. **316**(6027): p. 464-466.
8. Brenner, M.B., et al., *Identification of a putative second T-cell receptor.* Nature, 1986. **322**(6075): p. 145-149.
9. Bank, I., et al., *A functional T3 molecule associated with a novel heterodimer on the surface of immature human thymocytes.* Nature, 1986. **322**(6075): p. 179-181.
10. Borst, J., et al., *A T-cell receptor γ/CD3 complex found on cloned functional lymphocytes.* Nature, 1987. **325**(6106): p. 683-688.
11. Born, W., et al., *Recognition of a Peptide Antigen by Heat Shock--reactive γδ T Lymphocytes.* Science, 1990. **249**(4964): p. 67-69.
12. O'Brien, R.L., et al., *Stimulation of a major subset of lymphocytes expressing T cell receptor gamma delta by an antigen derived from Mycobacterium tuberculosis.* Cell, 1989. **57**(4): p. 667-74.
13. Janis, E.M., et al., *Activation of γδ T Cells in the Primary Immune Response to Mycobacterium tuberculosis.* Science, 1989. **244**(4905): p. 713-716.
14. Holoshttz, J., et al., *Isolation of CD4-CD8-mycobacteria-reactive T lymphocyte clones from rheumatoid arthritis synovial fluid.* Nature, 1989. **339**(6221): p. 226-229.
15. Takihara, Y., et al., *Diversity and structure of human T cell receptor delta chain genes in peripheral blood gamma/delta-bearing T lymphocytes.* J Exp Med, 1989. **169**(2): p. 393-405.
16. Groh, V., et al., *Human lymphocytes bearing T cell receptor gamma/delta are phenotypically diverse and evenly distributed throughout the lymphoid system.* J Exp Med, 1989. **169**(4): p. 1277-94.
17. Bigby, M., et al., *Most gamma delta T cells develop normally in the absence of MHC class II molecules.* J Immunol, 1993. **151**(9): p. 4465-75.
18. Vidović, D., et al., *Qa-1 restricted recognition of foreign antigen by a γδ T-cell hybridoma.* Nature, 1989. **340**(6235): p. 646-650.
19. Kozbor, D., et al., *Human TCR-gamma+/delta+, CD8+ T lymphocytes recognize tetanus toxoid in an MHC-restricted fashion.* Journal of Experimental Medicine, 1989. **169**(5): p. 1847-1851.
20. Pereira, P., et al., *Blockade of transgenic gamma delta T cell development in beta 2-microglobulin deficient mice.* Embo j, 1992. **11**(1): p. 25-31.
21. Wells, F.B., et al., *Requirement for positive selection of gamma delta receptor-bearing T cells.* Science, 1991. **253**(5022): p. 903-5.

22. Deseke, M. and I. Prinz, *Ligand recognition by the γδ TCR and discrimination between homeostasis and stress conditions.* Cellular & Molecular Immunology, 2020. **17**(9): p. 914-924.
23. Hayday, A.C., *γδ T Cells and the Lymphoid Stress-Surveillance Response.* Immunity, 2009. **31**(2): p. 184-196.
24. Pistoia, V., et al., *Human γδ T-Cells: From Surface Receptors to the Therapy of High-Risk Leukemias.* Front Immunol, 2018. **9**: p. 984.
25. Wang, L., et al., *The feature of distribution and clonality of TCR γ/δ subfamilies T cells in patients with B-cell non-Hodgkin lymphoma.* J Immunol Res, 2014. **2014**: p. 241246.
26. Deusch, K., et al., *A major fraction of human intraepithelial lymphocytes simultaneously expresses the gamma/delta T cell receptor, the CD8 accessory molecule and preferentially uses the V delta 1 gene segment.* Eur J Immunol, 1991. **21**(4): p. 1053-9.
27. Wu, Y., et al., *An innate-like Vδ1⁺ γδ T cell compartment in the human breast is associated with remission in triple-negative breast cancer.* Science Translational Medicine, 2019. **11**(513): p. eaax9364.
28. Wu, Y., et al., *A local human Vδ1 T cell population is associated with survival in nonsmall-cell lung cancer.* Nature Cancer, 2022. **3**(6): p. 696-709.
29. Falini, B., et al., *Distribution of T cells bearing different forms of the T cell receptor gamma/delta in normal and pathological human tissues.* J Immunol, 1989. **143**(8): p. 2480-8.
30. Ebert, L.M., S. Meuter, and B. Moser, *Homing and function of human skin γδ T cells and NK cells: relevance for tumor surveillance.* The journal of immunology, 2006. **176**(7): p. 4331-4336.
31. Grossi, C.E., et al., *Human T cells expressing the gamma/delta T-cell receptor (TcR-1): C gamma 1- and C gamma 2-encoded forms of the receptor correlate with distinctive morphology, cytoskeletal organization, and growth characteristics.* Proceedings of the National Academy of Sciences, 1989. **86**(5): p. 1619-1623.
32. De Paoli, P., et al., *A subset of gamma delta lymphocytes is increased during HIV-1 infection.* Clin Exp Immunol, 1991. **83**(2): p. 187-91.
33. Autran, B., et al., *T cell receptor gamma/delta+ lymphocyte subsets during HIV infection.* Clin Exp Immunol, 1989. **75**(2): p. 206-10.
34. Déchanet, J., et al., *Major expansion of gammadelta T lymphocytes following cytomegalovirus infection in kidney allograft recipients.* J Infect Dis, 1999. **179**(1): p. 1-8.
35. Munk, M.E., et al., *Onchocerca volvulus provides ligands for the stimulation of human gamma/delta T lymphocytes expressing V delta 1 chains.* J Infect Dis, 1993. **168**(5): p. 1241-7.
36. Giacomelli, R., et al., *Increase of circulating gamma/delta T lymphocytes in the peripheral blood of patients affected by active inflammatory bowel disease.* Clin Exp Immunol, 1994. **98**(1): p. 83-8.
37. Davey, M.S., et al., *Recasting Human Vδ1 Lymphocytes in an Adaptive Role.* Trends Immunol, 2018. **39**(6): p. 446-459.
38. Davey, M.S., et al., *Clonal selection in the human Vδ1 T cell repertoire indicates γδ TCR-dependent adaptive immune surveillance.* Nature Communications, 2017. **8**(1): p. 14760.
39. Mikulak, J., et al., *NKp46-expressing human gut-resident intraepithelial Vδ1 T cell subpopulation exhibits high antitumor activity against colorectal cancer.* JCI Insight, 2019. **4**(24).
40. Kierkels, G.J.J., et al., *Identification of a tumor-specific allo-HLA-restricted γδTCR.* Blood Adv, 2019. **3**(19): p. 2870-2882.
41. Bai, L., et al., *The majority of CD1d-sulfatide-specific T cells in human blood use a semiinvariant Vδ1 TCR.* Eur J Immunol, 2012. **42**(9): p. 2505-10.
42. Luoma, A.M., et al., *Crystal structure of Vδ1 T cell receptor in complex with CD1d-sulfatide shows MHC-like recognition of a self-lipid by human γδ T cells.* Immunity, 2013. **39**(6): p. 1032-42.

