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CD1D-γδ T CELL RECEPTOR INTERACTION: ADAPTATIONS AND FUNCTION IN TISSUE IMMUNITY

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For my mother, who taught me how to keep going.

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ABSTRACT

Tissue resident lymphocytes are critical sentinels of tissue homeostasis, capable of producing rapid memory responses upon detection of pathogens or stress. In humans, T cells bearing a Vδ1 TCR comprise a major portion of this tissue resident memory population. Despite this, their function and the role of antigen specificity in tissues remains largely unknown. In this work, CD1d was leveraged as a known Vδ1 TCR antigen to define and manipulate populations of αβ and γδ T cells. In the setting of umbilical cord blood and lung, CD1d-lipid antigen recognition critically delineated functional outcomes of T cells. CD1d-autoreactive T cells filled apparently opposing roles in asthma, either tissue repair or cytolytic effector function, depending on the immunogenicity of the self-lipids presented by CD1d. In separate but related studies, recently evolved features of γδ TCR constant region dampened TCR signal strength and reduced clonal expansion in vivo. Taken together, these results suggest that CD1d auto-reactivity—and more broadly, self-reactivity—by some T cells is an intrinsic feature of the TCR and could be important for detecting dysbiosis and maintaining tissue homeostasis.

CHAPTER 1

INTRODUCTION

1.1 γδ T cell subsets and localization

1.1.1 Features of human and mouse γδ T cells

γδ T cells are a subset of lymphocytes that are characterized by their expression of a γδ TCR. In many ways, this is their only unifying feature. While γδ TCR$^+$ T cells are evolutionarily ancient and are present in all vertebrate immune systems, this subset is rapidly evolving and has diversified to fill very different roles in different species[1]. Consequently, γδ T cells differ significantly between humans and mice, the model of choice for immunologists. However, in the absence of a comprehensive, systems-level understanding of human γδ T cells, careful comparisons to the mouse system can be instructive.

In both humans and mice, γδ T cells arise in the thymus as a result of productive V(D)J recombination at the TRA/TRD and TRG locus[2,3]. The rearrangement of a delta chain from the TRD locus has been postulated to be the defining lineage commitment step[3,4], however, functional TRD/TRG rearrangements do not preclude the possibility of αβ TCR expression[5]. It is unknown if thymic selection shapes γδ TCRs present in the mature repertoire of humans; in mice, positive selection on MHC-like molecules, such as T22, occurs[6]. Further, mouse γδ T cells undergo fate specification and terminal functional differentiation in the thymus as a function of TCR signaling during selection[6]. Mouse γδ T cell fates closely mirror those of conventional T cells in signature transcription factors (i.e. T-bet, ROR-γt) and function (i.e. type 1, 2 or 17 helper CD4$^+$ cells or CD8$^+$ cytotoxic lymphocytes)[6,7]. Human γδ T cells do not fall into these strict archetypal subsets.

General trends of tissue tropism of γδ T cells hold between human and mouse. Broadly, γδ T cells are rare when considered as a proportion of peripheral blood lymphocytes; however, they are overrepresented in barrier tissues like skin and intestines[8,9]. Other organs that harbor γδ T cells include brain[10,11], lung[12], and liver[13].

1.1.2 Subset ontogeny and localization

Though every combination of δ chains (TRDV1, TRDV2, TRDV3, TRAV14/TRDV4, TRAV29/TRDV5, TRAV23/TRDV6, TRAV36/TRDV7, TRAV38-2/TRDV8) and γ chains (TRGV2, TRGV3, TRGV4, TRGV5, TRGV8, TRGV9)[14] can be generated during thymic development[15,16], most γδ T cells can be categorized based on their chain usage as Vγ9Vδ2 or $V\delta 2^-$. These subsets occupy distinct functional roles and their generation is temporally separated[15,17].

Vγ9Vδ2 T cells are the γδ T cells most frequently found in peripheral blood of adults[17]. As their name indicates, the Vγ9Vδ2 subset uses a restricted set of semi-invariant public clonotypes, arising from a TRGV9-TRGJP-TRGC1 recombination event[18]. Generated by the fetal thymus and liver, this subset is the first to emerge during human thymic development, and undergoes major expansion after birth, likely due to activation by microbial signals[17,19] (discussed further in the next section, γδ TCR antigen recognition).

After birth, thymic output shifts to favor the production of $V\delta 2^-$ γδ T cells. These postnatally-derived cells are predominantly (but not exclusively) $V\delta 1^+$[17], but can also use Vδ3, or, less commonly, other shared α/δ chains. Despite this bias in chain usage, the TCR repertoire of $V\delta 2^-$ γδ T cells is much more diverse than the Vγ9Vδ2 subset[20]. Though the production of $V\delta 2^-$ γδ T cells surpasses Vγ9Vδ2 T cell output quickly and continues throughout life[19–21], $V\delta 2^-$ numbers remain low in the peripheral blood[17]. This is because $V\delta 2^-$ decamp rapidly from the blood to take up residence in tissues, where they are long-lived and prevalent[9,21].

1.1.3 Innate and adaptive characteristics of γδ T cells

Much effort has been put toward characterizing subsets of γδ T cells as "innate" and "adaptive." By definition, γδ T cells are adaptive lymphocytes because of their recombined antigen receptor. Considerable sequence diversity is present in the CDR3δ and CDR3γ sequences of both γδ T cell subsets. Further, γδ T cells proliferate upon TCR-ligand recognition, in a process called clonal expansion, to create a robust multicellular response to antigen[13,21–23]. Both of these features are

hallmarks of the adaptive arm of the immune system.

Despite their adherence to the textual definition of adaptive immunity, γδ T cells do have some shared features with innate immune cells. Vγ9Vδ2 T cells, in particular, have been cast in an innate role. The Vγ9Vδ2 TCR is produced by a stereotypical recombination event, resulting in germline-encoded residues mediating activation[24,25]. Prenatal Vγ9Vδ2 TCR sequences are largely predictable and shared across individuals[18,19]. In this way, Vγ9Vδ2 T cells are "hard-wired" for specificity toward a danger signal in a way that is reminiscent of other innate pattern recognition receptors. Additionally, both Vγ9Vδ2 and Vδ2^{-} γδ T cells commonly express natural killer receptors (NKR), named for their expression on innate natural killer cells[26–28]. The activating receptor, NKG2D, is the most often discussed γδ-associated NKR. It is expressed on most mature γδ T cells and recognizes the stress-induced MHC-like proteins MICA and MICB to trigger γδ T cell activation[29]. Other innate features of γδ T cells include their ability to present peptide MHC to prime CD4^{+} and CD8^{+} T cells[30], expression of other innate pattern recognition receptors[31–33], and their ability to respond rapidly to stimulation.

Despite historical definitions of Vγ9Vδ2 T cells as "innate" and Vδ2^{-} as "adaptive", both subsets have innate-like and adaptive-like features. Because γδ T cells can make use of a wide array of innate and adaptive functions, responses are heterogeneous and highly context dependent. Stimulus, disease status, tissue locale, and age all factor into what type of functional niche a γδ T cell will occupy.

1.2 γδ TCR antigen recognition

1.2.1 Architecture of the γδ TCR

γδ T cell development and ensuing V(D)J recombination ultimately yields a γδ TCR protein complex capable of trafficking to the surface, binding antigen, and transmitting an activating signal across the membrane. Understanding the structure-function relationships embedded in the γδ TCR

complex helps contextualize the data presented herein. Both the γ and δ chains are comprised, from N terminus to C terminus, two Ig domains, a connecting peptide linker, and a membrane-spanning alpha helix[14]. The first Ig domain is the direct product of V(D)J recombination and contains the complementarity determining regions (CDRs) that contact antigen. The constant gene (either TRGC1/TRGC2/TRDC) makes up the second Ig domain, linker, and transmembrane helix. The γ and δ chains associate such that they are aligned from N to C termini, with their CDRs forming a contiguous variable molecular surface for antigen recognition. CD3ε, γ, δ, and ζ are essential components for TCR signal transduction and are typically incorporated in the TCR complex as one ε:δ heterodimer, one ε:γ heterodimer, and one ζ:ζ homodimer[34]. The complex structure of an αβ TCR with associated CD3 subunits reveals the TCR Ig domains in close association, ringed by CD3 subunits[35]. The structure of the γδ TCR complex has not been experimentally determined, but it is thought to be highly similar to that of the the αβ TCR, with few exceptions. Studies suggest that γδ TCRs may be more tolerant of deviations from the standard complex assembly, such absence of CD3ε/γ subunits in the complex[36] and incorporation of DAP10 or FcRγ for signaling instead of CD3ζ[37–43].

1.2.2 Vγ9Vδ2-BTN2A1/3A1 axis

The best-characterized ligands for the γδ TCR are butyrophilin (BTN) proteins that activate Vγ9Vδ2 T cells. Current evidence suggests that BTN3A1 binds to intracellular phosphoantigens and undergoes a conformation change, heterodimerizing with BTN2A1[44–46]. In turn, BTN2A1 extracellular domains are thought to mediate binding to the HV4 of the Vγ9 chain to stimulate Vγ9Vδ2 T cells[47]. Phosphoantigens are produced by stressed or infected cells[48], and, in a strict sense, are not true “antigens” at all because they are not directly recognized by the TCR. This axis is a prime example of innate-like triggering of the γδ TCR, as evidence accrued so far suggests involvement of germline-encoded receptors.

1.2.3 Vγ4-Btnl3/8 axis

A similar system of dimer recognition by Vγ4$^+$ TCRs has recently been uncovered. Like the BTN2A1/3A1 axis, homologous butyrophilin-like (Btnl) proteins, Btnl3/8, heterodimerize to activate γδ TCRs that contain the Vγ4 gene segment[49–51]. These TCRs are typically Vδ1$^+$. Unlike the BTNs, binding of an additional activating component, like phosphoantigen, does not seem to be required. Expression dynamics of the Btnl3/8 proteins appear to be sufficient to control activation[51,52]. It is hypothesized that Btnl3/8 expression decreases with tissue dysbiosis and leads to turnover of Vγ4$^+$ T cells in favor of more pro-inflammatory subsets of γδ T cells.

1.2.4 Historical antigens for Vδ1 T cells

The αβ TCR's CDR loops are crucial for recognition of peptide-MHC complexes. In contrast, the γδ TCR's recombined CDRs do not have a clear purpose in the BTN/Btnl systems. This raises the intriguing possibility of the existence of true adaptive "antigens" that engage the γδ TCR's recombined CDRs. Suggested ligands for individual γδ T cell clones include MICA, R-PE, tRNA synthetase, Annexin A2, and heat shock proteins (reviewed thoroughly here[53]). In much of the early work on γδ antigens, it was unclear if responses were truly TCR specific, although the activation in the presence of only certain V genes suggests a level of TCR specificity. Additionally, links between microbe exposure and Vγ9Vδ2 expansion, as well as CMV infection and Vδ1$^+$ T cell expansion, point to infections as a source of antigen for these subsets. However, the past decade, MHC and MHC-like molecules have emerged decisively as antigen presenting molecules for the γδ TCR. γδ T cells specific for HLA[54,55], CD1[56–58], and MR1[59] have been identified by investigators worldwide as a small but highly reproducible proportion of the γδ population.

1.2.5 Emergence of CD1 molecules as ligands for Vδ1 T cells

We and others have shown that antigens presented by CD1d, an MHC-like protein that presents sphingolipids rather than peptides, could represent a major class of adaptive γδ TCR ligands[60,61]. In structural studies of two different $V\delta1^+$ TCRs, the highly variable CDR3 loops were shown to interact with CD1d-lipid complexes. The DP10.7 TCR uses all three CDR loops of the δ chain to contact CD1d loaded with sulfatide, resulting in δ chain biased footprint of the TCR on CD1d[62]. The direct interaction with sulfatide is mediated entirely by non-templated residues in the CDR3δ: this is reminiscent of αβ TCR recognition of peptide. Similarly, the structure of the 9C2 TCR revealed that both its CDR3δ and CDR3γ are involved in recognition of the lipid antigen, α-galactosylceramide[63]. This work has been extended to show that γδ TCRs bind to all isoforms of CD1 (CD1a, CD1b, CD1c, and CD1d)[64–66], which, due to slight differences in their groove structures, present a diverse, semi-overlapping array of lipid antigens[67,68].

1.3 CD1d and CD1d-restricted T cells

1.3.1 Biochemistry of CD1d

CD1d is perhaps the most prominent member of the CD1 protein family. The subject of decades of study, its structure, trafficking properties, and lipid antigen repertoire have been thoroughly investigated[69]. CD1d shares gross structural features with classical class I MHC, namely, the α1 and α2 domains make up an antigen-binding groove, and this "platform" structure rests atop an α3 domain that associates with beta-2-microglobulin (β2m)[70]. However, CD1d has two deep hydrophobic pockets in the platform (referred to as A' and F') which accomodate alipathic alkyl chains, distinguishing it from peptide-presenting MHCs[71]. CD1d also has an intracellular tail with amino acid motifs that enable sorting in cellular compartments and signaling. Once CD1d associates with β2m in the ER, it recycles between the membrane and the endosome in a manner dependent on its intracellular sorting motif (YXXN/E)[72]. Together, the structure of the A'/F' pockets and the route

of intracellular trafficking work in concert to define the pool of lipid antigens presented on CD1d. Mass spectrometry studies of lipids eluted from CD1d are typically performed with truncated constructs lacking the sorting motif and, therefore, may not represent the true abundance and diversity of ligands acquired by CD1d recycling between the endosome and plasma membrane. Still, these studies have provided insight into general properties of CD1d ligands, and demonstrated CD1d's general specificity for phospholipids, characterized by a phosphate head group joining two hydrophobic fatty acid tails, and sphingolipids, characterized by a sphingosine base modified with two hydrophobic fatty acid tails[67,68].

1.3.2 CD1d-restricted T cells

Perhaps what cemented CD1d's importance as an antigen presenting molecule was the subsequent discovery of a corresponding conserved, semi-invariant T cell population, invariant natural killer T (iNKT) cells[73]. iNKT cells are an innate-like CD1d-restricted T cell population that express an αβ TCR (TRAV10-TRAJ18/TRBV25 in humans) as well as NKRs[74]. The iNKT literature is too vast to summarize in its entirety here, but as the "original" CD1d-specific T cells, a brief discussion of iNKT biology and antigen specificity is germane to our understanding of other CD1d-specific populations. iNKT cells are selected by homotypic interactions and emerge fully functional from the thymus. Upon stimulation, they respond rapidly, producing IFNγ, TNFα, and cytolytic factors, as well as IL-17 and IL-4 in mouse[7]. It is hypothesized that their function is wholly innate-like, with their invariant TCR acting as a pattern recognition receptor, drawing parallels to the Vγ9Vδ2 T cell subset. The stereotypical lipid ligand for iNKT cells is α-galactosylceramide, discovered serendipitously in a screen for T-cell-stimulating compounds[75]. Though α-galactosylceramide is the canonical lipid ligand for iNKT cells, synthesized analogs and several endogenous glycolipids[76] have been shown to stimulate iNKT cells as well, and will be discussed more thoroughly in the next passage concerning CD1 autoreactivity (1.3.3).

Other subsets of T cells that recognize CD1d have emerged, and in particular, the "type II

NKT" subset has gained traction in the field. The "type II" designation was initially assigned to a narrow subset of T cells specific for CD1d loaded with sulfatide without cross-reactivity to CD1d-α-galactosylceramide[77]. However, more T cell populations were found to bind CD1d-sulfatide, including γδ T cells and the aforementioned DP10.7 and 9C2 clones, as well as αβ T cells with promiscuous lipid specificity that included, but was not limited to, sulfatide[72,77–79]. As a result, type II NKT cells can be more accurately referred to as non-invariant CD1d-restricted T cells.