43. Roy, S., et al., *Molecular Analysis of Lipid-Reactive Vδ1 γδ T Cells Identified by CD1c Tetramers.* The Journal of Immunology, 2016. **196**(4): p. 1933-1942.
44. Zeng, X., et al., *γδ T cells recognize a microbial encoded B cell antigen to initiate a rapid antigen-specific interleukin-17 response.* Immunity, 2012. **37**(3): p. 524-34.
45. Le Nours, J., et al., *A class of γδ T cell receptors recognize the underside of the antigen-presenting molecule MR1.* Science, 2019. **366**(6472): p. 1522-1527.
46. Davey, M.S., et al., *Vδ2(+) T Cells-Two Subsets for the Price of One.* Front Immunol, 2018. **9**: p. 2106.
47. Yang, Y., et al., *A Structural Change in Butyrophilin upon Phosphoantigen Binding Underlies Phosphoantigen-Mediated Vγ9Vδ2 T Cell Activation.* Immunity, 2019. **50**(4): p. 1043-1053.e5.
48. Gay, L., et al., *Role of Vγ9vδ2 T lymphocytes in infectious diseases.* Front Immunol, 2022. **13**: p. 928441.
49. Oliaro, J., et al., *Vγ9Vδ2 T cells use a combination of mechanisms to limit the spread of the pathogenic bacteria Brucella.* Journal of Leukocyte Biology, 2005. **77**(5): p. 652-660.
50. Ryan-Payseur, B., et al., *Multieffector-functional immune responses of HMBPP-specific Vγ2Vδ2 T cells in nonhuman primates inoculated with Listeria monocytogenes ΔactA prfA*.* J Immunol, 2012. **189**(3): p. 1285-93.
51. Gay, L., et al., *BTN3A Targeting Vγ9Vδ2 T Cells Antimicrobial Activity Against Coxiella burnetii-Infected Cells.* Front Immunol, 2022. **13**: p. 915244.
52. Roussilhon, C., et al., *Human TcR gamma delta+ lymphocyte response on primary exposure to Plasmodium falciparum.* Clin Exp Immunol, 1994. **95**(1): p. 91-7.
53. Chang, W.L., et al., *Subset heterogeneity among gamma delta T cells found in peripheral blood during Plasmodium falciparum malaria.* Immunol Lett, 1992. **32**(3): p. 273-4.
54. Kunzmann, V., E. Bauer, and M. Wilhelm, *Gamma/delta T-cell stimulation by pamidronate.* N Engl J Med, 1999. **340**(9): p. 737-8.
55. Scotet, E., et al., *Tumor Recognition following Vγ9Vδ2 T Cell Receptor Interactions with a Surface F1-ATPase-Related Structure and Apolipoprotein A-I.* Immunity, 2005. **22**(1): p. 71-80.
56. Eagle, R.A. and J. Trowsdale, *Promiscuity and the single receptor: NKG2D.* Nature Reviews Immunology, 2007. **7**(9): p. 737-744.
57. Davey, M.S., et al., *The human Vδ2(+) T-cell compartment comprises distinct innate-like Vγ9(+) and adaptive Vγ9(-) subsets.* Nat Commun, 2018. **9**(1): p. 1760.
58. Bruder, J., et al., *Target Specificity of an Autoreactive Pathogenic Human γδ T Cell Receptor in Myositis* Journal of Biological Chemistry, 2012. **287**(25): p. 20986-20995.
59. Kenna, T., et al., *Distinct subpopulations of gamma delta T cells are present in normal and tumor-bearing human liver.* Clin Immunol, 2004. **113**(1): p. 56-63.
60. Dunne, M.R., et al., *Persistent changes in circulating and intestinal γδ T cell subsets, invariant natural killer T cells and mucosal-associated invariant T cells in children and adults with coeliac disease.* PLoS One, 2013. **8**(10): p. e76008.
61. Déchanet, J., et al., *Implication of γδ T cells in the human immune response to cytomegalovirus.* The Journal of clinical investigation, 1999. **103**(10): p. 1437-1449.
62. Mangan, B.A., et al., *Cutting Edge: CD1d Restriction and Th1/Th2/Th17 Cytokine Secretion by Human Vδ3 T Cells.* The Journal of Immunology, 2013. **191**(1): p. 30-34.
63. Falk, M.C., et al., *Predominance of T cell receptor V delta 3 in small bowel biopsies from coeliac disease patients.* Clin Exp Immunol, 1994. **98**(1): p. 78-82.
64. Kabelitz, D., et al., *Clonal expansion of Vγ3/Vδ3-expressing γδ T cells in an HIV-1/2-negative patient with CD4 T-cell deficiency.* British journal of haematology, 1997. **96**(2): p. 266-271.
65. Marlin, R., et al., *Sensing of cell stress by human γδ TCR-dependent recognition of annexin A2.* Proceedings of the National Academy of Sciences, 2017. **114**(12): p. 3163-3168.

66. Rice, M.T., et al., *Recognition of the antigen-presenting molecule MR1 by a Vδ3(+) γδ T cell receptor.* Proc Natl Acad Sci U S A, 2021. **118**(49).
67. Willcox, C.R., et al., *Butyrophilin-like 3 Directly Binds a Human Vγ4(+) T Cell Receptor Using a Modality Distinct from Clonally-Restricted Antigen.* Immunity, 2019. **51**(5): p. 813-825.e4.
68. Willcox, C.R., et al., *Cytomegalovirus and tumor stress surveillance by binding of a human γδ T cell antigen receptor to endothelial protein C receptor.* Nature Immunology, 2012. **13**(9): p. 872-879.
69. Shibata, K., et al., *IFN-γ–producing and IL-17–producing γδ T cells differentiate at distinct developmental stages in murine fetal thymus.* The Journal of Immunology, 2014. **192**(5): p. 2210-2218.
70. Ma, Y., et al., *Contribution of IL-17-producing gamma delta T cells to the efficacy of anticancer chemotherapy.* J Exp Med, 2011. **208**(3): p. 491-503.
71. Chabab, G., et al., *Pro-tumor γδ T Cells in Human Cancer: Polarization, Mechanisms of Action, and Implications for Therapy.* Front Immunol, 2020. **11**: p. 2186.
72. Harmon, C., et al., *γδ T cell dichotomy with opposing cytotoxic and wound healing functions in human solid tumors.* Nat Cancer, 2023. **4**(8): p. 1122-1137.
73. Künkele, K.-P., et al., *Vγ9Vδ2 T Cells: Can We Re-Purpose a Potent Anti-Infection Mechanism for Cancer Therapy?* Cells, 2020. **9**(4): p. 829.
74. Gaballa, A., et al., *Revisiting the Role of γδ T Cells in Anti-CMV Immune Response after Transplantation.* Viruses, 2021. **13**(6).
75. Tuengel, J., et al., *Characterization of Adaptive-like γδ T Cells in Ugandan Infants during Primary Cytomegalovirus Infection.* Viruses, 2021. **13**(10).
76. De Paoli, P., et al., *Gamma delta T cell receptor-bearing lymphocytes during Epstein-Barr virus infection.* J Infect Dis, 1990. **161**(5): p. 1013-6.
77. Déchanet, J., et al., *Implication of gammadelta T cells in the human immune response to cytomegalovirus.* J Clin Invest, 1999. **103**(10): p. 1437-49.
78. Boullier, S., et al., *CDR3-independent gamma delta V delta 1+ T cell expansion in the peripheral blood of HIV-infected persons.* The Journal of Immunology, 1995. **154**(3): p. 1418-1431.
79. Petersen, J.L., C.R. Morris, and J.C. Solheim, *Virus evasion of MHC class I molecule presentation.* J Immunol, 2003. **171**(9): p. 4473-8.
80. Cornel, A.M., I.L. Mimpen, and S. Nierkens, *MHC Class I Downregulation in Cancer: Underlying Mechanisms and Potential Targets for Cancer Immunotherapy.* Cancers (Basel), 2020. **12**(7).
81. Carena, I., et al., *Major histocompatibility complex class I molecules modulate activation threshold and early signaling of T cell antigen receptor-gamma/delta stimulated by nonpeptidic ligands.* J Exp Med, 1997. **186**(10): p. 1769-74.
82. Airò, P., et al., *Characterization of gammadelta T cells expressing CD158b, a killer cell inhibitory receptor, in a patient with chronic CD4(+) lymphocytopenia and disseminated Mycobacterium intracellulare infection.* Clin Immunol, 2000. **96**(1): p. 67-75.
83. Pende, D., et al., *Killer Ig-Like Receptors (KIRs): Their Role in NK Cell Modulation and Developments Leading to Their Clinical Exploitation.* Front Immunol, 2019. **10**: p. 1179.
84. Middleton, D. and F. Gonzelez, *The extensive polymorphism of KIR genes.* Immunology, 2010. **129**(1): p. 8-19.
85. Bruijnesteijn, J., N.G. de Groot, and R.E. Bontrop, *The Genetic Mechanisms Driving Diversification of the KIR Gene Cluster in Primates.* Front Immunol, 2020. **11**: p. 582804.
86. Rajagopalan, S., J. Fu, and E.O. Long, *Cutting edge: induction of IFN-gamma production but not cytotoxicity by the killer cell Ig-like receptor KIR2DL4 (CD158d) in resting NK cells.* J Immunol, 2001. **167**(4): p. 1877-81.
87. Rajagopalan, S. and E.O. Long, *A human histocompatibility leukocyte antigen (HLA)-G-specific receptor expressed on all natural killer cells.* J Exp Med, 1999. **189**(7): p. 1093-100.

88. Garcia-Beltran, W.F., et al., *Open conformers of HLA-F are high-affinity ligands of the activating NK-cell receptor KIR3DS1.* Nature immunology, 2016. **17**(9): p. 1067-1074.
89. Djaoud, Z. and P. Parham, *HLAs, TCRs, and KIRs, a Triumvirate of Human Cell-Mediated Immunity.* Annu Rev Biochem, 2020. **89**: p. 717-739.
90. Kang, X., et al., *Inhibitory leukocyte immunoglobulin-like receptors: Immune checkpoint proteins and tumor sustaining factors.* Cell Cycle, 2016. **15**(1): p. 25-40.
91. Cosman, D., et al., *A novel immunoglobulin superfamily receptor for cellular and viral MHC class I molecules.* Immunity, 1997. **7**(2): p. 273-282.
92. Goodridge, J.P., et al., *Remodeling of secretory lysosomes during education tunes functional potential in NK cells.* Nature Communications, 2019. **10**(1): p. 514.
93. Sivori, S., et al., *Human NK cells: surface receptors, inhibitory checkpoints, and translational applications.* Cell Mol Immunol, 2019. **16**(5): p. 430-441.
94. Duan, S., et al., *Natural killer group 2D receptor and its ligands in cancer immune escape.* Molecular Cancer, 2019. **18**(1): p. 29.
95. Borrego, F., et al., *Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis.* J Exp Med, 1998. **187**(5): p. 813-8.
96. Braud, V.M., et al., *HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C.* Nature, 1998. **391**(6669): p. 795-9.
97. Kaiser, B.K., et al., *Interactions between NKG2x immunoreceptors and HLA-E ligands display overlapping affinities and thermodynamics.* The Journal of Immunology, 2005. **174**(5): p. 2878-2884.
98. Tamm, A. and R.E. Schmidt, *IgG binding sites on human Fc gamma receptors.* Int Rev Immunol, 1997. **16**(1-2): p. 57-85.
99. Yeap, W.H., et al., *CD16 is indispensable for antibody-dependent cellular cytotoxicity by human monocytes.* Scientific Reports, 2016. **6**(1): p. 34310.
100. Li, W., H. Yang, and D.S. Dimitrov, *Identification of high-affinity anti-CD16A allotype-independent human antibody domains.* Exp Mol Pathol, 2016. **101**(2): p. 281-289.
101. Pinto, S., et al., *Reimagining antibody-dependent cellular cytotoxicity in cancer: the potential of natural killer cell engagers.* Trends Immunol, 2022. **43**(11): p. 932-946.
102. Narni-Mancinelli, E., et al., *Complement factor P is a ligand for the natural killer cell–activating receptor NKp46.* Science immunology, 2017. **2**(10): p. eaam9628.
103. Mandelboim, O., et al., *Recognition of haemagglutinins on virus-infected cells by NKp46 activates lysis by human NK cells.* Nature, 2001. **409**(6823): p. 1055-1060.
104. Garg, A., et al., *Vimentin expressed on Mycobacterium tuberculosis-infected human monocytes is involved in binding to the NKp46 receptor.* The Journal of Immunology, 2006. **177**(9): p. 6192-6198.
105. Vitenshtein, A., et al., *NK cell recognition of Candida glabrata through binding of NKp46 and NCR1 to fungal ligands Epa1, Epa6, and Epa7.* Cell host & microbe, 2016. **20**(4): p. 527-534.
106. Mavoungou, E., et al., *A Duffy Binding—Like Domain Is Involved in the NKp30-Mediated Recognition of Plasmodium falciparum—Parasitized Erythrocytes by Natural Killer Cells.* The Journal of Infectious Diseases, 2007. **195**(10): p. 1521-1531.
107. Chaushu, S., et al., *Direct recognition of Fusobacterium nucleatum by the NK cell natural cytotoxicity receptor NKp46 aggravates periodontal disease.* PLoS Pathog, 2012. **8**(3): p. e1002601.
108. Bloushtain, N., et al., *Membrane-Associated Heparan Sulfate Proteoglycans Are Involved in the Recognition of Cellular Targets by NKp30 and NKp461.* The Journal of Immunology, 2004. **173**(4): p. 2392-2401.

109. Arnon, T.I., et al., *Recognition of viral hemagglutinins by NKp44 but not by NKp30.* European journal of immunology, 2001. **31**(9): p. 2680-2689.
110. Barrow, A.D., et al., *Natural killer cells control tumor growth by sensing a growth factor.* Cell, 2018. **172**(3): p. 534-548. e19.
111. Baychelier, F., et al., *Identification of a cellular ligand for the natural cytotoxicity receptor NKp44.* Blood, The Journal of the American Society of Hematology, 2013. **122**(17): p. 2935-2942.
112. Jarahian, M., et al., *Activation of natural killer cells by newcastle disease virus hemagglutinin-neuraminidase.* Journal of virology, 2009. **83**(16): p. 8108-8121.
113. Parodi, M., et al., *NKp44-NKp44 ligand interactions in the regulation of natural killer cells and other innate lymphoid cells in humans.* Frontiers in immunology, 2019. **10**: p. 453174.
114. Brandt, C.S., et al., *The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans.* J Exp Med, 2009. **206**(7): p. 1495-503.
115. Binici, J., et al., *A soluble fragment of the tumor antigen BCL2-associated athanogene 6 (BAG-6) is essential and sufficient for inhibition of NKp30 receptor-dependent cytotoxicity of natural killer cells.* J Biol Chem, 2013. **288**(48): p. 34295-303.
116. Welte, S., et al., *Mutual activation of natural killer cells and monocytes mediated by NKp80-AICL interaction.* Nature Immunology, 2006. **7**(12): p. 1334-1342.
117. Freud, A.G., et al., *NKp80 Defines a Critical Step during Human Natural Killer Cell Development.* Cell Reports, 2016. **16**(2): p. 379-391.
118. Chen, S. and Z. Dong, *NK cell recognition of hematopoietic cells by SLAM-SAP families.* Cellular & Molecular Immunology, 2019. **16**(5): p. 452-459.
119. Hislop, A.D., et al., *Impaired Epstein-Barr virus-specific CD8+ T-cell function in X-linked lymphoproliferative disease is restricted to SLAM family-positive B-cell targets.* Blood, 2010. **116**(17): p. 3249-57.
120. Brandes, M., K. Willimann, and B. Moser, *Professional antigen-presentation function by human gammadelta T Cells.* Science, 2005. **309**(5732): p. 264-8.
121. Muto, M., et al., *Myeloid molecular characteristics of human γδ T cells support their acquisition of tumor antigen-presenting capacity.* Cancer Immunology, Immunotherapy, 2015. **64**(8): p. 941-949.
122. Mao, C., et al., *Tumor-activated TCRγδ$^+$ T cells from gastric cancer patients induce the antitumor immune response of TCRαβ$^+$ T cells via their antigen-presenting cell-like effects.* J Immunol Res, 2014. **2014**: p. 593562.
123. Bansal, R.R., et al., *IL-21 enhances the potential of human γδ T cells to provide B-cell help.* European Journal of Immunology, 2012. **42**(1): p. 110-119.
124. Petrasca, A., et al., *Human Vδ3+ γδ T cells induce maturation and IgM secretion by B cells.* Immunology Letters, 2018. **196**: p. 126-134.
125. Münz, C., R.M. Steinman, and S.-i. Fujii, *Dendritic cell maturation by innate lymphocytes : coordinated stimulation of innate and adaptive immunity.* Journal of Experimental Medicine, 2005. **202**(2): p. 203-207.
126. Conti, L., et al., *Reciprocal Activating Interaction Between Dendritic Cells and Pamidronate-Stimulated γδ T Cells: Role of CD86 and Inflammatory Cytokines1.* The Journal of Immunology, 2005. **174**(1): p. 252-260.
127. Walwyn-Brown, K., et al., *Phosphoantigen-Stimulated γδ T Cells Suppress Natural Killer-Cell Responses to Missing-Self.* Cancer Immunol Res, 2022. **10**(5): p. 558-570.
128. Maniar, A., et al., *Human gammadelta T lymphocytes induce robust NK cell-mediated antitumor cytotoxicity through CD137 engagement.* Blood, 2010. **116**(10): p. 1726-33.
129. Peters, C., D. Kabelitz, and D. Wesch, *Regulatory functions of γδ T cells.* Cellular and Molecular Life Sciences, 2018. **75**(12): p. 2125-2135.