1.3.3 CD1 autoreactivity

The search for endogenous lipid agonists for the iNKT TCR spurred investigation self lipids presented by CD1d. While α-galactosylceramide remains a useful tool for identifying iNKT cells, it seems unlikely that this lipid isolated from the marine sponge *Agelas mauritianus* has a direct physiological role in immunity. Exogenous ligands derived from actual human pathogens, including an array of ceramides and diacylglycerols with α-linked sugar head groups, have also been shown to activate iNKT cells[80]. Though this clarified a potential role for the iNKT/CD1d axis in antimicrobial responses, it did not explain observed iNKT cell responses in the absence of exogenous lipid antigens. Eventually, endogenous lipid agonists for the iNKT TCR emerged. Importantly, mammalian cells produce glycosphingolipids with β-linkages. The β-linkage changes the positioning of the sugar head group in the context of CD1d, generally resulting in lower affinity of the iNKT TCR to endogenous lipid antigens[80]. Despite this weak binding, a handful of endogenous lipids have been confirmed as agonists for iNKT cells, and several have structural and/or biochemical evidence behind them, including lysophosphatidylcholine (LPC)[78,81], isoglobotrihexosylceramide (iGb3)[82,83], β-galactosylceramide[83], and phosphatidylinositol[84]. It is worth noting that TCRs with engineered CDR3s (to enhance interactions with CD1d) were used in the studies of iGB3, β-galactosylceramide, and phosphatidylinositol. Further, though the LPC-CD1d-iNKT TCR complex was solved using a naturally occurring iNKT sequence, binding data between the LPC complex and the iNKT TCR was not achieved. Perhaps because of the difficulty of captur-

ing information about endogenous ligands the iNKT TCR, focus has shifted toward responses to exogenous ligands or synthetic ligands with therapeutic applications in mind.

However, in some ways, the focus on biochemical evidence and iNKT cell literature complicates a long record of functional studies that suggest that, generally, CD1d-restricted T cells are autoreactive[76,85]. In mice deficient of MHCI/II-restricted T cells, the residual T cells had overt autoreactivity to CD1d.[85] Hybridomas of autoreactive clones were generated and subjected to further study, yielding two key findings that set the stage for Chapters 4 and 5 of this dissertation: 1) CD1d-autoreactive T cells can use diverse TCRs, not only the iNKT TCR, 2) some clones showed selective activation based on the endogenous lipid repertoire presented by CD1d, but some activated indiscriminately[72]. Altogether, this motivates a comprehensive redefinition of TCR-CD1d recognition that encompasses self and nonself lipid antigens, is inclusive of invariant and diverse TCRs, and contains lipid-selective as well as broadly autoreactive TCRs.

Here I define "autoreactivity" as TCR recognition of any self lipid in the context of CD1d. The DP10.7 TCR, discussed briefly above (section 1.2.5), is a prime example of this type of autoreactivity. DP10.7 is exquisitely specific for CD1d-sulfatide complexes. Other lipids presented by CD1d do not stimulate DP10.7. While the germline-encoded regions of the DP10.7 TCR, such as the CDR1δ, participate in contacts with CD1d, the adaptive CDR3δ interacts with the antigen, presumably conferring lipid selectivity[86]. In essence, this is co-recognition of a self antigen presenting molecule (CD1d) and a self lipid (sulfatide), hence: autoreactivity. To underscore this point, autoreactivity, as I have defined, includes specificity for self lipids (Figure 1.1).

However, we also know that some autoreactive CD1d-restricted T cells do not display lipid selectivity. Recall the non-selective CD1d autoreactive hybridomas mentioned above. The TCR 9C2 (also discussed previously in section 1.2.5) binds indiscriminately to many different lipid ligands in the context of CD1d, including α-galactosylceramide and β-glucosylceramide[62]. Though, technically, this TCR can bind foreign lipids, as evidenced by its recognition of α-galactosylceramide, I contend that this is still technically "autoreactivity." The interaction is dominated by TCR binding

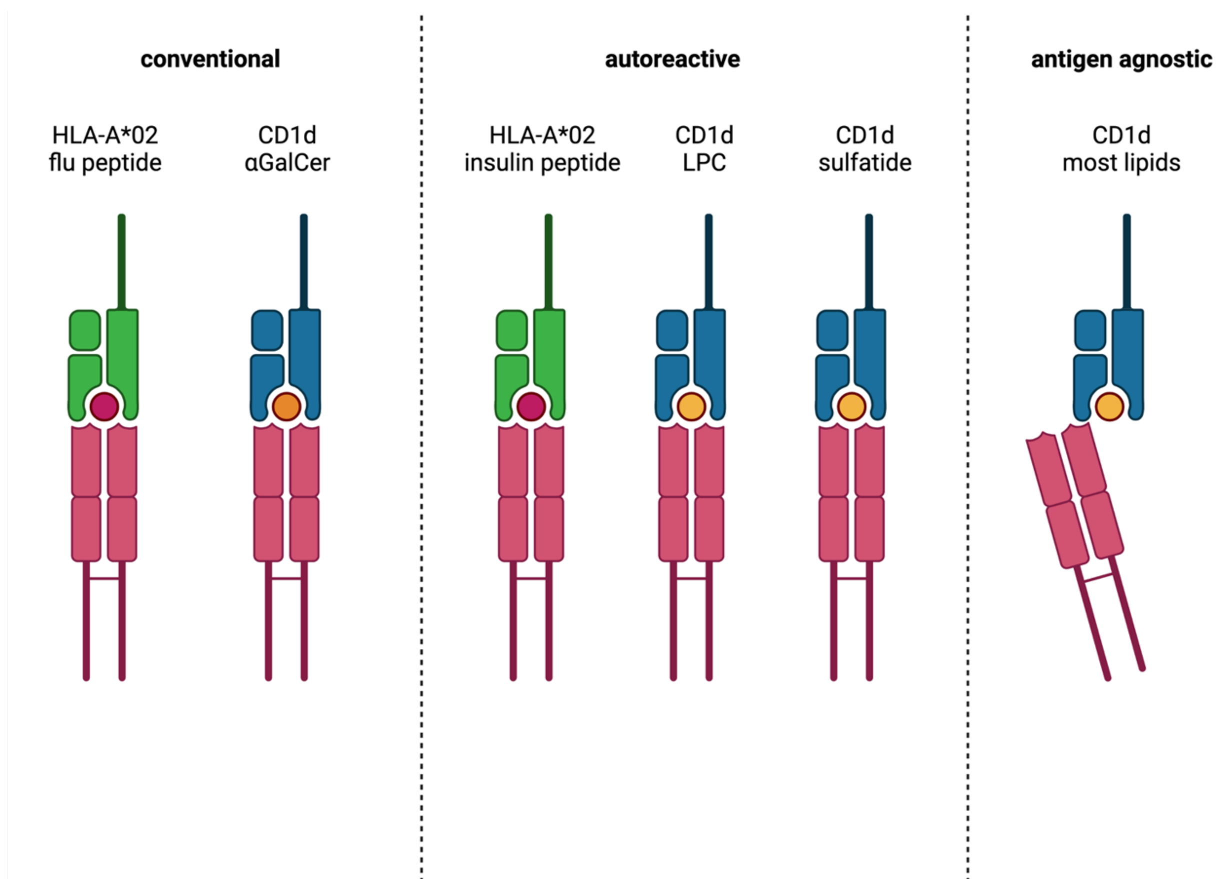


Figure 1.1: Defining TCR reactivity in the context of CD1d
Left: conventional TCRs recognize foreign peptides in the context of MHC. Similarly, some CD1d-restricted TCRs recognize foreign lipids in the context of CD1d. Middle: Autoreactivity, illustrated as specific recognition of self-derived ligands in the context of an antigen-presenting molecule. In conventional T cells, autoreactivity to self antigens, such as to an insulin peptide, can lead to autoimmunity. CD1d-restricted T cells can also be reactive to self antigens, such as lysophosphatidylcholine (LPC) or sulfatide, though this autoreactivity seems to not produce autoimmune pathology. Finally, antigen-agnostic recognition is a special case of autoreactivity. Here, the TCR binding footprint is thought to be dominated by contacts with the antigen-presenting molecule itself, resulting in co-recognition of a wide variety of possible antigens. TCRs with these characteristics are typically eliminated from the conventional TCR repertoire during thymic selection. For reasons yet unknown, broadly autoreactive/lipid-agnostic recognition seems to be permitted in CD1d-restricted TCRs.

a self molecule, CD1d, with minimal direct recognition of the lipid presented. This type of recognition, which I term "broad autoreactivity" or "lipid agonistic" recognition, is a special case of CD1d autoreactivity that accounts for lack of lipid selectivity (Figure 1.1). Promiscuous TCR recognition of lipids, however, is not unbounded. Studies with CD1a-restricted TCRs show that TCR activation is possible with a wide variety of endogenous lipids, but lipids with head groups that are highly glycosylated and interfere with the TCR-CD1a interface can break the interaction[87,88]. These are termed "permissive" and "non-permissive" lipids, respectively.

To certain extent, all T cells have some degree of autoreactivity and degeneracy in antigen recognition. Why are these distinctions of "autoreactivity" and "lipid agnostic" recognition important here? These are useful constructs for categorizing patterns of CD1d reactivity in the periphery, which I link to differences in function in Chapter 4. It is important to note that autoreactivity, as defined here, does not result in autoimmunity. Rather, both lipid-specific and lipid-selective recognition have a role in CD1d-restricted T cell function and corresponding regulation mechanisms. With antigen-specific autoreactivity, dominant negative blockers like sphingomyelin occupy the groove of CD1d and are proposed to prevent antigen-specific activation. In the case of broad autoreactivity, the balance of permissive or non-permissive lipids presented modulates cellular activation[87,88].

1.4 Vδ1$^+$ T cell responses in disease

The functional niche of Vδ2$^-$ T cells in human immunity is largely unclear. Individual vignettes have emerged illustrating their expansion and phenotype in many diseases (both of xeno- and endo-origin) but due to lack of an applicable mouse model, the necessity and/or sufficiency of Vδ2$^-$ T cells in disease outcomes is unproven. Nonetheless, many correlations have been drawn between disease status and Vδ2$^-$/Vδ1$^+$ T cell frequency and functional profile. Here, focus is drawn to diseases eliciting clonal expansion (such as viral infection), taking place in the lung (such as mycobacterium tuberculosis infection and allergy/asthma), and giving rise to population-level functional plasticity (celiac disease, cancer).

1.4.1 Viral infections

One of the earliest functions ascribed to the still-enigmatic Vδ1$^+$ T cells is the expansion of this subset in response to human cytomegalovirus (CMV). Notably, CMV seropositive adults have lasting expansions of Vδ1$^+$ T cells[89–91]. EPCR, an MHC-like protein that is upregulated in CMV, has been put forward as a mediator of this response[92], as has HLA-DR[54]. However, it is unclear what role, if any, Vδ1$^+$ cells play in protective immunity against CMV[93]. Similarly, studies have described expansions of Vδ1$^+$ T cells in the blood and gut of HIV seropositive individuals, though it is controversial whether changes in Vδ1$^+$ numbers are produced by clonal or nonspecific expansion, and unclear if the expansion impacts the course of disease[94,95]. This provides an example of Vδ1$^+$ T cells expanding as a result of antigenic challenge and persisting, leaving the Vδ1$^+$ compartment with signs of previous antigenic experience.

1.4.2 Mycobacterium tuberculosis (Mtb)

Tuberculosis is an infection of the lung, characterized by the formation of granulomas, fibrous lesions in the lung caused by the immune response to Mtb. Vδ1$^+$ T cells have been convincingly shown to undergo clonal expansion in the lung of tuberculosis patients and are correlated with control of the disease[96]. In infected individuals, there is minimal overlap between blood and lung γδ TCR sequences, indicating that the γδ T cells expanded by the Mtb infection were non-recirculating and lung resident[96]. Mycobacterium lipid-dependent clonal expansion of γδ T cells in cord blood has also been reported. It has been hypothesized that the observed clonal expansions in lung and cord blood are mediated by CD1 molecules presenting glycolipids from Mtb[97].

1.4.3 Asthma and allergy

Asthma is a chronic airway inflammatory disease, often tied to a vigorous T_H2 response that enhances pathogenic eosinophil recruitment. Oligoclonal expansions of Vδ1$^+$ T cells are thought

to occur in the lungs of asthmatic subjects, based on their frequency in bronchoalveolar lavage fluid[98,99]. It is unclear if there is any accompanying change in frequency or phenotype in blood[100], again pointing to a compartmentalized tissue resident lung γδ T cell population. Some V$\delta1^+$ T cell clones from lung were demonstrated to be CD1d-specific, and activated vigorously in response to CD1d loaded with exogenous lipids from cypress, a common allergen[99,101,102]. Functionally, V$\delta1^+$ T cells are capable of taking on a T_H2-like phenotype and producing IL-4[101]. However, many V$\delta1^+$ T cells in lung are cytolytic and produce IFNγ, hallmarks of T_H1-like response[103]. It is unclear how the typically opposed T_H1 and T_H2 responses are combined in the V$\delta1^+$ population and if this has an impact on the course of the disease.

1.4.4 Celiac disease

The gut V$\delta1^+$ intra-epithelial lymphocytes (IELs) compartment undergoes drastic remodeling with celiac disease. Celiac disease is characterized by a vigorous immune response to dietary gluten and subsequent tissue destruction. In celiac disease, expression of Btnl3/8 is lost, and the V$\gamma4^+$ T cells resident in the gut disappear[52]. The niche then fills with pro-inflammatory, cytolytic V$\delta1^+$ T cells that persist as IELs, even as patients with celiac disease adopt a gluten-free diet[52]. The antigen that drives the expansion and persistence of the pro-inflammatory cells is unclear, but it is hypothesized that the new IELs amplify inflammation in the gut. Inflammation-induced turnover of V$\gamma4^+$ T cells represents a unique paradigm in human γδ T cell biology, whereby the absence of a certain subset signifies inflammatory history.

1.4.5 Cancer

Cancer is a disease setting where V$\delta1^+$ T cells have been extensively investigated, though their role is still nebulous. In colorectal cancer, γδ T cells have variably been described as agents of tumorigenesis[104,105] and cytolytic anti-tumor immunity[105–107]. Several groups have proposed factors to help delineate these opposing functions, including V-gene clonotype[105], NKR expression[106], and

amphiregulin production[107]. Plasticity or in situ polarization has been put forward as an explanation to tie together these observations. Namely, features such cytokines, nutrient availability, and hypoxia could contribute to functional skewing of $V\delta1^+$ T cells in the tumor micro-environment, giving rise to the diverse and sometimes contradictory functions described[108].

1.5 T cell memory differentiation

1.5.1 Memory subsets in conventional T cells

Memory, that is, the ability to mount a rapid immune response to a previously encountered stimulus, is a key feature of the adaptive immune system. Both γδ T cells and their conventional T cell counterparts, CD4+ and CD8+ T cells, have this capacity. The memory response of conventional T cells has been dissected into subsets with distinct roles: T central memory cells (T_{CM}), T effector memory cells (T_{EM}), T effector memory cells re-expressing CD45RA (T_{EMRA}), and T resident memory cells(T_{RM}). T_{CM},T_{EM}, and T_{EMRA} survey the body for changes in homeostasis by traversing the lymphatic and blood circulatory systems. On the other hand, T_{RM} patrol the tissues, especially at barrier sites, but are thought to be another "branch" of the same memory differentiation process that gives rise to other T conventional memory subsets, unlocked by the presence of certain cytokines, such as IL-7, IL-15, and/or TGFβ[109].

The main memory subsets of conventional T cells, naïve T cells, T_{CM},T_{EM}, and T_{EMRA}, are classified based on their expression of CD45RA and CCR7/CD62L. CD45RA and CD45RO are isoforms of CD45. CD45RA is expressed on the surface of naïve T cells. Once a T cell has encountered antigen, CD45RO instead becomes the predominant isoform of CD45 expressed by the cell and CD45RA expression decreases. CCR7 and CD62L both play a role in trafficking to secondary lymphoid organs, such as lymph nodes. Thus, these markers logically delineate the function of the memory subsets[110]. Naïve T cells are $CD45RA^+$ and $CCR7/CD62L^+$, as they have not yet experienced a TCR signal and travel to the lymph node to scan for antigen. T_{CM} directly result

from TCR engagement, so are CD45RA$^-$, but continue still home to lymph nodes and are highly proliferative. T_{EM}, which are CD45RA$^-$ and CCR7/CD62L$^-$ are the prime functional memory subset that arise. They have encountered antigen, are functionally armed, and can access tissue sites other than the lymph node or continue to circulate. T_{EMRA} are terminally differentiated memory cells that remain CCR7/CD62L$^-$, but begin to re-express CD45RA, possibly due to time elapsed since antigen encounter. The T_{EMRA} subset represents the end of the line for conventional T cell memory differentiation. With reduced proliferative capacity, many of these cells are senescent, and furthermore, often undergo apoptosis with additional stimulation[111].