130. Marlin, R., et al., *Sensing of cell stress by human γδ TCR-dependent recognition of annexin A2.* Proc Natl Acad Sci U S A, 2017. **114**(12): p. 3163-3168.
131. Gu, S., et al., *Phosphoantigen-induced conformational change of butyrophilin 3A1 (BTN3A1) and its implication on Vγ9Vδ2 T cell activation.* Proc Natl Acad Sci U S A, 2017. **114**(35): p. E7311-e7320.
132. Roy Chowdhury, R., et al., *NK-like CD8(+) γδ T cells are expanded in persistent Mycobacterium tuberculosis infection.* Sci Immunol, 2023. **8**(81): p. eade3525.
133. Junqueira, C., et al., *γδ T cells suppress Plasmodium falciparum blood-stage infection by direct killing and phagocytosis.* Nat Immunol, 2021. **22**(3): p. 347-357.
134. Sabbaghi, A., et al., *Role of γδ T cells in controlling viral infections with a focus on influenza virus: implications for designing novel therapeutic approaches.* Virol J, 2020. **17**(1): p. 174.
135. Pauza, C.D., et al., *γδ T Cells in HIV Disease: Past, Present, and Future.* Front Immunol, 2014. **5**: p. 687.
136. Djaoud, Z., et al., *Two alternate strategies for innate immunity to Epstein-Barr virus: One using NK cells and the other NK cells and γδ T cells.* J Exp Med, 2017. **214**(6): p. 1827-1841.
137. von Massow, G., et al., *Gamma Delta T Cells and Their Involvement in COVID-19 Virus Infections.* Front Immunol, 2021. **12**: p. 741218.
138. Goldberg Michael, F., K. Saini Neeraj, and A. Porcelli Steven, *Evasion of Innate and Adaptive Immunity by Mycobacterium tuberculosis.* Microbiology Spectrum, 2014. **2**(5): p. 10.1128/microbiolspec.mgm2-0005-2013.
139. Junqueira, C., et al., *γδ T cells suppress Plasmodium falciparum blood-stage infection by direct killing and phagocytosis.* Nature Immunology, 2021. **22**(3): p. 347-357.
140. von Borstel, A., et al., *Repeated Plasmodium falciparum infection in humans drives the clonal expansion of an adaptive γδ T cell repertoire.* Sci Transl Med, 2021. **13**(622): p. eabe7430.
141. Hviid, L., C. Smith-Togobo, and B.E. Willcox, *Human Vδ1(+) T Cells in the Immune Response to Plasmodium falciparum Infection.* Front Immunol, 2019. **10**: p. 259.
142. Rincon-Orozco, B., et al., *Activation of V gamma 9V delta 2 T cells by NKG2D.* J Immunol, 2005. **175**(4): p. 2144-51.
143. Dong, P., et al., *γδ T Cells Provide Protective Function in Highly Pathogenic Avian H5N1 Influenza A Virus Infection.* Front Immunol, 2018. **9**: p. 2812.
144. Robinson, J.E., *The biology of circulating B lymphocytes infected with Epstein-Barr virus during infectious mononucleosis.* Yale J Biol Med, 1982. **55**(3-4): p. 311-6.
145. Wong, Y., et al., *Estimating the global burden of Epstein–Barr virus-related cancers.* Journal of Cancer Research and Clinical Oncology, 2022. **148**(1): p. 31-46.
146. Shannon-Lowe, C. and A. Rickinson, *The Global Landscape of EBV-Associated Tumors.* Front Oncol, 2019. **9**: p. 713.
147. Epstein, M.A., B.G. Achong, and Y.M. Barr, *Virus particles in cultured lymphoblasts from burkitt's lymphoma.* Lancet, 1964. **1**(7335): p. 702-3.
148. Raab-Traub, N., et al., *The differentiated form of nasopharyngeal carcinoma contains Epstein-Barr virus DNA.* Int J Cancer, 1987. **39**(1): p. 25-9.
149. Raab-Traub, N., et al., *Epstein-Barr virus infection in carcinoma of the salivary gland.* J Virol, 1991. **65**(12): p. 7032-6.
150. Bjornevik, K., et al., *Longitudinal analysis reveals high prevalence of Epstein-Barr virus associated with multiple sclerosis.* Science, 2022. **375**(6578): p. 296-301.
151. Cannon, M.J., D.S. Schmid, and T.B. Hyde, *Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection.* Reviews in Medical Virology, 2010. **20**(4): p. 202-213.