1.5.2 Memory subsets in γδ T cells

Whereas recirculation to secondary lymphoid organs is a central element of conventional memory T cell differentiation, γδ T cells, especially the Vδ2$^-$ subset, are generally sparse in lymph nodes and have a propensity to traffic directly to tissues without apparent priming. Despite this known divergence, the field remains beholden to using the same markers, CD45RA and CD62L/CCR7, to classify memory acquisition of γδ T cells and conventional T cells. Unsurprisingly, γδ T cells and conventional T cells expressing the same memory markers do not respond the same way to activation. The clearest example of this is the T_{EMRA} (CD45RA$^+$CCR7/CD62L$^-$) phenotype. In the context of CMV, Vδ1 T cells bearing this phenotype display cytotoxic potential, producing high levels of perforin and granzyme B, and also have robust production of interferon-γ[89,90,93]. Intriguingly, expansion of T_{EMRA}-phenotype Vδ1 T cells in the blood has also been reported in a variety of other inflammatory conditions[94,112]. Accordingly, designations such as "T_{EMRA}" or "T_{RM}", borrowed from conventional T cell subsets, gloss over major differences, and indeed, major open questions, in the development of γδ T cell memory.

1.6 Summary

Even though γδ T cells were discovered only shortly after αβ T cells, our understanding of their antigen specificity, biological role, and participation in disease has lagged behind. Findings from conventional T cells and mouse γδ T cells have not easily been extended to human γδ T cells, and attempts to draw parallels between these distinct cell types can obfuscate the unusual, but meaningful, features of γδ T cells. With the discovery of γδ TCRs that recognize CD1 molecules, methodical dissection of antigen-specific γδ T cell function becomes possible. More broadly, the CD1 autoreactivity exemplified by γδ T cells, but also present in some αβ T cell populations, can shine light on an emerging paradigm of intentional self recognition in the adaptive immune system.

CHAPTER 2

METHODS

2.1 Protein production and purification

CD1d-β2m was produced in Hi5 cells using the baculoviral expression system, described previously. The ectodomain of human CD1d with a C-terminal BirA biotinylation sequence, human rhinovirus 3C protease cleavage site, and hexa-histidine tag was co-expressed with BirA and untagged human β2m. The complex was purified over Nickel NTA resin (Qiagen), then treated overnight with human rhinovirus 3C protease to remove the hexa-histidine tag. CD1d-β2m was buffer exchanged and, where indicated, incubated with an 10-fold molar excess of lipid. The following lipids and conditions were used:

Table 2.1: Lipid Loading Conditions

Lipid	Product Number (Vendor)	Temperature	Duration
Lysophosphatidylcholine	845875 (Avanti Polar Lipids)	37ºC	2 hour
Mixed brain sulfatides	131305 (Avanti Polar Lipids)	37ºC	1 hour
Ganglioside GT1b	860059 (Avanti Polar Lipids)	25ºC	overnight
PBS-57	Courtesy of Dr. Paul Savage	25ºC	overnight

After loading, CD1d-β2m was further purified by size exclusion chromatography using a SuperDex 200i column (Cytivia). Biotinylation was verified by size shift on an SDS-PAGE gel following a brief co-incubation of CD1d-β2m and streptavidin. MR-1 loaded with microbial components was produced as previously reported.

2.2 Tetramer generation

Streptavidin-PE (Agilent) was combined with biotinylated CD1d-β2m or MR1-β2m at a molar ratio of 1 streptavidin (monomer) to 1.1 biotinylated monomer to produce tetramers. Oligonucleotide

barcoded tetramers were produced in the same manner, but with barcoded-conjugated streptavidin PE. Oligonucleotides barcode sequences were selected from the 10X Genomics Barcode whitelist. We used a HyNic/4FB conjugation system (Protein-Oligonucleotide Conjugation Kit, Vector Labs) to couple Streptavidin PE and the barcodes. Barcodes were synthesized with a 5' amine (IDT) to enable modification with 4-FB. Streptavidin PE was then functionalized with HyNic. The two functionalized molecules were incubated overnight at 25ºC, then conjugates were purified by gel filtration size exclusion.

2.3 Flow cytometry and sorting

Cells were resuspended in FACS buffer made of 2% fetal bovine serum (FBS, Gemini) in sterile phosphate-buffer saline free of magnesium and calcium ions (Corning). Cells were blocked with 10% heat-inactivated human serum in FACS buffer to prevent nonspecific binding. If applicable, cells were stained with 100 nM of each tetramer, diluted in 10% human serum, for 30 minutes at 25ºC. Finally, cells were stained with the indicated antibody panels diluted in FACS buffer for 30 minutes at 4ºC. Data was collected on a BD Fortessa X-20 or LSR II 4-15. For most sorting, a BD FACSAria Fusion with a 100 µm nozzle was used.

Table 2.2: Antibody Panels for Flow Cytometry

Cord Blood T Cell Phenotyping/Activation Panel			
Marker/Target	**Clone**	**Fluorophore**	**Product Number (Vendor)**
αβ TCR	IP26	PE-Cy7	25-9986-42 (Thermo Fisher Scientific)
Vδ1	TS8.2	APC	17-5679-42 (Invitrogen)
Vδ2	B6	BV711	331412 (BioLegend)
CCR7	G043H7	PerCP-Cy5.5	353220 (BioLegend)
CD45RA	HI100	BV421	582885 (BD)
CD69	FN50	APC-Fire750	310945 (BioLegend)

Table 2.2 Antibody Panels for Flow Cytometry (continued)

CD161	HP-3G10	FITC	339905 (BioLegend)
Fixable Live Dead	-	Aqua	L34957 (Thermo Fisher Scientific)

Cord Blood T Cell Sorting Panel

Marker/Target	**Clone**	**Fluorophore**	**Product Number (Vendor)**
CD3	UCHT1	APC-Fire750	300469 (BioLegend)
αβ TCR	IP26	PE-Cy7	25-9986-42 (Thermo Fisher Scientific)
γδ TCR	B1	FITC	331217 (BioLegend)
CCR7	G043H7	PerCP-Cy5.5	353220 (BioLegend)
Fixable Live Dead	-	Aqua	L34957 (Thermo Fisher Scientific)

Lung Phenotyping Panel

Marker/Target	**Clone**	**Fluorophore**	**Product Number (Vendor)**
CD45	HI30	BV510	304036 (BioLegend)
CD14	M5E2	PerCP-Cy5.5	301824 (BioLegend)
CD3	UCHT1	Pe-Cy7	300420 (BioLegend)
Vδ1	TS8.2	FITC	TCR2730 (Invitrogen)
Vδ2	B6	BV711	331412 (BioLegend)
Vα7.2	3C10	BV605	351720 (BioLegend)
CD45RA	HI100	BV421	582885 (BD)
CD62L	DREG-56	AlexaFluor-700	304820 (BioLegend)

Lung Lymph Node Phenotyping Panel

Marker/Target	**Clone**	**Fluorophore**	**Product Number (Vendor)**
CD3	UCHT1	PE-Cy7	300420 (BioLegend)

Table 2.2 Antibody Panels for Flow Cytometry (continued)

Vδ1	TS8.2	FITC	TCR2730 (Invitrogen)
Vδ2	B6	BV711	331412 (BioLegend)
CD45RA	HI100	BV421	582885 (BD)
GPR183	SA313E4	APC	368907 (Biolegend)
Fixable Live Dead	-	Near IR	L10119 (Thermo Fisher Scientific)

10X Sample Preparation Sort Panel			
Marker/Target	**Clone**	**Fluorophore**	**Product Number (Vendor)**
CD3	UCHT1	APC	300412 (BioLegend)
CD14	M5E2	BV421	301830 (BioLegend)
Fixable Live Dead	-	Blue	L23105 (Thermo Fisher Scientific)

Cell Line Activation Panel			
Marker/Target	**Clone**	**Fluorophore**	**Product Number (Vendor)**
CD3	UCHT1	FITC	300406 (BioLegend)
CD69	FN50	APC-Fire750	310945 (BioLegend)
Fixable Live Dead	-	Near IR	L10119 (Thermo Fisher Scientific)

2.4 Lung mononuclear cell isolation

Cells were isolated from dissociated lung tissue as described previously. Briefly, human lung samples were obtained from Gift of Hope Regional Organ Bank of Illinois, from organ donations that were not used for transplantation. Samples were denoted as “asthmatic” or “nonasthmatic” based on self-reported asthma status. Therefore, “nonasthmatic” samples cannot be considered “healthy controls” because they were not verified to be free of all lung disease. Lung tissue was perfused with

FBS and PBS. Lymph nodes attached to the lungs dissected and pooled. Lymph nodes were mechanically dissociated into a single cell suspension and cryopreserved. Likewise, lung lobes were mechanically dissociated, enzymatically digested, and cryopreserved. For use in experiments, the lung and lymph node samples were thawed and subjected to density gradient centrifugation with Ficoll to remove dead cells and debris before proceeding to flow cytometry staining or sample preparation for single-cell RNA sequencing.

2.5 Sample preparation for single-cell RNA sequencing

Cells were stained with the relevant flow cytometry antibody panel, and additionally incubated with the following TotalSeqC antibodies (Biolegend): IgG1 K (C0090), γδ TCR (C0139), CD45RA (C0063), CCR7 (C0148), CD62L (C0147), CD4 (C0045), CD8 (C0046). Live $CD3^+$ $CD14^-$ $tetramer^+$ cells were then sorted on a BD FACSymphony cell sorter and immediately subjected to GEM generation and library preparation using the 10X Genomics Chromium v2 5' Single Cell Immune Profiling Solution (10X Genomics). In addition, 5 ng of cDNA was put toward γδ TCR transcript amplification, which was amplified with custom primers and processed otherwise according to the manufacturer's instructions for the αβ library. Libraries for all samples were pooled and sequenced on a NovaSeq6000 (Illumina), and resultant FASTQs were demultiplexed by the University of Chicago Genomics Facility.

2.6 Single-cell RNA sequencing data analysis

FASTQ files were preprocessed using the Cellranger pipeline (3.0 or 7.0) developed by 10X Genomics. Reads were aligned to the GRCh38 reference genome. For alignment of the γδ sequences, we used a custom reference gene set consisting of only TRG/TRD sequences from IMGT. Alternatively, previously published datasets were accessed from the GEO portal (GSE205720). Unfiltered TCR contigs were paired, quantified, and analyzed using scRepertoire (v 1.7.2). scRepertoire was

also used to quantify clonal expansion by cell count and diversity indices. Only cells with paired TCR sequences were included in repertoire analyses; rare cells with both αβ and γδ TCR sequences called were excluded. TCR sequence data was further assessed for biochemical characteristics using Tcrdist3 (0.2.2) and Gliph2 (accessible online http://50.255.35.37:8080/). Processed count matrices were analyzed with Seurat (4.3.0.1). Quality control steps were performed to filter out low-quality cells based on total gene counts, unique molecular identifiers (UMIs), and mitochondrial gene expression. Data from each sample was normalized with SCTransform (0.3.5) and integrated using Harmony (0.1.1) to correct for batch effects from samples processed on different days. Differentially expressed genes were identified by Wilcoxon rank-sum test. GO Term enrichment was performed using enrichR (3.2). Pseudotime trajectory was applied to model differentiation using Monocle (2.24.1). Genes that varied significantly over pseudotime were identified with Moran's I test and grouped into modules with a Louvain method. All analyses were done in R (4.2.1) and Python (3.7.6). Graphs were made using ggplot2 (3.4.3) and cowplot (1.1.1) or Prism 7.

2.7 Cell lines

C1R lymphoblastoid cells were maintained in RPMI-1640 with 10% FBS (Corning). C1R-CD1d cells were previously generated, briefly: C1R cells were transfected with empty or full-length-CD1d pcDNA 3.1 vectors; following drug selection, C1R-CD1d cells had high and uniform surface CD1d expression verified by flow cytometry staining with anti-CD1d-PE (clone 51.1, Biolegend). TCRβ deficient Jurkat J.RT3-T3.5 cells were obtained from ATCC and cultured in RPMI-1640 with 10% FBS (ATCC). For activation assays dependent on CD1d, CD1d deficient J.RT3-T3.5 cells were used. Lenti-X 293T cells (Takara) were maintained in DMEM with 10% FBS. For polyclonal IEL lines, cells isolated from human colon samples were sorted as indicated on the basis of Vδ1 and Vγ9 expression. Sorted cells were co-cultured with irradiated B-lymphoblastoid cell lines and heterologous PBMC, and grown in RMPI-1640 with 10% Human Serum supplemented with 300 U/mL IL-2 (Biolegend) and 1 μg/mL phytohemagglutinin (EMD Millipore) to expand. All cell

lines were verified to be free of mycoplasma prior to experiments.

2.8 Lentiviral constructs, cloning, and transduction

Full TCR sequences were cloned into the pHIV-EGFP backbone, a gift from Bryan Welm and Zena Werb (Addgene plasmid # 21373). In brief, each TCR sequence was amplified using primers with overhangs to add restriction cut sites to the 5' and 3' ends. Fragments were then digested with XbaI and XmaI restriction enzymes (NEB) and ligated into pHIV-EGFP. Ligations were transformed into Stbl3 competent cells (Invitrogen). Single colonies were inoculated, miniprepped, and validated by restriction digest and sanger sequencing. Lentivirus were produced using Lenti-X 293T cells. In brief, packaging cells were plated, grown to 85% confluence, and then transfected with Lipofectamine 3000 (Invitrogen). An equal molar ratio of a previously cloned TCR transfer plasmid, pMD2.G envelope plasmid, and psPAX2 packaging plasmid were used. Viral supernatant was collected 48 hours post-transfection, passed through a 0.45 µm low protein-binding membrane filter (Fisher Scientific), aliquoted, and stored at -80ºC. JRT.3-T3.5 cells or JRT.3-T3.5 CD1d-/- cells were transduced with viral supernatant through spinoculation for 1 hour at 800g at 33ºC in the presence of polybrene (Fisher Scientific).

2.9 Cell line activation assays

The day before seeding, JRT.3-T3.5 Jurkat cell lines were resuspended in fresh media at a concentration of 5.0×10^5 cells per mL. Where indicated, C1R cell lines were loaded by overnight incubation with an excess of lipid. After overnight incubation, lipids were removed from the C1R cells were suspended in fresh media. C1R cells were seeded into round bottom 96 well tissue culture treated plates (Corning or Genesee) at the 5.0×10^4 cells per well, unless otherwise noted. Jurkat cell lines were resuspended in fresh media and added at a density of 5.0×10^4 cells per well. After 14-18 hours, the plates were centrifuged to collect the cells, and the cells were stained with the cell line

activation panel and analyzed.

2.10 Stimulation of primary cord blood T cells

Autoreactive T cells from cord blood were sorted on Live $CD3^+$ $\alpha\beta TCR^+$ $\gamma\delta TCR^-$ CD1d-endo $tetramer^+$ $CCR7^{high}$. Cells were seeded at $1.0\text{-}2.0\times10^4$ cells per well per well in a 96 well plate with CD1d-coated beads or in wells coated with anti-CD3, then centrifuged briefly to encourage contact with the stimulus. Cells were incubated for 5 days, then analyzed with the Primary Cell Activation antibody panel.

2.11 Phylogenetics and alignments

Full length DNA and amino acid sequences were obtained from IMGT[14]. Multi-sequence alignments were generated using the web-based Clustal Omega software using default parameters.

2.12 Connecting peptide structural prediction

Connecting peptide amino acid sequences corresponding to TRGC1 (DVITMDPKDNCSKDANDTL-LLQLTNTSAYYMY), TRGC2*01 (DVTTVDPKDSYSKDANDVITMDPKDNWSKDANDTL-LLQLTNTSAYYMY), and TRGC2*05 (DVTTVDPKDSYSKDANDVTTVDPKYNYSKDAND-VITMDPKDNWSKDANDTLLLQLTNTSAYYMY) were obtained from IMGT. Modeled structures and quality assessment metrics (pLDDT) were obtained using AlphaFold (2.0) in monomer mode[113].