152. McGeoch, D.J., F.J. Rixon, and A.J. Davison, *Topics in herpesvirus genomics and evolution.* Virus Res, 2006. **117**(1): p. 90-104.
153. Rölle, A., et al., *Effects of human cytomegalovirus infection on ligands for the activating NKG2D receptor of NK cells: up-regulation of UL16-binding protein (ULBP) 1 and ULBP2 is counteracted by the viral UL16 protein.* The Journal of Immunology, 2003. **171**(2): p. 902-908.
154. Wilkinson, G.W., et al., *Modulation of natural killer cells by human cytomegalovirus.* J Clin Virol, 2008. **41**(3): p. 206-12.
155. Béziat, V., et al., *Polyclonal Expansion of NKG2C(+) NK Cells in TAP-Deficient Patients.* Front Immunol, 2015. **6**: p. 507.
156. Haroun-Izquierdo, A., et al., *Adaptive single-KIR(+)NKG2C(+) NK cells expanded from select superdonors show potent missing-self reactivity and efficiently control HLA-mismatched acute myeloid leukemia.* J Immunother Cancer, 2022. **10**(11).
157. Goodridge, J.P., et al., *Remodeling of secretory lysosomes during education tunes functional potential in NK cells.* Nat Commun, 2019. **10**(1): p. 514.
158. Gentles, A.J., et al., *The prognostic landscape of genes and infiltrating immune cells across human cancers.* Nat Med, 2015. **21**(8): p. 938-945.
159. Ma, L., Y. Feng, and Z. Zhou, *A close look at current γδ T-cell immunotherapy.* Front Immunol, 2023. **14**: p. 1140623.
160. Martini, F. and E. Champagne, *The Contribution of Human Herpes Viruses to γδ T Cell Mobilisation in Co-Infections.* Viruses, 2021. **13**(12): p. 2372.
161. Corces, M.R., et al., *An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues.* Nat Methods, 2017. **14**(10): p. 959-962.
162. McMurray, J.L., et al., *Transcriptional profiling of human Vδ1 T cells reveals a pathogen-driven adaptive differentiation program.* Cell Rep, 2022. **39**(8): p. 110858.
163. Prinz, I. and C. Koenecke, *Antigen-specific γδ T cells contribute to cytomegalovirus control after stem cell transplantation.* Curr Opin Immunol, 2023. **82**: p. 102303.
164. Faint, J.M., et al., *Memory T Cells Constitute a Subset of the Human CD8+CD45RA+ Pool with Distinct Phenotypic and Migratory Characteristics1.* The Journal of Immunology, 2001. **167**(1): p. 212-220.
165. Béziat, V., et al., *NK cell terminal differentiation: correlated stepwise decrease of NKG2A and acquisition of KIRs.* PLoS One, 2010. **5**(8): p. e11966.
166. Kaplan, M.H., et al., *Impaired IL-12 responses and enhanced development of Th2 cells in Stat4-deficient mice.* Nature, 1996. **382**(6587): p. 174-177.
167. Gil, M.P., et al., *Regulating type 1 IFN effects in CD8 T cells during viral infections: changing STAT4 and STAT1 expression for function.* Blood, 2012. **120**(18): p. 3718-3728.
168. Gerlach, C., et al., *The Chemokine Receptor CX3CR1 Defines Three Antigen-Experienced CD8 T Cell Subsets with Distinct Roles in Immune Surveillance and Homeostasis.* Immunity, 2016. **45**(6): p. 1270-1284.
169. Gordon, C.L., et al., *Induction and maintenance of CX3CR1-intermediate peripheral memory CD8+ T cells by persistent viruses and vaccines.* Cell Reports, 2018. **23**(3): p. 768-782.
170. Lino, C.N.R., et al., *Eomes expression reports the progressive differentiation of IFN-γ-producing Th1-like γδ T cells.* Eur J Immunol, 2017. **47**(6): p. 970-981.
171. Rodríguez-Gómez, I.M., et al., *Expression of T-Bet, Eomesodermin, and GATA-3 Correlates With Distinct Phenotypes and Functional Properties in Porcine γδ T Cells.* Front Immunol, 2019. **10**: p. 396.
172. Pitard, V., et al., *Long-term expansion of effector/memory Vδ2− γδ T cells is a specific blood signature of CMV infection.* Blood, The Journal of the American Society of Hematology, 2008. **112**(4): p. 1317-1324.

173. Prinz, I., et al., *Donor Vδ1+ γδ T cells expand after allogeneic hematopoietic stem cell transplantation and show reactivity against CMV-infected cells but not against progressing B-CLL.* Experimental hematology & oncology, 2013. **2**: p. 1-4.
174. Prinz, I., et al., *Donor Vδ1+ γδ T cells expand after allogeneic hematopoietic stem cell transplantation and show reactivity against CMV-infected cells but not against progressing B-CLL.* Experimental Hematology & Oncology, 2013. **2**(1): p. 14.
175. Lewis, D., et al., *Cytomegalovirus Infection Is Associated with Expansions of CD8 T Cells and Highly Oligoclonal Vdelta1 Gamma/Delta T Cells in Patients Treated with Dasatinib for Chronic Myelogenous Leukaemia.* Blood, 2014. **124**(21): p. 1814-1814.
176. Khairallah, C., J. Déchanet-Merville, and M. Capone, *γδ T Cell-Mediated Immunity to Cytomegalovirus Infection.* Front Immunol, 2017. **8**: p. 105.
177. Davey, M.S., et al., *The human Vδ2+ T-cell compartment comprises distinct innate-like Vγ9+ and adaptive Vγ9- subsets.* Nature Communications, 2018. **9**(1): p. 1760.
178. de Vries, N.L., et al., *γδ T cells are effectors of immunotherapy in cancers with HLA class I defects.* Nature, 2023. **613**(7945): p. 743-750.
179. Beersma, M.F., M.J. Bijlmakers, and H.L. Ploegh, *Human cytomegalovirus down-regulates HLA class I expression by reducing the stability of class I H chains.* J Immunol, 1993. **151**(9): p. 4455-64.
180. Sandoz, P.A., et al., *Modulation of lytic molecules restrain serial killing in γδ T lymphocytes.* Nature Communications, 2023. **14**(1): p. 6035.
181. Raziorrouh, B., et al., *The immunoregulatory role of CD244 in chronic hepatitis B infection and its inhibitory potential on virus-specific CD8+ T-cell function.* Hepatology, 2010. **52**(6): p. 1934-47.
182. Schlaphoff, V., et al., *Dual function of the NK cell receptor 2B4 (CD244) in the regulation of HCV-specific CD8+ T cells.* PLoS Pathog, 2011. **7**(5): p. e1002045.
183. Ezinne, C.C., et al., *HTLV-1 specific CD8+ T cell function augmented by blockade of 2B4/CD48 interaction in HTLV-1 infection.* PLoS One, 2014. **9**(2): p. e87631.
184. Wang, Y., et al., *Long noncoding RNA derived from CD244 signaling epigenetically controls CD8+ T-cell immune responses in tuberculosis infection.* Proc Natl Acad Sci U S A, 2015. **112**(29): p. E3883-92.
185. Gustafson, C.E., et al., *Immune Checkpoint Function of CD85j in CD8 T Cell Differentiation and Aging.* Front Immunol, 2017. **8**: p. 692.
186. Braakman, E., et al., *CD16 on human γδ T lymphocytes: Expression, function, and specificity for mouse IgG isotypes.* Cellular Immunology, 1992. **143**(1): p. 97-107.
187. Couzi, L., et al., *Antibody-dependent anti-cytomegalovirus activity of human γδ T cells expressing CD16 (FcγRIIIa).* Blood, 2012. **119**(6): p. 1418-1427.
188. de Witte, M.A., et al., *Early Reconstitution of NK and γδ T Cells and Its Implication for the Design of Post-Transplant Immunotherapy.* Biol Blood Marrow Transplant, 2018. **24**(6): p. 1152-1162.
189. Forconi, C.S., et al., *A New Hope for CD56(neg)CD16(pos) NK Cells as Unconventional Cytotoxic Mediators: An Adaptation to Chronic Diseases.* Front Cell Infect Microbiol, 2020. **10**: p. 162.
190. Vahedi, G., et al., *STATs Shape the Active Enhancer Landscape of T Cell Populations.* Cell, 2012. **151**(5): p. 981-993.
191. Böttcher, J.P., et al., *Functional classification of memory CD8(+) T cells by CX3CR1 expression.* Nat Commun, 2015. **6**: p. 8306.
192. Almeida, A.R., et al., *Delta One T Cells for Immunotherapy of Chronic Lymphocytic Leukemia: Clinical-Grade Expansion/Differentiation and Preclinical Proof of Concept.* Clin Cancer Res, 2016. **22**(23): p. 5795-5804.
193. Guo, J., et al., *γδ T cell antigen receptor polyspecificity enables T cell responses to a broad range of immune challenges.* Proc Natl Acad Sci U S A, 2024. **121**(4): p. e2315592121.