2.13 TRGC transcript PCR

Healthy PBMCs from 2 donors were sorted as described above and resuspended in 1mL TRI-zol reagent (Thermo Scientific). Cryopreserved polyclonal IEL lines were thawed, rested overnight in

complete media and resuspended in 1mL TRI-zol. RNA was extracted by chloroform/isopropanol precipitation. cDNA was generated using the Maxima cDNA synthesis kit (Thermo Scientific). The TCR gamma constant region was amplified from the cDNA with DreamTaq (Thermo Scientific). The size of the amplicon was visualized with gel electrophoresis and TRGC gene usage was determined visually.

2.14 Oligonucleotide sequences

Oligonucleotides were synthesized by Integrated DNA Technology. 10X Barcodes were ordered with a 5' amino modifier, /5AmMC6/, for conjugation.

Table 2.3: Oligonucleotide Sequences

ID	Method	Sequence
10X Barcode 2	Barcoded tetramer generation	CTCCAGCTCGAGCTC
10X Barcode 3	Barcoded tetramer generation	GGACGCAACTTAAGA
10X Barcode 18	Barcoded tetramer generation	TAGGTTACGAGTGTG
10X Barcode 19	Barcoded tetramer generation	CAAGCGCGGCTTCCG
10X R1 Forward	γδ TCR library preparation	CTACACGACGCTCTTCCGATCT
10X TRG outer Reverse	γδ TCR library preparation	AGTCTTCATGGTGTTCCCCTCC
10X TRG inner Reverse	γδ TCR library preparation	AAAATAGTGGGCTTGGGGGA
10X TRD outer Reverse	γδ TCR library preparation	GTCGTGTTGAACTGAACATGTCAC
10X TRD inner Reverse	γδ TCR library preparation	CAGACAAGCGACATTTGTTCCA
TRGC Exon 2 Forward	TRGC transcript PCR	TTCCCCCAAGCCCACTATTT
TRGC Exon 2 Reverse	TRGC transcript PCR	GTAATATGCAGAGGTGTTTG

CHAPTER 3

TCRγ CONSTANT REGION USAGE TUNES ANTIGEN RESPONSIVENESS OF THE γδ TCR

3.1 Introduction

T lymphocytes are an integral component of the adaptive immune system. These cells undergo genetic recombination to yield diverse antigen receptors capable of recognizing varied molecular surfaces with precise specificity. TRB or TRG genetic loci can be recombined in combination with TRA/TRD to make an αβ or γδ T cell receptor (TCR) respectively[2]. The contribution of V, D, and J genes to the diversity and function of the TCR is well-documented, as they comprise the highly variable CDR3 loops responsible for antigen recognition. However, the incorporation of different constant region genes (in primates: TRBC1/TRBC2 for αβ or TRGC1/TRGC2 for γδ) is an underappreciated source of additional heterogeneity in the TCR sequence repertoire.

Previous work has shown that TRBC1/TRBC2 usage does not impact antigen recognition nor signal transduction through the αβ TCR. TRBC1 and TRBC2 are nearly identical in sequence and differ by only four residues in their extracellular domains. During thymic development, one of the TRBC genes is irreversibly incorporated into the recombined αβ TCR at random. TRBC1 and TRBC2 are represented in the TCR repertoire of healthy individuals at consistent ratios, and this ratio does not significantly change during infection, suggesting that both forms of the αβ TCR are equally capable of responding to antigen.

In addition to a handful of conservative substitutions in the extracellular domain, TRGC1 and TRGC2 significantly differ in the connecting peptide sequence[16,114]. Notably, TRGC2 lacks a cysteine in the connecting peptide that allows TRGC1 to form a disulfide linkage with TRDC[115]. The overall divergence of the TRGC connecting peptides is the result of replication of an exon. TRGC1 has a single copy (DVITMDPKDNCSKDAN). Foundational work demonstrated that TRGC2 has at least two, and occasionally three copies of this exon in the locus[115–117].

Beyond differences in sequence, little is known about the consequences of TRGC1/TRGC2 usage in the γδ TCR. Several lines of evidence hint that they may have distinct functional roles. TRGC1 and TRGC2 are associated with different subsets of γδ T cells[118,119]. Vγ9Vδ2 T cells, an innate-like subset that patrols the peripheral blood of adults, are reported to be predominantly TRGC1^{+}. In contrast, Vδ2^{-} T cells, which reside in barrier tissues, have been shown to be mostly TRGC2^{+}. There is a temporal shift in thymic output from TRGC1 to TRGC2 usage in the perinatal period that coincides with the production of these subsets[19,119]. Skewed variable gene usage, differences in antigen specificity, and separate locations in the body complicate direct comparisons between TRGC1^{+} and TRGC2^{+} cells. Thus, the consequence of TRGC1 vs TRGC2 incorporation into the γδ TCR remain unknown.

In this chapter, I discuss my experiments testing whether there is a functional consequence of TRGC1 versus TRGC2 domain usage and explore the usage of these domains in the human immune system. To approach this question, we generated TRGC domain swapped versions of Vγ9Vδ2 and Vδ2^{-} TCRs. In these reductionist systems, the TRGC2 constant domain reduced sensitivity to stimulus compared to TRGC1. Leveraging naturally-occurring human genetic variation in the TRGC locus, we show that activation strength is dependent upon the number of exon repeats in the connecting peptide, which differs between TRGC1 and TRGC2. We demonstrate that the weakest variant we tested, TRGC2(3x), despite its attenuated signal strength, is found in TCRs in the periphery. Finally, we show that TCRs with either TRGC1 or TRGC2 can expand and mediate anti-tumor immunity, but clones using TRGC2 appear hypofunctional. Together, these results demonstrate that TRGC2 usage can modify signaling of γδ TCR.

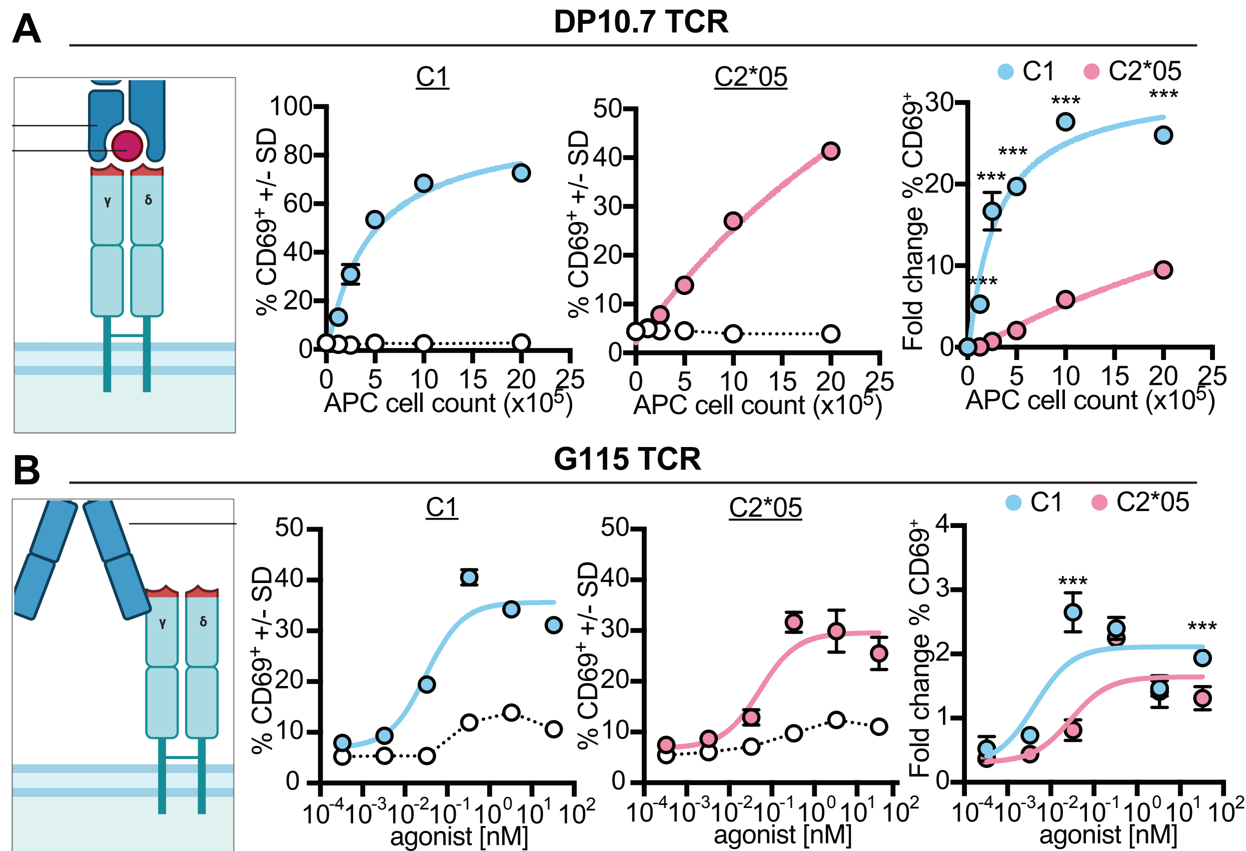


Figure 3.1: TRGC2 reduces sensitivity to antigen compared to TRGC1

(A) Left: diagram of DP10.7 TCR recognition of CD1d presenting sulfatide. Middle left: DP10.7 TRGC1 CD69 induction by coculture with increasing number of CD1d+ C1R cells loaded sulfatide (blue filled circles) or unloaded (white circles). Best fit curve (used to estimate EC50) in blue. Middle right: DP10.7 TRGC2*05 CD69 induction by coculture with increasing number of CD1d+ C1R cells loaded sulfatide (pink filled circles) or unloaded (white circles). Best fit curve (used to estimate EC50) in pink. Right: Comparison of DP10.7 TRGC1 (C1-blue) and DP10.7 TRGC2*05 (C2*05- pink) CD69 induction normalized to unloaded CD1d+ C1R for each cell line. **(B)** Left: diagram of G115 TCR recognition of BTN2A1 heterodimer. Middle left: G115 TRGC1 CD69 induction by coculture with increasing number of CD1d+ C1R cells loaded sulfatide (blue filled circles) or unloaded (white circles). Best fit curve (used to estimate EC50) in blue. Middle right: G115 TRGC2*05 CD69 induction by coculture with increasing number of CD1d+ C1R cells loaded sulfatide (pink filled circles) or unloaded (white circles). Best fit curve (used to estimate EC50) in pink. Right: Comparison of G115 TRGC1 (C1-blue) and G115 TRGC2*05 (C2*05- pink) CD69 induction normalized to unloaded CD1d+ C1R for each cell line.

3.2 Results

3.2.1 TRGC2 attenuates γδ TCR signaling

I hypothesized that TRGC1 and TRGC2 could impart differences in activation through the γδ TCR. To test this, I engineered constructs of DP10.7, a Vδ1 TCR with exquisite antigen specificity to the lipid sulfatide presented by CD1d[62]. One construct was designed with the full length DP10.7 γ and δ sequence and the endogenous constant region, TRGC1. The other construct was identical, except TRGC2 was substituted for TRGC1. We transduced CD1d-/- JRT.3-T3.5 Jurkat lines with the DP10.7 TCR constructs, referred to as C1 and C2*05. The C1 and C2*05 constructs generated approximately equal TCR expression, indicating similar surface stability of the DP10.7 TCR complex for both constant regions. The two lines also upregulated CD69 expression and fluxed Ca^{2+} similarly with anti-CD3 stimulation. From these data, I concluded that the C1 and C2*05 lines were equivalent downstream of the TCR-proximal steps of activation.

Next I tested if there was a difference between DP10.7 C1 and C2*05 with antigen-specific activation, again using CD69 expression as a readout of activation. I co-cultured C1 and C2*05 with an antigen presenting cell (APC) line with ectopic CD1d expression, loaded with an excess of sulfatide to promote activation through the DP10.7 TCR. In this assay, when compared to C1, C2*05 required a higher target:effector ratio to induce CD69 expression. Additionally, the maximum % CD69 achieved by C2*05 was lower than C1, and never saturated (Figure 3.1a). This suggests that the C2*05 constant domain reduces the sensitivity to activation. Indeed, when the C1 and C2*05 lines were directly compared, the C2*05 line required more sulfatide antigen to enable signal transduction.

The Vδ2⁻ TCRs (including DP10.7) mediate activation by binding antigen with their highly variable CDR loops. Vδ2Vγ9 TCRs, in contrast, are triggered through a distinct mechanism that depends on germline-encoded residues in the γ chain. To see if this other mode of activation was also affected by TRGC1/TRGC2 usage, I again engineered C1 and C2*05 constant domain con-

structs for a known Vδ2Vγ9 TCR, G115[120]. In this system, we also found that the C2*05 was less effective at mediating activation through the G115 TCR (Figure 3.1b). Taken together, the data suggest that C1 and C2*05 could impart differences in the ability of the γδ TCR to transform extracellular ligand binding to signal transduction.

3.2.2 *Genetic variation at the TRGC locus modulates TCR signal strength*

I reasoned that the disulfide present in the TRGC1 connecting peptide could strengthen the intermolecular interaction between the γ and δ chain, resulting in stronger TCR signaling. I generated additional DP10.7 TCR mutant lines: a TRGC1 connecting peptide C->A mutant to remove the interchain disulfide bond, and a TRGC2*05 connecting peptide SY->NC mutant to attempt to introduce a disulfide bond with TRDC. The DP10.7 lines were tested in the assay, and surprisingly, the C1 and C1 C->A mutant activated equivalently (Figure 3.2a). Correspondingly, addition of a cysteine to the connecting peptide to recruit a disulfide bond did not improve the activation of C2*05, but did affect the overall stability of the DP10.7 TCR. Thus, I conclude that the difference in activation between C1 and C2*05 is not merely due to the presence or absence of the interchain disulfide bond.

I hypothesized that the repeats present in the allelic forms of TRGC2 could be tuning signal strength (Figure 3.2b). Since our previous experiments were done with TRGC2*05, a TRGC2(3x) allele, I included a DP10.7 mutant with the TRGC2*01 constant domain to see if TRGC2(2x) would produce an intermediate phenotype. Here, I saw a dose-dependent decrease in activation based on exon 2 repeats, with TRGC1 (1 copy) activation exceeding TRGC2(2x) (2 repeats), which still enabled a more activation than TRGC2(3x) (3 repeats) (Figure 3.2c). In an attempt to understand the relationship between the connecting peptide sequence and signaling ability, I generated structural models with Alphafold[113]. Notably, the TRGC1 connecting peptide adopted a helical conformation, whereas the TRGC2*05 has no clear secondary nor tertiary structure (Figure 3.2d). Alphafold generates a measure of model accuracy for each residue, predicted local distance difference test

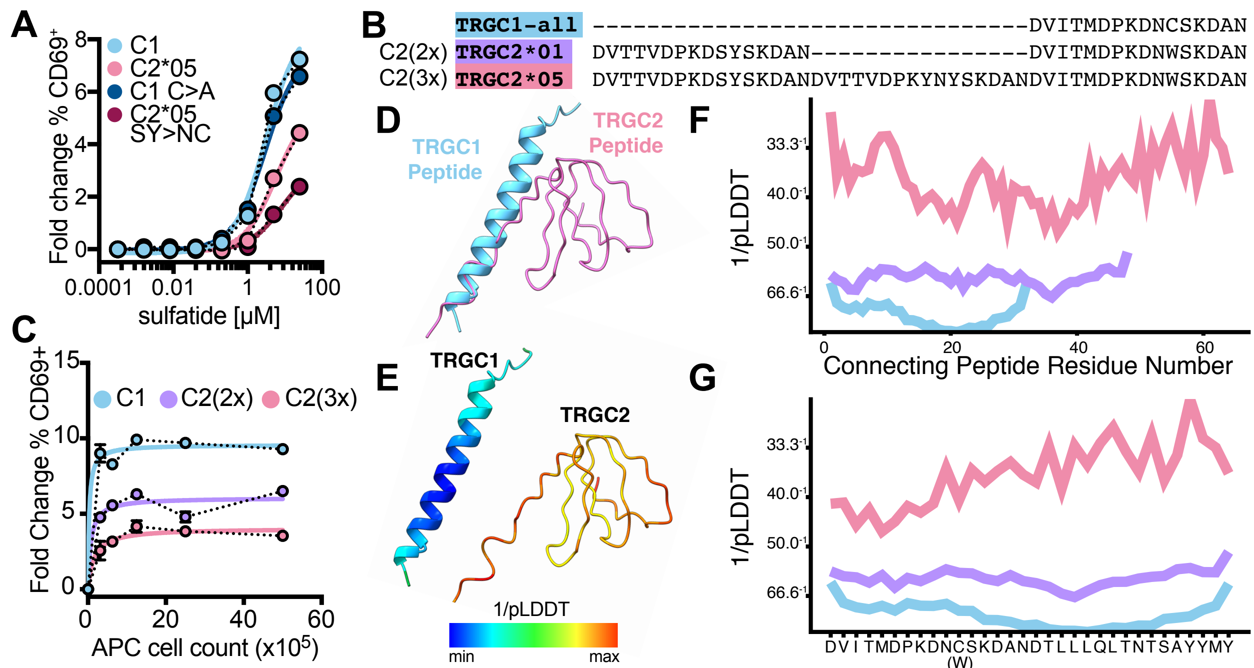


Figure 3.2: Genetic variation at the TRGC locus modulates TCR signal strength
A) Comparison of CD69 induction with increasing sulfatide concentration among DP10.7 TRGC1 (C1-blue), DP10.7 TRGC2*05 (C2*05- pink), and disulfide mutants (DP10.7 TRGC1 C->A, navy; DP10.7 TRGC2*05 SY->NC, maroon). **B)** Connecting peptide sequences of TRGC1 and selected TRGC2 allelic variants, aligned manually by exon sequence similarity. **C)** Comparison of DP10.7 TRGC1 (C1-blue), DP10.7 TRGC2*01 (C2(2x) - purple), and DP10.7 TRGC2*05 (C2(3x) - pink) CD69 induction normalized to unloaded CD1d+ C1R for each DP10.7 line. Best fit curves (used to estimate EC50) in corresponding colors. **D)** Modeled structures of the TRGC1 (blue) and TRGC2*05 (pink) connecting peptide with TRGC2*05 connecting peptide aligned to TRGC1. **E)** Modeled structures of the TRGC1 (left) and TRGC2*05 (right) colored by the inverse of their predicted local distance difference test (1/pLDDT). Lowest pLDDT/highest intrinsic disorder is colored in red, highest pLDDT/lowest intrinsic disorder is colored in blue. **F)** 1/pLDDT values by residue for TRGC1 (blue), TRGC2*01 (purple), and TRGC2*05 (pink) connecting peptides. **G)** 1/pLDDT values for the conserved terminal 33 amino acid residues of the TRGC1 (blue), TRGC2*01 (purple), and TRGC2*05 (pink) connecting peptides.

score (pLDDT), which inversely correlates with intrinsic disorder[121]. To visualize the local intrinsic disorder of the connecting peptides, I superimposed the inverse pLDDT score onto the models. I observed that the TRGC2*05 connecting peptide was predicted to be globally intrinsically disordered, while the model confidence for the TRGC1 connecting peptide helix was high (Figure 3.2e). These results were consistent when the TRGC connecting peptides and the TRDC connecting peptide were modeled together in multimer mode, or when full length γ and δ sequences were used in the model.

I quantified the intrinsic disorder of the connecting peptides of TRGC1, TRGC2(2x) and TRGC2(3x) by plotting 1/pLDDT per residue for each model. Strikingly, the predicted disorder of the entire sequence increased as a function of the number of exon 2 repeats (Figure 3.2f). This suggests that the context of the repeats can destabilize the whole length of the connecting peptide. To further illustrate this point, I focused on the 33 connecting peptide residues most proximal to the membrane (Figure 3.2g). Though these amino acids are highly conserved in all of the connecting peptides (save for the cysteine, which I have shown to be dispensable), the presence of exon repetitions is predicted to introduce disorder to the sequence – the more repeats, the more disorder.

Next, I sought to understand if the TRGC2(3x) allele, which significantly impairs T cell activation, is actually present in human populations. Previous studies depending on RFLP technology suggested that the TRGC2(3x) alleles are not rare, occurring in various populations at a frequency of 13-68%[116]. With the advent of short-read NextGen sequencing, it has become challenging to call structural variants such as TRGC2(2x) and TRGC2(3x). I re-analyzed the long-read sequencing dataset from Sudmant et al[122] and indeed, I found genomic structural variants that correspond to the 2x and 3x alleles of TRGC2. This study is not unbiased, but was instead designed to provide a picture of global genetic variation, which indicates that TRGC2(3x) alleles are significantly represented in indigenous human populations across the globe (Figure 3.3a). Of the two ancient human DNA samples included in this study, both the Denisovan and Neanderthal individuals were homozygous for TRGC2(3x). Additionally, I can see that TRGC2 with exonic repeats is present

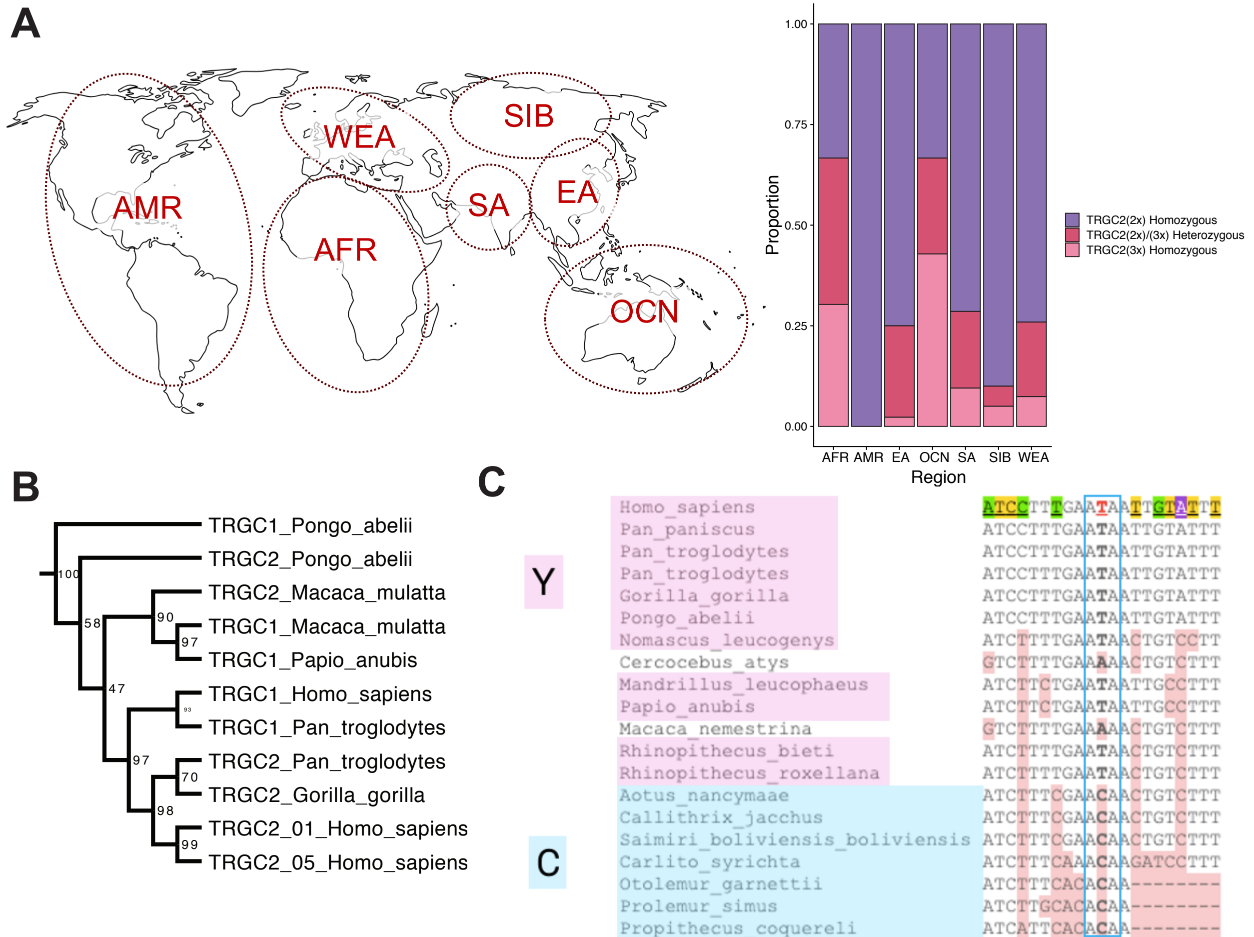


Figure 3.3: Evolution of TRGC2 and alleles

A) Analysis of allelic variation in TRGC2 exon 2 across indigenous global populations. **B)** Phylogenetic tree showing relatedness of human and ape TRGC2 genes. **C)** Sequence analysis of TRGC2 exon 2 repeat 1 ”SYS” among primates.

in old world primates and apes as well as humans, but absent in new world monkeys (Figure 3.3b, Figure 3.3c). In depth sequence analysis of alleles of TRGC2 expressed in primates reinforces this finding, showing that old world monkeys, apes, and human populations have a similar ”SYS” containing exon 2 TRGC2 sequences, like TRGC2*05. This suggests that the TRGC2(3x) allelic variant arose relatively recently in evolutionary time and is under selective pressure[123].

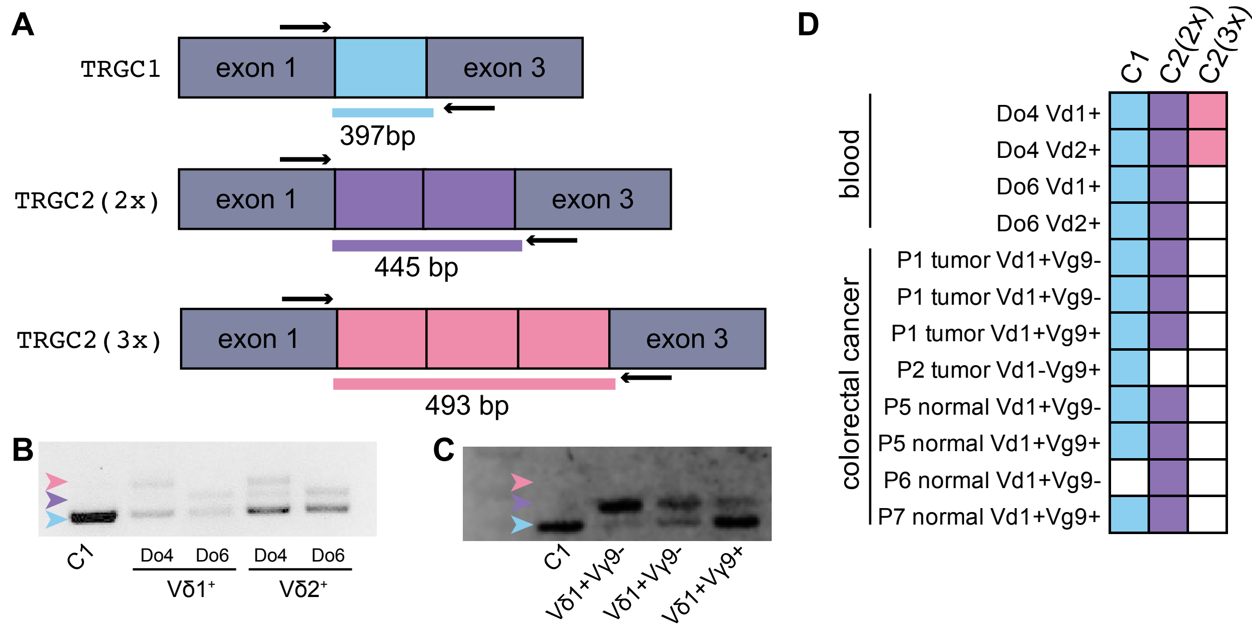


Figure 3.4: TRGC1, TRGC2(2x), and TRGC2(3x) are all used by TCRs in vivo
A) Schematic indicating amplification strategy for distinguishing TRGC1, TRGC2(2x), and TRGC2(3x) from human samples with expected product size indicated. Black arrows represent approximate primer binding sites. **B)** Agarose gel visualization of TRGC amplicon from sorted PBMC γδ T cells. Expected amplicon size highlighted with arrows: C1, blue; C2(2x), purple; C2(3x), pink. Lane 1, JR2 a monoclonal cell line uniformly expressing C1 (positive control). Sorted Vδ1+ T cells from Donor 4 (Do4), lane 2, and Donor 6 (Do6), lane 3. Sorted Vδ2+ T cells from Donor 4 (Do4), lane 4, and Donor 6 (Do6), lane 5. **C)** Agarose gel visualization of TRGC amplicon from CRC IEL cell lines from P1. Expected amplicon size highlighted with arrows: C1, blue; C2(2x), purple; C2(3x), pink. Lane 1, JR2 a monoclonal cell line uniformly expressing C1 (positive control). Lane 2, line sorted on Vδ1+Vγ9-; Lane 3, sorted on Vδ1+Vγ9-; Lane 4 sorted on Vδ1+Vγ9+. **D)** Schematic of PCR results, presence of corresponding band in each sample is indicated by shading.

3.2.3 TRGC1, TRGC2(2x), and TRGC2(3x) are all used by TCRs in vivo

In addition to evolution within the human population, selection occurs on an individual level in the T cell repertoire. A weaker version of the TCR could be maintained in the global population, but eliminated during thymic selection in an individual host. To assay for TRGC usage in the periphery of human subjects, not just presence of the gene or allele in the genome, I developed a PCR protocol that produced distinct bands corresponding to C1, C2(2x), or C2(3x) usage (Figure 3.4a). Peripheral blood mononuclear cells (PBMCs) were sorted on the basis of Vδ1 vs Vδ2 staining, then assayed

in bulk for TRGC usage by PCR. This semi-quantitative measure suggested that TCRs expressed by γδ T cells in PBMC do not follow a simple Vδ1-TRGC2 and Vδ2-TRGC1 pairing rule (Figure 3.4b). Rather, Vδ1 and Vδ2 have heterogeneous TRGC usage. Based on band intensity, TRGC1 usage is more common, though far from exclusively used, in the Vδ2 population. This PCR also revealed the use of both TRGC2(2x) and TRGC2(3x) from an apparently heterozygous individual.

To ascertain if additional repertoire shaping could occur in the tissues, we performed the same TRGC amplification assay on polyclonal γδ T cell lines, derived from colorectal cancer (CRC) tumor intra-epithelial lymphocytes (Figure 3.4c). We looked at lines that had been previously sorted on the basis of Vδ1 or Vδ2 TCR expression and non-specifically expanded in the presence of PHA and IL-2. Again, we noted promiscuous TRGC usage, suggesting that both TRGC1- and TRGC2- containing TCRs are able to traffic to tissues and infiltrate the tumor microenvironment. In summary, we found that TRGC1 and TRGC2 were present in human blood and barrier tissue γδ T cell populations, suggestive of both heterogeneity and persistence of TRGC1 and TRGC2 clonotypes (Figure 3.4d).

3.2.4 Both TRGC1 and TRGC2 clones are present in colorectal tissue and can infiltrate tumors, but TRGC2 clones are hypofunctional in tumors

Next, I wanted to explore TRGC1 and TRGC2 usage in unmanipulated γδ cells to get a more concrete idea of their respective frequencies. I reanalyzed a single cell RNA sequencing dataset of γδ T cells sorted from colorectal cancer tumors (T) or adjacent normal tissue (N), deposited by Reis et al[105]. Cells were designated as TRGC1 or TRGC2 based on their cellranger V(D)J call. Like to our CRC IEL lines, I found that all samples analyzed contained T cell populations using TRGC1 and TRGC2 (Figure 3.5a). Interestingly, γδ T cells derived from the tumor and from adjacent normal tissue from the same patient tended to have similar ratios of TRGC1:TRGC2. No other clear patterns emerged when TRGC1 and TRGC2 instances were compared between tumor and normal; the number of γδ T cells in the tumors was reduced for both TRGC1 and TRGC2 (Figure

3.5b).