194. Deniger, D.C., et al., *Activating and propagating polyclonal gamma delta T cells with broad specificity for malignancies.* Clin Cancer Res, 2014. **20**(22): p. 5708-19.

Supplementary Materials

Figure S1:

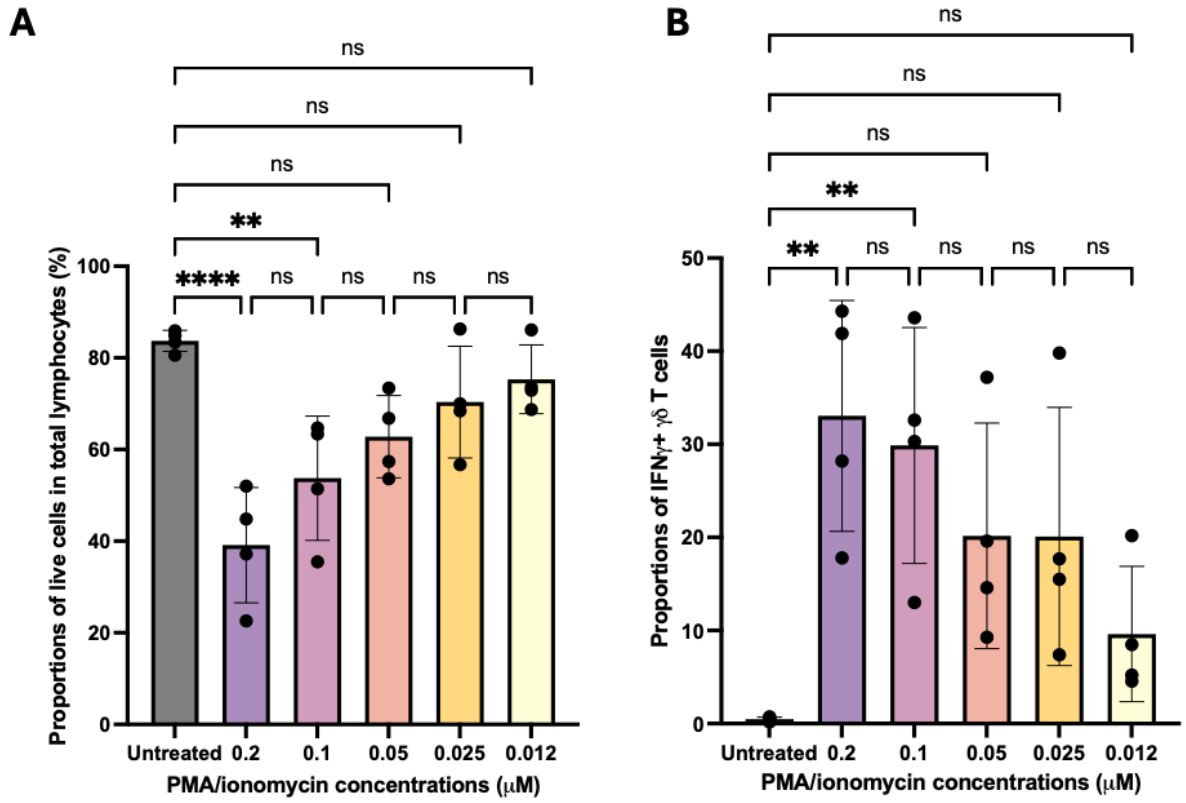
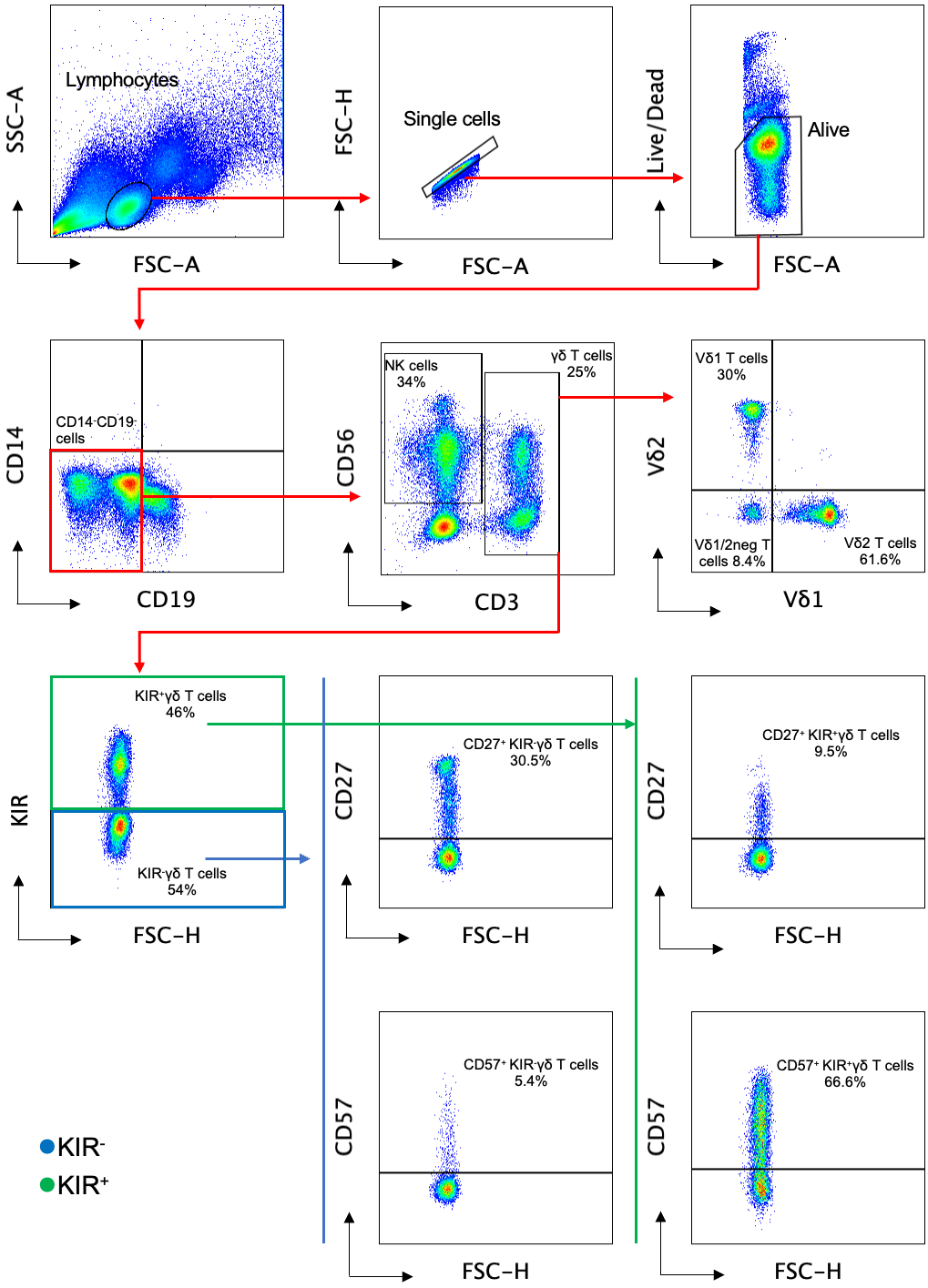
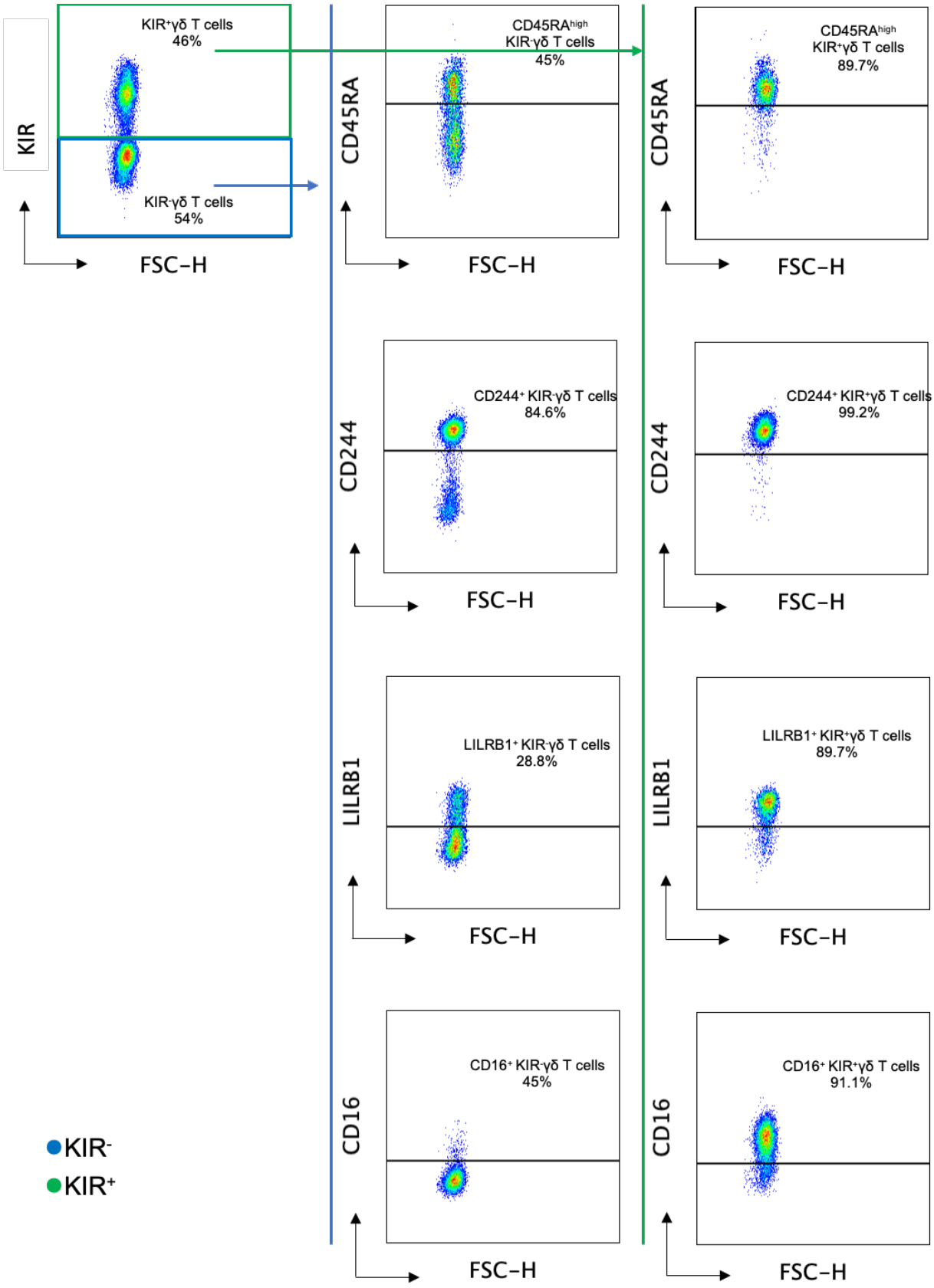