Next, I broke down TRGC1 and TRGC2 on a clonal level. Clonotypes were defined as cells having identical TCR by CDR3γ amino acid sequence. I excluded a single, hyperexpanded clone from p989N. I found that within clonotypes (or clones) using the same CDR3γ, TRGC usage was not uniform, but typically skewed heavily toward either TRGC1 or TRGC2 (Figure 3.5c). It is unclear if this variation within clones reflects cells switching from TRGC1 and TRGC2 during clonal expansion, or if it is due to wobble in the short-read alignments for the highly similar TRGC1 and TRGC2 sequences. Regardless, when I focused on individual expanded clones shared between tumor and normal on a per-patient level, TRGC1 clones were more expanded than TRGC2 clones (Figure 3.5d). This was especially apparent in the tumor, where most TRGC2 clones were singletons (Figure 3.5e). These γδ repertoire data imply that though TRGC1 and TRGC2 TCRs are both present in the colon and can both infiltrate tumors, TRGC2 clones expand less readily in the tumor, in line with the idea that TRGC2 is less sensitive to stimulation.

Finally, I checked for functional consequences of weaker TCR signaling by TRGC2, this time using an in-house generated single cell RNA sequencing dataset of γδ T cells sorted from colorectal cancer samples. When we assessed differentially expressed genes between TRGC1 and TRGC2 clonotypes, the TRGC2 clones appeared to be hypofunctional relative to the TRGC1 clones (Figure 3.5f). Apart from TRGC2 transcript itself, only *ZBP1*, a cytosolic pattern recognition receptor, emerges. In contrast, TRGC1 clones had higher expression of genes associated with TCR signaling (*CD6, SLA*) and response to cancer (*FASL, CSF1*) and proliferation (*NPAT*), and appear to be more active overall.

3.3 Discussion

In this report, we used antigen specific stimulation of domain-swapped TCRs to demonstrate how variation in TRGC genes and alleles alter sensitivity to antigen. We show that TRGC1 transmits the strongest signal strength, whereas TRGC2(2x) requires more antigen to trigger a lower-amplitude

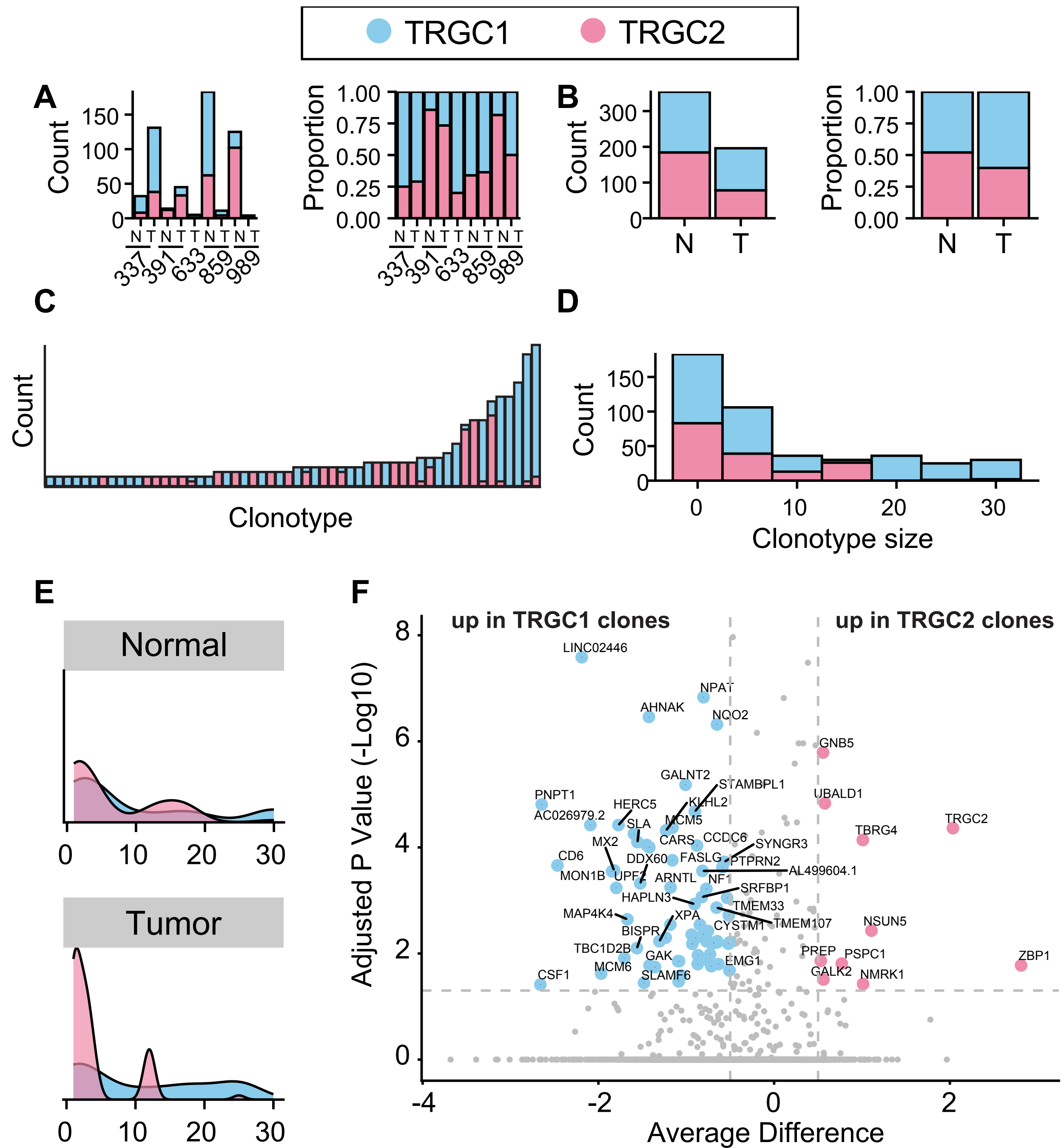


Figure 3.5: Both TRGC1 and TRGC2 clones are present in colorectal tissue and can infiltrate tumors, but TRGC2 clones are hypofunctional in tumors

Figure 3.5 (continued)

A) Bar chart showing the number (left) or proportion (right) of γ chain sequences in each colorectal tissue sample using TRGC1 (blue) or TRGC2 (pink). N = Normal, T = Tumor. **B)** Bar chart showing the number (left) or proportion (right) of γ chain sequences by disease state using TRGC1 (blue) or TRGC2 (pink). N = Normal, T = Tumor. **C)** Bar chart of clonotypes with a size > 1, colored by TRGC1 (blue) or TRGC2 (pink). **D)** Histogram of clone sizes for TRGC1 (blue) and TRGC2 (pink) subset on clones shared between both tumor and adjacent normal samples for each patient. **E)** Histogram of clone sizes of TRGC1 (blue) and TRGC2 (pink) subset on clones shared between both tumor and adjacent normal samples for each patient, normalized by disease status, normal (top) or tumor (bottom). **F)** Volcano plot of differentially expressed genes between cells using TRGC1 and cells using TRGC2. Genes that are significantly expressed in TRGC1 over TRGC2 are colored blue, up in TRGC2 over TRGC1 are colored pink. Grey dots fall below the threshold of effect size (Average Difference) and significance (Adjusted P Value), indicated with grey dashed lines.

response. The TRGC2(3x) allele makes TCR even more resistant to antigen stimulation. Despite this weaker response, TCRs in human tissues use TRGC2(2x) and TRGC2(3x), and T cells bearing TRGC2 TCRs can clonally expand to participate in an immune response. Notably, TRGC2(2x) and TRGC2(3x) are both conserved among primates, suggesting a meaningful biological role. In addition to its persistence on an evolutionary timescale, the TRGC2 gene also maintains its representation in vivo in individual hosts. TCRs using the TRGC2 gene can clonally expand and mediate anti-tumor immunity, though not to the same extent as TRGC1 clones.

It remains unclear how exactly the exon 2 repeats reduce TCR signal strength. Notably, the connecting peptide of TRGC2(3x) has many charged residues and is predicted to be intrinsically disordered and unstructured. This leads to the intriguing possibility that the extended connecting peptide of TRGC2(2x) and TRGC2(3x) could mediate condensation of TCR complexes. This phase separation could render the TCR less sensitive to antigenic stimulation while maintaining tonic signaling. This tracks with our observation that TRGC2 lines have modestly but reproducibly higher baseline CD69%, suggesting low level activation without antigen.

Our findings call into question how TRGC1 or TRGC2 is selected in receptor recombination. Other reports suggested a pre-natal preference for the "outer" option of recombination (TRDV2, TRGV9, TRGC1), which switches after birth to enable more variable gene usage. But why have this

“switch” at all? I hypothesize that since many γδ TCRs recognize self-antigens or stress-induced ligands, dialing back sensitivity to a highly expressed self-antigen could be beneficial for γδ T cell function. In contrast to how αβ TCRs use catch bonds to bind and signal in response to extremely rare peptide-MHC complexes, it has been suggested that γδ TCRs do not[124]. Taken together, these γδ TCR features could be adaptations to reduce chronic stimulation of γδ T cells by ubiquitous antigens, preventing exhaustion or autoimmunity.

CHAPTER 4

RECOGNITION OF SELF-DERIVED LIPIDS MARKS A SUBSET OF FUNCTIONALLY DISTINCT UMBILICAL CORD BLOOD T CELLS

4.1 Introduction

Natural Killer T (NKT) cells are a group of T lymphocytes that recognize lipids presented by CD1d. Invariant NKT (iNKT) cells, which are defined, in humans, by expressing a T cell receptor (TCR) exclusively using a TRAV10-TRAJ18 rearranged alpha chain and a TRBV25 β chain, are the most extensively studied, and are usually identified by their reactivity to CD1d presenting αGalCer. Following their development in the thymus, iNKT cells acquire an effector program, enabling them to adopt a T_H1-, T_H2-, or T_H17-like fate[125?] . They are generally considered to be innate lymphocytes, due to their expression of NK receptors and rapid production of cytokines upon stimulation.

Thus far, all other CD1d-binding αβ T cells have been designated "type II" NKT cells. The first identified type II NKT TCRs displayed a distinctly different docking strategy on CD1d than iNKT TCRs. Encompassing diverse sequences, this broadly defined class of type II NKT cells also recognize a variety of lipid antigens in the context of CD1d with varying degrees of specificity: sulfatide, LPC, and more have been reported to be recognized by these cells[79]. In addition, many subsets and functional roles have been attributed to these cells in health and disease[77,126].

Beyond the invariant and type II archetypes, we do not have a comprehensive understanding of CD1d-restricted T cells and their antigen receptors at large. This is due, in part, to the reliance on studying in vitro expanded individual clones, which most likely do not fully represent the in vivo population due to factors such as uneven expansion, effector skewing based on culture conditions, and survival bias. I sought to extend our understanding of CD1d-specific cells using single cell RNA sequencing (scRNAseq). I employed a modified CITE-Seq approach[127], using barcoded CD1d tetramers to mark antigen specificity of T cells directly isolated from human umbilical cord blood. Cord blood T cells have a maximally diverse TCR repertoire that is typically not yet skewed

by immune challenge. This approach provides a more extensive assessment of TCR repertoire representation, antigen specificity and function of CD1d-specific T cells.

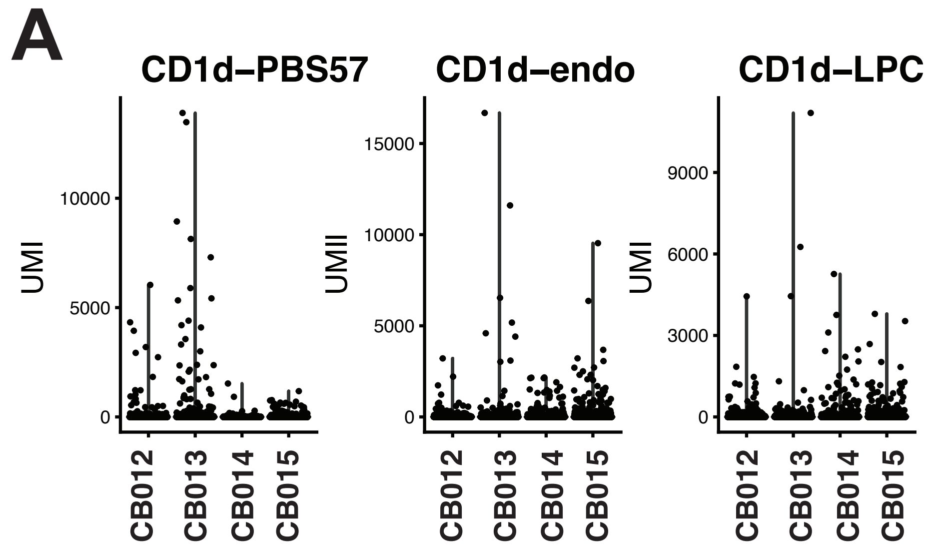
In our analysis of the scRNAseq data, I discovered diverse CD1d-restricted TCRs with distinct patterns of lipid reactivity. These CD1d-specific cells were functionally and transcriptionally distinct from the iNKT population. Surprisingly, I also identified a population of "autoreactive" cells that were labeled by multiple CD1d tetramers. Using flow cytometry, I confirmed the presence of the CD1d-autoreactive T cells in adult peripheral blood as well as cord blood. Finally, I found that these cells have characteristics of stem-like T cells, unlike most iNKT cells.

4.2 Results

4.2.1 Identification of CD1d-restricted iNKT cells by scRNAseq with CITE-Seq

To understand the TCR repertoire and biological function of the nascent CD1d-restricted T cell compartment, I subjected total $CD3^{+}$ T cells from umbilical cord blood to single cell RNA sequencing. To assay antigen specificity as part of our sequencing experiment, I stained cells with pool of barcoded tetramers during sample preparation[127,128]. These tetramers included CD1d loaded with either lyso-phosphatidylcholine (LPC), PBS57 (a synthetic analogue of the canonical iNKT lipid antigen), or a mixture of endogenous lipids (referred to as ”endo” or ”unloaded”). Each tetramer had a distinct barcode sequence to enable classification of cells based on CD1d-lipid specificity. Also included was an MR1 tetramer to control for interactions with human B2m (which associates with both the CD1d and MR1) and more general non-specific binding.

After initial filtering steps, 25,743 cells and 4,216 paired αβ TCR sequences were obtained and analyzed. Cells with paired αβ TCR sequences were assessed for CD1d specificity based on tetramer UMI density; cells with tetramer signal ten times higher than background were considered tetramer positive. The barcoded CD1d tetramers revealed rare, but distinct tetramer positive cells among the cord blood T cells (Figure 4.1a).



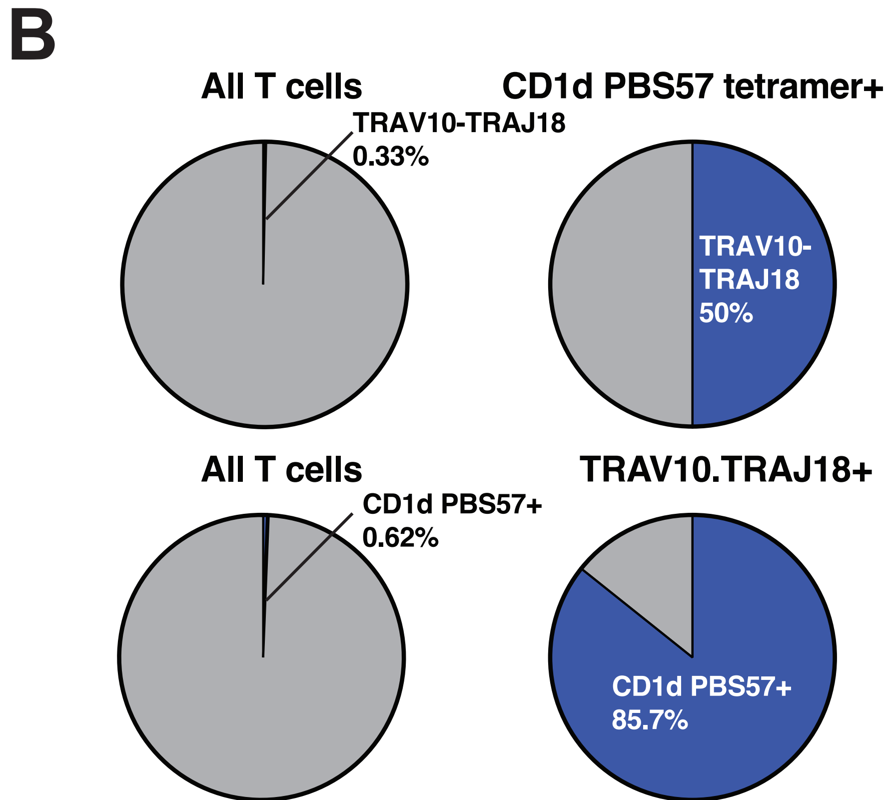


Figure 4.1: Identification of CD1d-specific cells by CITE-Seq

A) CD1d Tetramer UMI signal, shown by sample. **B)** Frequency of TRAV10-TRAV18 rearrangement in all T cells compared to CD1d PBS57 tetramer positive cells (top) and frequency of PBS57 tetramer positive cells among all T cells compared to TRAV10-TRAV18^{+} T cells (bottom).

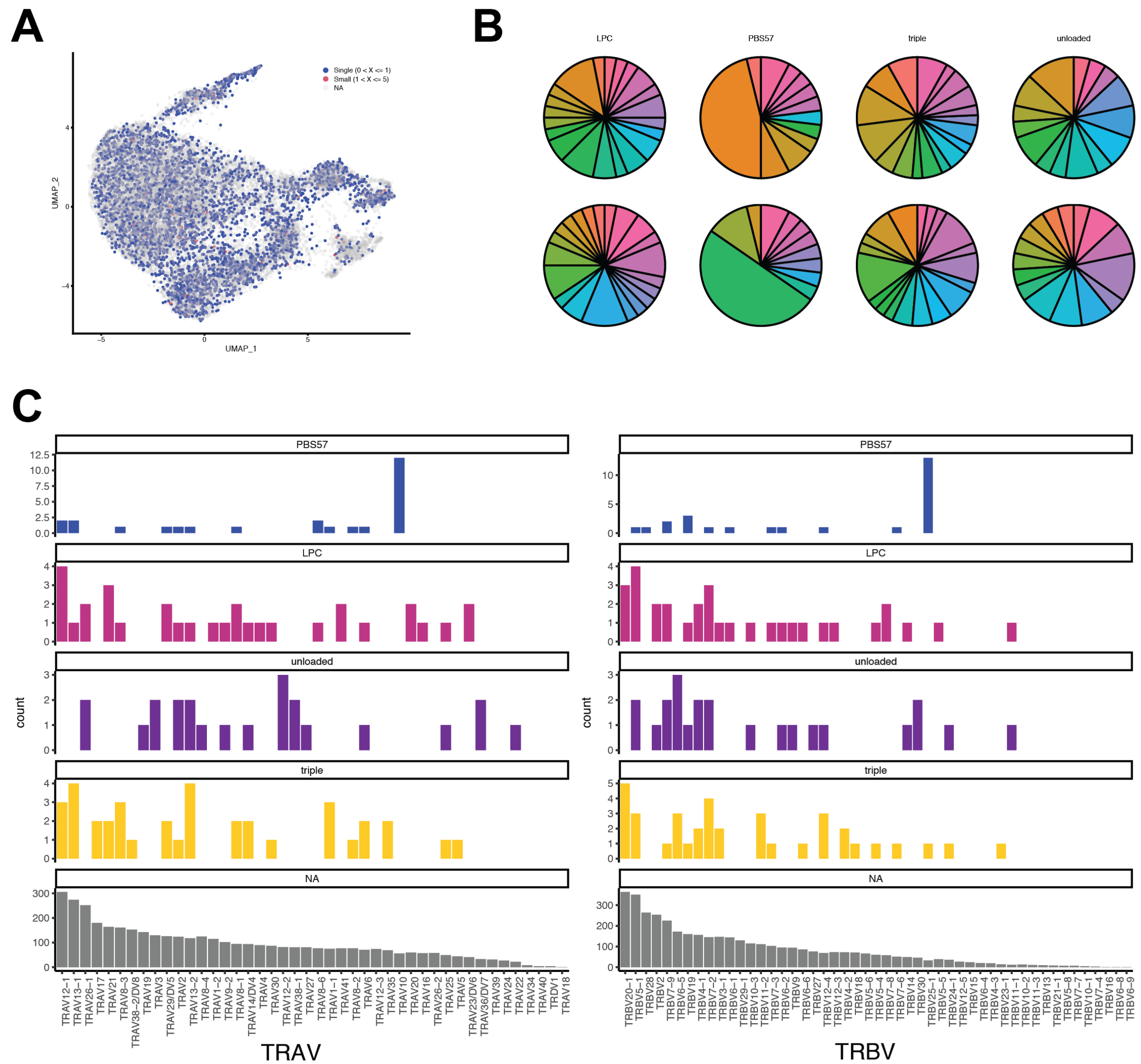


Figure 4.2: Non-invariant CD1d-specific TCRs have a distinct and diverse repertoire
A) UMAP of transcriptome data, with 4,216 cells bearing TCR sequences highlighted. Blue indicates a single instance of a TCR, pink indicates reoccurring TCRs. **B)** Pie charts displaying TRAV (top) and TRBV (bottom) gene usage for each CD1d-lipid tetramer binding population. **C)** Frequency of TRAV (left) and TRBV (right) gene usage detailed by CD1d-lipid tetramer specificity.

I turned next to the well-defined iNKT population to evaluate the specificity of labeling with the barcoded CD1d tetramers. I assessed the presence of the stereotypical iNKT α chain rearrangement, TRAV10-TRAJ18, in the CD1d-PBS57 tetramer$^+$ cells (Figure 4.1b). This rearrangement was enriched among the CD1d-PBS57 tetramer$^+$ subset compared to all T cells. Similarly, 85.7% of cells with TRAV10-TRAJ18 TCRs were identified as CD1d-PBS57 tetramer positive. These data imply that specific labeling was occurring with the tetramer and that the classification method applied was capable of detecting true positives.

4.2.2 CD1d-restricted cells express diverse TCRs with conserved features that predict specificity

Next, I focused on all CD1d tetramer$^+$ cells to define patterns of lipid specificity and conserved sequence features. To this end, I subset on cells with paired αβ TCR sequences (Figure 4.2a). I categorized cells according to their pattern of tetramer specificity. Our analysis uncovered cells positive for a single lipid-CD1d complex; LPC, PBS-57, or unloaded. Notably, a small population of T cells bound all three CD1d tetramers, but not the control MR1 tetramer. In this analysis, they are designated ”triple” for their triple tetramer reactivity. The TCR sequences of the LPC, unloaded, and triple cells were highly diverse compared to CD1d-PBS57 tetramer positive cells, with no clonal expansions or invariant rearrangements apparent in the TCR sequences analyzed (Figure 4.2b,c)

I next analyzed the TCR sequence characteristics to ascertain if there are conserved binding determinants that confer lipid specificity (or autoreactivity) to CD1d-restricted TCRs. Hierarchical clustering performed the α/β chain sequences revealed two broad groups of TCRs within the CD1d-specific group (Figure 4.3). The left-most branch included TCRs biochemically similar to the canonical iNKT TCR sequences, illustrated by cluster of nodes colored in blue. Several triple reactive and endo TCR sequences were enriched within this branch as well. One of the features underpinning this cluster was the TRBJ2-7 segment, ETQTY. Interestingly, a distinct strategy for

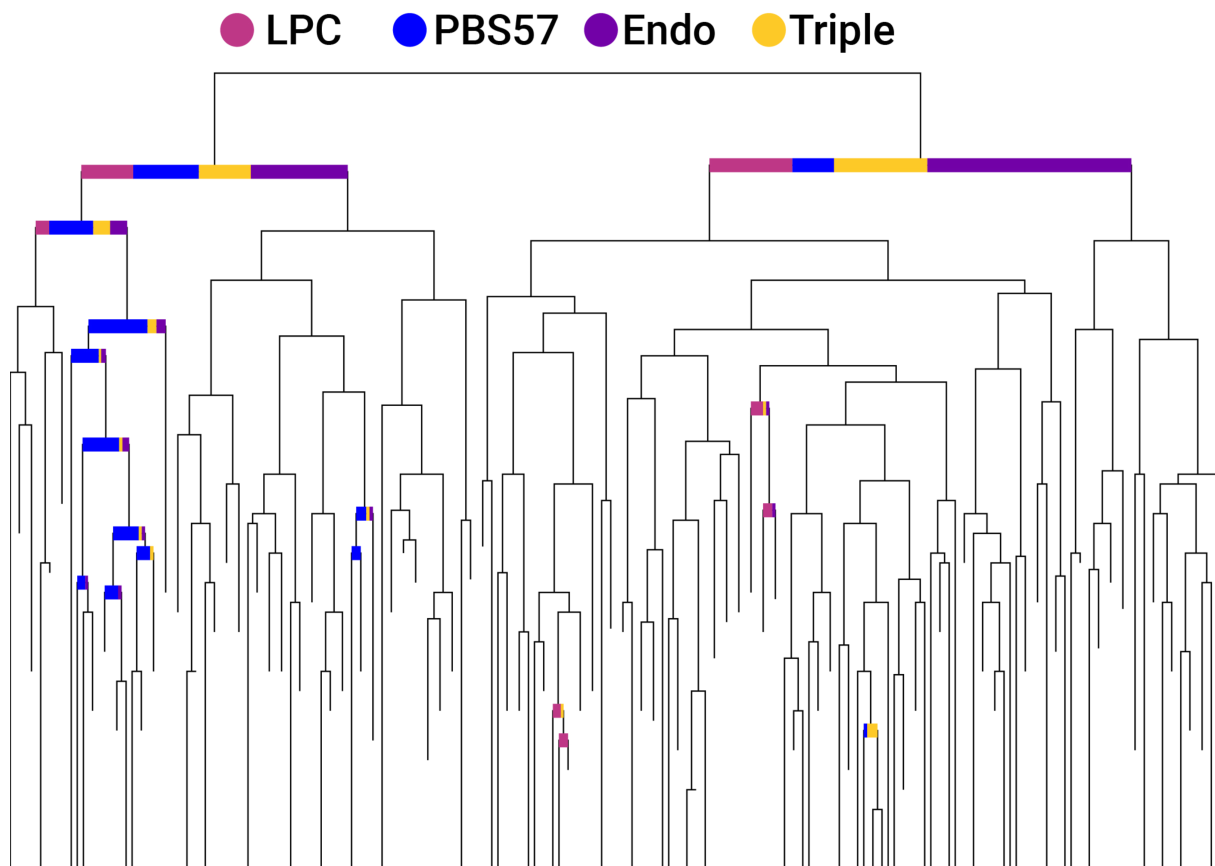


Figure 4.3: Sequence characteristics of CD1d binding TCRs
Hierarchical clustering of α chain sequences, colored by tetramer specificity. Colored bars indicate statistically significant enrichment for the indicated specificity.

recognizing CD1d can also be employed, shown in the right branch. Here, TRBV7 was commonly used, and TRBV28 marks the small enriched triple/PBS57 cluster, shown by the blue and yellow "leaf" toward the bottom of this branch.

I hypothesized that these cells have TCRs that are broadly autoreactive to CD1d. Instead of binding to CD1d-lipid in a manner that permits lipid antigen discrimination, they might instead recognize CD1d in a manner more dependent on contacts with CD1d. To test this hypothesis, I selected a TCR sequence from a cell that bound all three CD1d tetramers, T5, and an iNKT TCR sequence. I produced these TCRs recombinantly and tested binding to CD1d loaded with various lipid ligands with bilayer interferometry (Figure 4.4). It is prudent to note that this assay was not

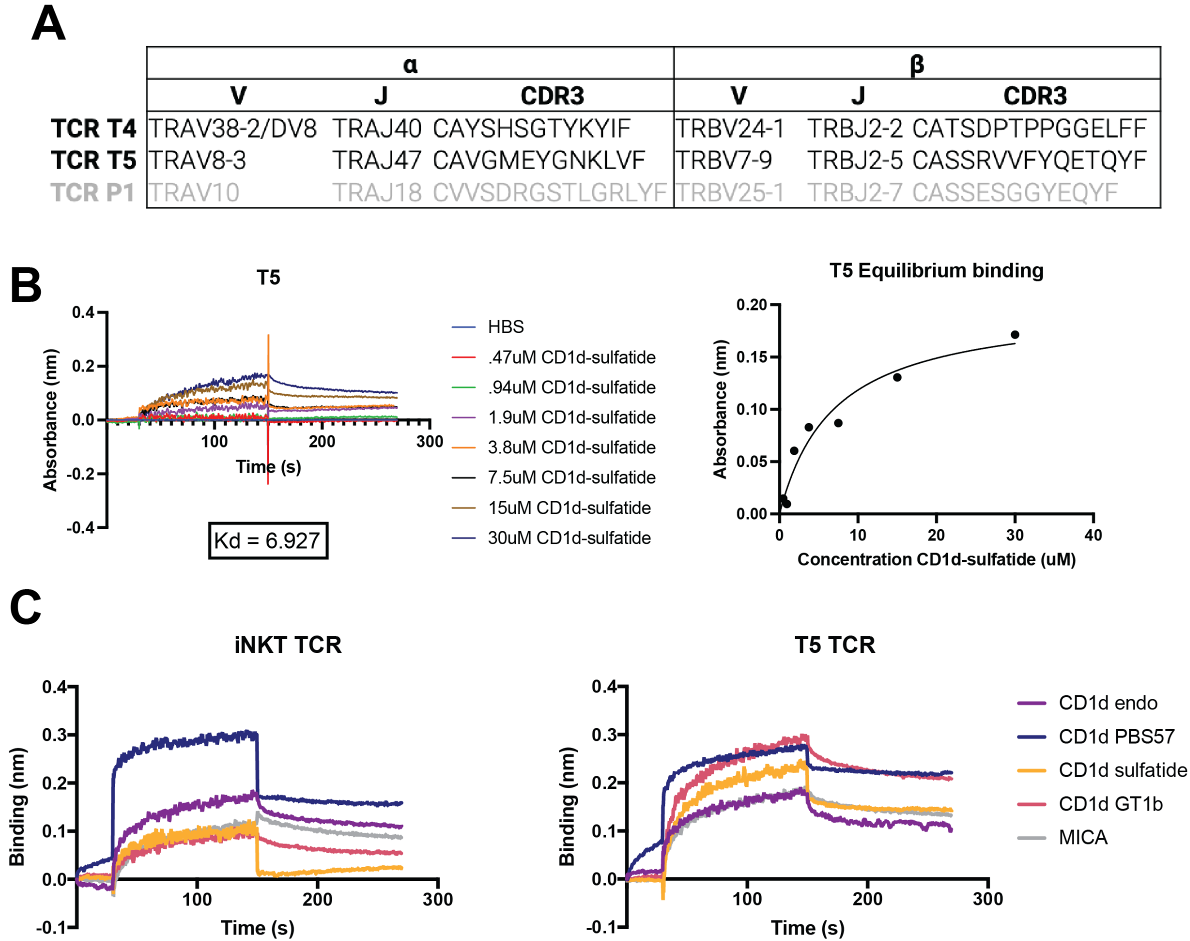


Figure 4.4: Autoreactive TCR T5 binds CD1d irrespective of lipid presented
A) TCR sequences selected for further study. **B)** T5 TCR binding measurements with CD1d loaded with sulfatide (left). T5 TCR was immobilized. Equilibrium binding curve of T5 TCR and CD1d at maximum absorbance (left). **C)** Binding measurements between iNKT TCR (left) or T5 TCR (right), and immobilized CD1d loaded with indicated lipids.

optimized, and the signal to noise ratio is subpar. However, this data hints that while the iNKT TCR displayed ordinary specificity for CD1d-PBS57, T5 did not bind preferentially to any particular lipid-CD1d complex. Though the signal is minimal, CD1d loaded with ganglioside GT1b, which has a complex sugar moiety that extends out of the groove of CD1d, bound comparably to CD1d-PBS57. Making the assumption that T5 is representative of the entire class of tetramer cross-reactive TCRs, this would suggest that the observed triple-reactive tetramer binding patterns are due to lipid-agnostic binding of TCRs to CD1d.