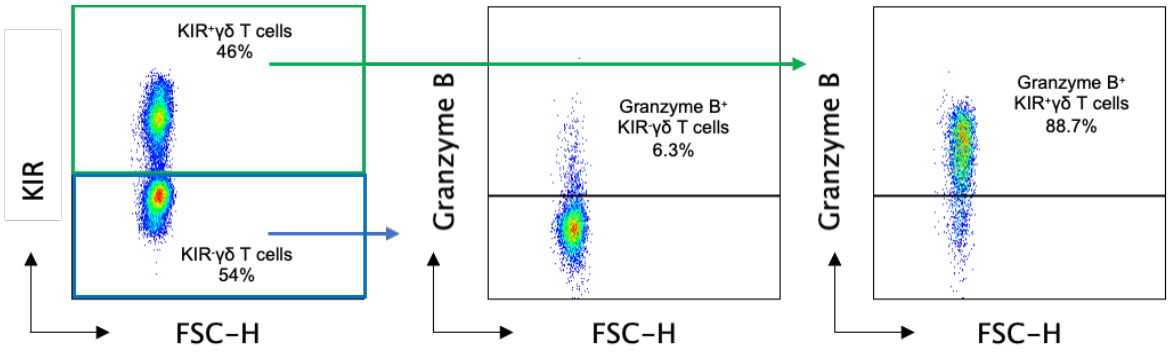
Figure S1: Optimal concentration of PMA/Ionomycin. (A-B) Histograms displaying the viability of αβ-T depleted lymphocytes (**A**) and the IFN-γ expression in γδ T cells (**B**) in different concentration of PMA/Ionomycin. The concentration of 0.2μM is recommended by manufacturer. Paired t-tests were performed to compare variables. Statistical significance is denoted by asterisks: *= p<0.05, **= p<0.01, ***= p<0.001, ****= p<0.0001, ns = non-significant.

Figure S2: Gating strategy

1. Immunophenotyping assay




KIR
KIR+γδ T cells
46%
KIR-γδ T cells
54%
FSC-H
CD45RA
CD45RAhigh
KIR-γδ T cells
45%
FSC-H
CD45RA
CD45RAhigh
KIR+γδ T cells
89.7%
FSC-H
CD244
CD244+ KIR-γδ T cells
84.6%
FSC-H
CD244
CD244+ KIR+γδ T cells
99.2%
FSC-H
LILRB1
LILRB1+ KIR-γδ T cells
28.8%
FSC-H
LILRB1
LILRB1+ KIR+γδ T cells
89.7%
FSC-H
CD16
CD16+ KIR-γδ T cells
45%
FSC-H
CD16
CD16+ KIR+γδ T cells
91.1%
FSC-H
KIR-
KIR+



2. Functional assay

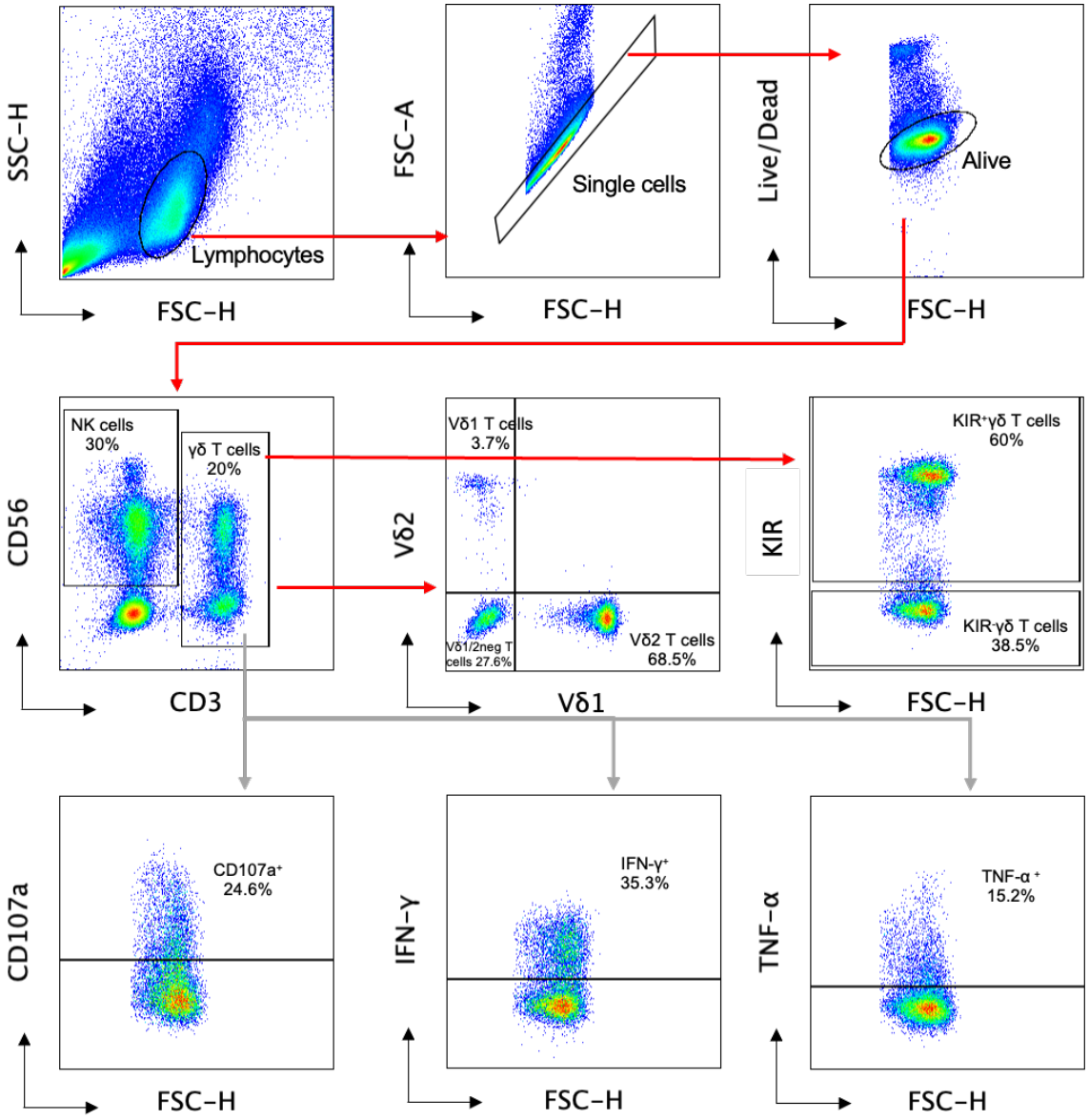


Figure S2: The sequential gating strategy for identification of NK cells and γδ T cells from unstimulated αβ T-depleted samples. NK cells were defined as CD3−CD56+, while γδ T cells were defined as CD3+. Additionally, to ensure purity, these cells were confirmed to be $CD14^{-}CD19^{-}$ in immunophenotyping assay. Subsequently, γδ T cells were further characterized by gating on the Vδ1 and Vδ2 TCRs to distinguish Vδ1, Vδ2 and Vδ1/2neg subsets. Within each subset, cells were gated based on the absence (KIR^{-}) or presence of KIRs (KIR^{+}), followed by further gating on each subset to assess various markers including CD27, CD57, CD45RA, CD244, LILRB1, CD16, Granzyme B, CD107a, IFN-γ, and TNF-α. This consistent gating strategy was applied across different subsets of γδ T cells and NK cells to ensure accuracy and reliability in the analysis.

Figure S3:

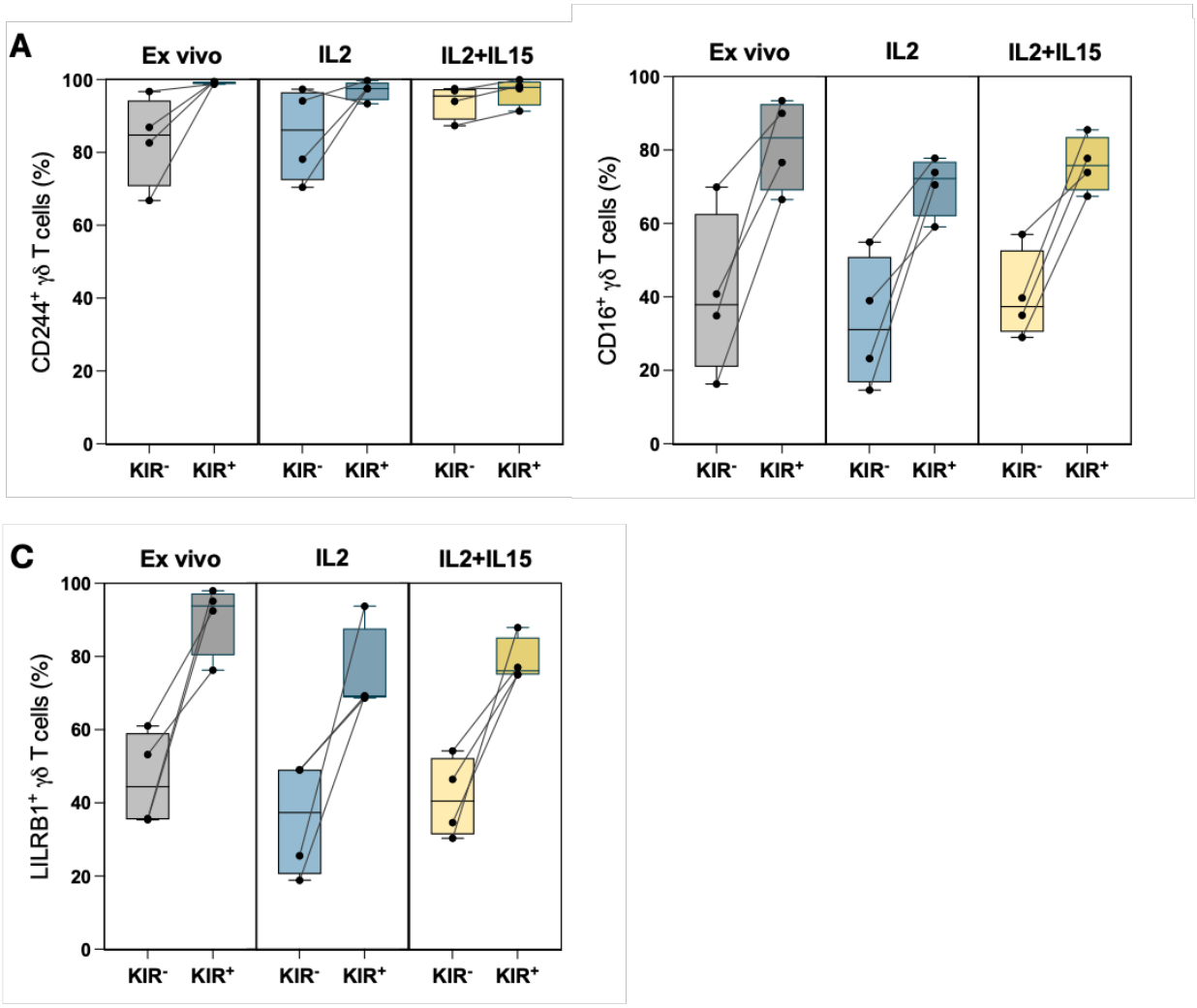


Figure S3: Expressions of NKRs are higher in expanded KIR$^+$γδ T cells. (**A-C**) Graphs comparing the proportion of CD244$^+$(**A**), CD16$^+$(**B**) and LILRB1+ (**C**) between KIR$^-$ and KIR$^+$γδ T cells in different condition of *ex vivo*, IL2, and IL2+IL15 conditions at day 17. Paired t-tests were performed to compare variables. Statistical significance was denoted by asterisks: * = p<0.05, **= p<0.01, ns= non-significant.