4.2.3 Autoreactive CD1d-restricted T cells differ from iNKT cells and express high levels of CCR7

Next, I sought to understand functional correlates of CD1d autoreactive T cells by examining their transcriptome. Globally, CD1d tetramer binding does not appear to predict function: except for iNKT cells, CD1d-restricted T cells appear to cluster primarily with all other αβ T cells (Figure 4.5a). However, when iNKT cells and lipid-agnostic CD1d-restricted T cells are compared directly, several genes mark these lineages as distinct. iNKT cells express ZBTB16 (which encodes PLZF), KLRB1, and CCR4, whereas autoreactive T cells have higher expression of SELL, CCR7, and TCF7 (Figure 4.5b). In particular, CCR7 expression is a distinguishing factor between the autoreactive subset and canonical iNKT cells in cord blood. I further investigated the expression pattern of several surface markers using bar-coded antibody CITE-Seq and confirmed high expression of CCR7 in the autoreactive T cells (Figure 4.5c). Most of the broadly autoreactive CD1d-specific T cells were $CD45RA^{-}CCR7^{+}$, indicating an unexpected central memory phenotype, distinct from CD1d-PBS57 specific iNKT cells and other T cells that bound the LPC and endo tetramers.

4.2.4 Autoreactive CD1d-restricted T cells have stem-like TCR-directed function

To verify the CD1d autoreactive phenotype observed in our sequencing experiment, I analyzed several additional cord blood samples for the presence of CD1d-endo tetramer positive cells that were also $CCR7^{high}$. In line with the scRNAseq results, flow cytometric analysis of additional cord blood samples confirmed the presence of this subset (Figure 4.6a). Likewise, CD1d-endo $tetramer^{+}$ cells harbored a distinct $CCR7^{high}$ population, similar to conventional cord blood αβ T cells and in contrast with CD1d-PBS57 $tetramer^{+}$ iNKT cells. CD1d-autoreactive T cells (CD1d-endo $tetramer^{+}$ $CCR7^{high}$) were also observed in adult peripheral blood, suggesting that this subset is not unique to cord blood (Figure 4.6b).

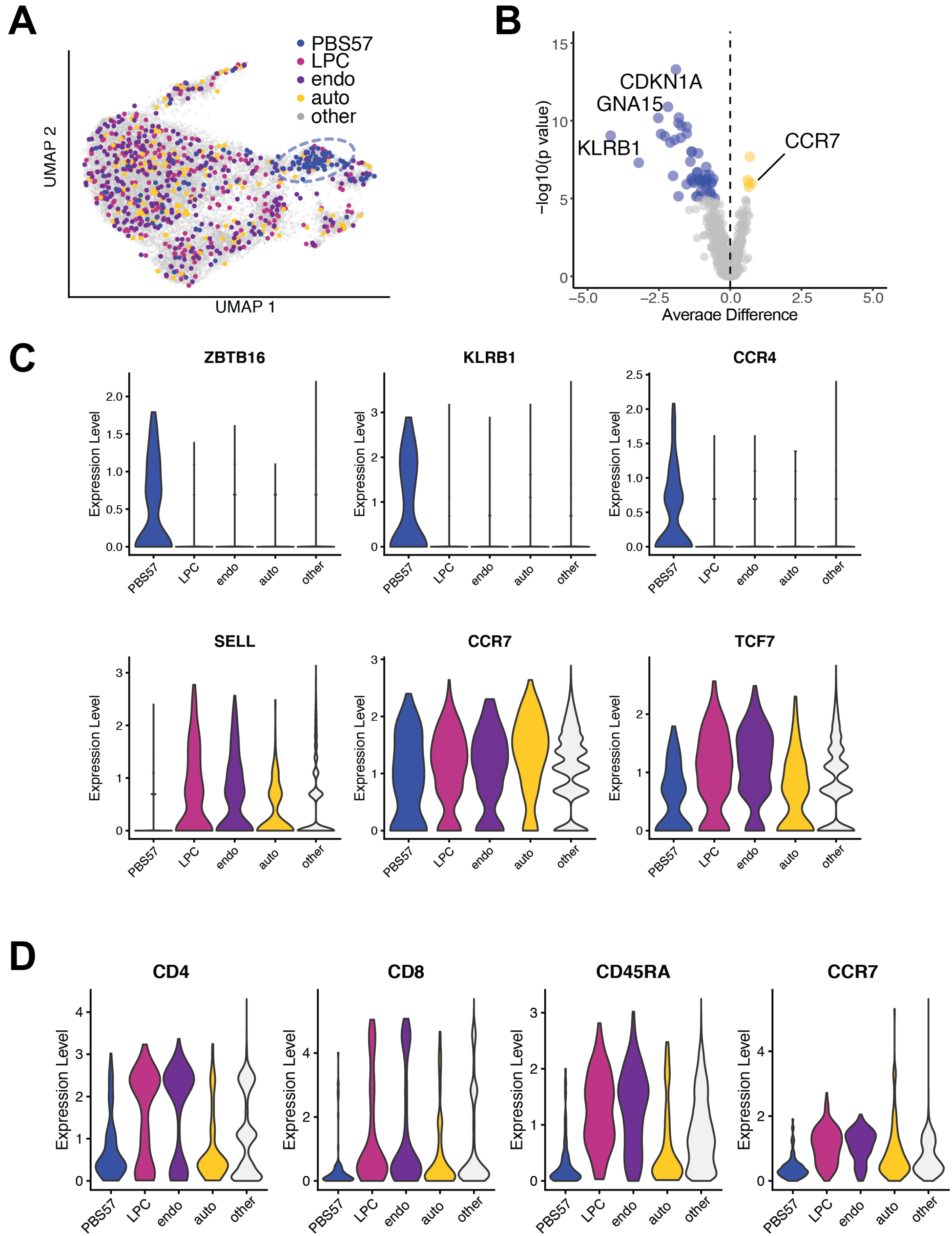


Figure 4.5: CD1d-specific T cells are transcriptionally distinct from invariant NKT cells

Figure 4.5 (continued)

A) UMAP representation of transcriptome data from 25,743 UCB T cells, colored by CD1d-lipid specificity. Each point represents a single cell. **B)** Volcano plot indicating significantly differentially expressed genes between CD1d-PBS57$^+$ T cells and CD1d-autoreactive T cells. Transcripts relatively enhanced in CD1d-PBS57$^+$ T cells are colored in blue, those enhanced in T cells that bind all CD1d tetramers are colored in yellow; genes below the threshold of significance are colored in grey. **C)** Violin plots comparing NKT signature genes and memory-status genes across CD1d-lipid specificities. **D)** Violin plots comparing surface expression of CD4, CD8, and memory markers across CD1d-lipid specificities.

I hypothesized that TCF1 (encoded by *TCF7*) could be playing a role in maintaining CCR7 expression and imparting the divergent phenotype of CD1d autoreactive T cells. CCR7 expression is controlled by TCF1, and TCF1 has been implicated generally in maintaining stem-ness in T cell populations[129]. Additional cord blood samples were stained for TCF1 and Tbet. In all samples analyzed, the autoreactive T cells had higher TCF-1 expression than the iNKT population (Figure 4.6c). This suggests that the CD1d-autoreactive cells have a central memory or stem-like function in the body.

Next, I performed functional experiments to confirm that the TCF1 results were truly indicative of stem-ness. Using CD1d-endo tetramer$^+$ CCR7$^+$ as a proxy for the autoreactive population identified previously, I sorted based on these markers and provided antigen-specific stimulation for 72 hours. PHA, which crosslinks the TCR along with other surface receptors, spurred differentiation into an effector memory program, as expected. Strikingly, administration of recombinant CD1d lead to an increase in CCR7^{high} cells and a concomitant increase of CD69 positive cells (Figure 4.6d). A CD3 agonist antibody treatment also elicited this response. These results imply that CD1d-autoreactive cells in cord blood are able to self-renew in the with “signal 1” antigenic stimulation, a canonical stem-like T cell function.

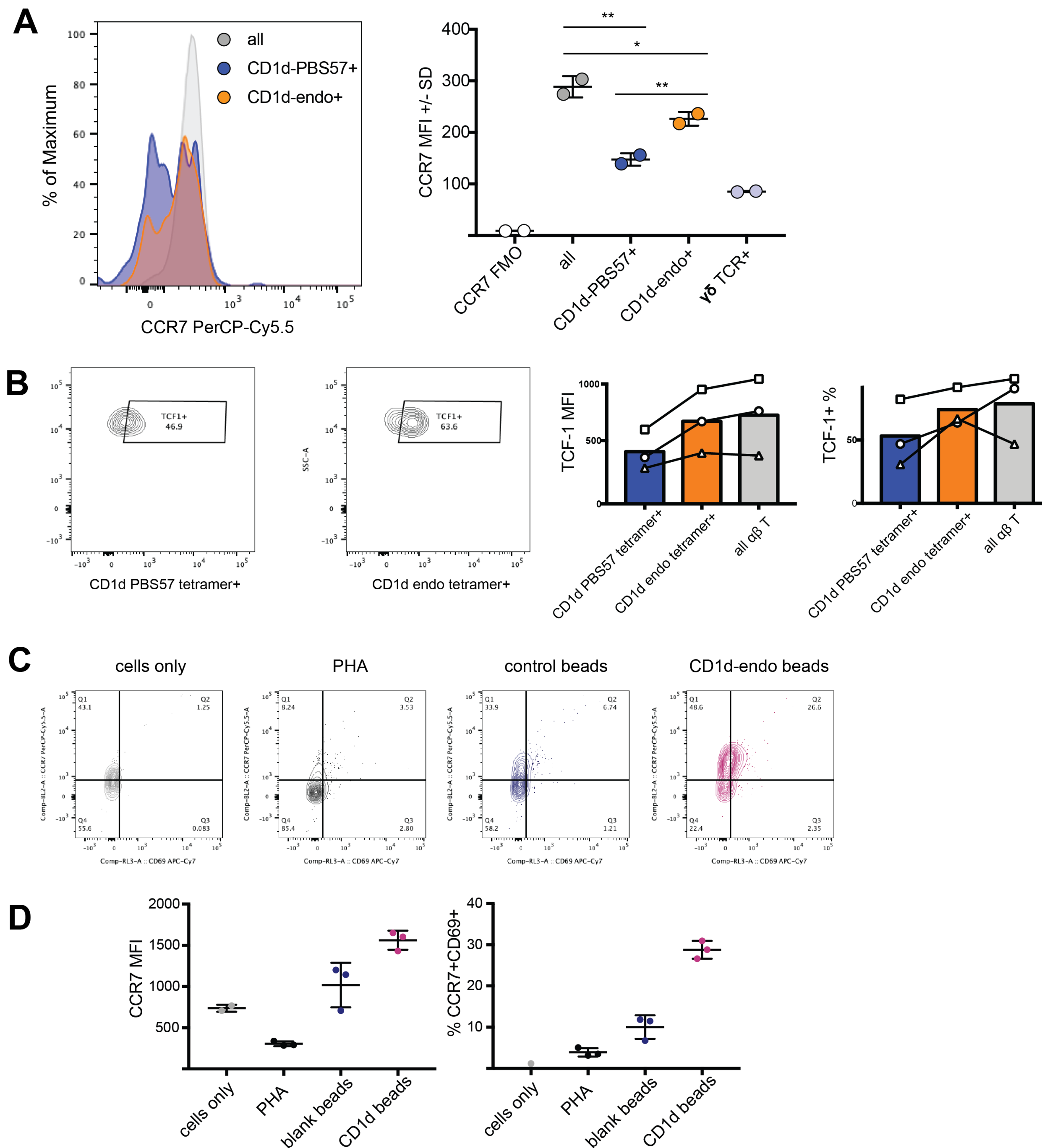


Figure 4.6: CD1d-autoreactive T cells highly express CCR7 and are functionally distinct from invariant NKT cells

A) Flow cytometric comparison of CCR7 expression on all αβ T cells and selected tetramer$^+$ cells isolated from UCB. **B)** Flow cytometric comparison of TCF-1 expression of all αβ T cells and selected tetramer$^+$ cells isolated from UCB, plotted with CCR7 expression. **C)** CCR7 and CD69 expression on sorted CD1d autoreactive T cells cultured for 72 hours with the indicated stimuli. **D)** Increase in CCR7 and CD69 in response to 72 hour stimulation with recombinant bead-bound CD1d-endo.

4.3 Discussion

In summary, I have identified a distinct subset of CD1d-restricted T cells, marked by broad TCR recognition of CD1d and a distinct functional phenotype from both iNKT cells and conventional αβ T cells. Using oligo-conjugated tetramers, I find the αβ T cells that engage CD1d tetramers loaded with diverse lipid antigens express diverse TCRs; some of these cells have fine specificity to a single lipid antigen, and others appear to be CD1d-centric and lipid-permissive. I show that germline encoded β chain regions are likely important for this atypical autoreactive CD1d recognition, and, using recombinant TCRs, demonstrate CD1d centric, lipid-agnostic binding similar to what is seen with other human CD1 isoforms[88]. Additionally, I demonstrate that these cells have role in cord blood that is skewed toward stem-ness, proliferation, and lymph node trafficking.

This work focuses on cord blood, providing a snapshot of the immune repertoire before exposure to external stimuli. The absence of clonal expansion maximizes the diversity T cell sequences available for biochemical analysis. In this unbiased, unexpanded state, I was unable to uncover any sequence trends that definitively predicted TCR binding to CD1d.

It remains undetermined if these CD1d-autoreactive T cells would participate in an immune response, or if they are outcompeted, become anergic, or are otherwise rendered nonfunctional – all possible outcomes for MHC-restricted autoreactive cells. However, the identification of similar CD1d-endo tetramer^{+} CCR7^{high} cells in adult peripheral blood suggests that they may persist and potentially play a role in the immune system throughout life. More studies are needed to ascertain the function of CD1d autoreactivity in immune defense or potentially tissue homeostasis.

Using our panel of tetramers, I uncovered several classes of lipid ligand recognition in the context of CD1d. These tetramers represent only a small subset of possible lipid antigens, but the use of PBS57 and LPC allowed me to dissect TCR interactions with structurally divergent lipid ligands. Generally, cells either bound a single CD1d-lipid tetramer or bound all three. Recognition of CD1d-PBS57 was dominated by iNKT TCRs, as predicted. TCRs that bound CD1d-LPC, CD1d-endo or all three tetramers were similar in diversity and had minimal overlap in gene usage.

Potentially, a high affinity lipid ligand for these TCRs could exist, and low affinity interactions with self ligands could sustain tonic signaling to these cells for survival. This seems likely for CD1d-endo and CD1d-LPC specific cells identified, which predominantly maintain a naïve phenotype ($CD45RA^{+}CCR7^{+}$). In contrast, CD1d autoreactive cells are more likely to have a stem-like TCM phenotype ($CD45RA^{+}CCR7^{+}$), in keeping with the idea that they have already encountered their antigen, CD1d, and have high lymph node trafficking and proliferative capacity. When considering the functional consequences of antigen specificity, it is worth bearing in mind that the sequencing data collected here is at single timepoint, early in life. Perhaps with pathogen experience, these antigen-specific populations will become more discrete, or (more likely) converge to have a more similar distribution of effector phenotypes.

Overall, our data suggests that CD1d autoreactive TCRs bind far from the lipid portal, imparting broad specificity. Even lipids with large head groups can be accommodated, implying that the "permissive lipid" model that is useful for categorizing TCR recognition of other CD1 molecules may not apply to CD1d. Our findings are in line with previous findings in mouse, which demonstrated that CD1d specific TCRs can have diverse sequences and lipid cross-reactivity. Our work defines a heterogeneous pool of CD1d-specific T cells that expands beyond iNKT or "type II" designations. Diverse antigen receptors enable a variety of patterns of lipid specificity, and could enable a wider range of functions for CD1d-specific T cells.

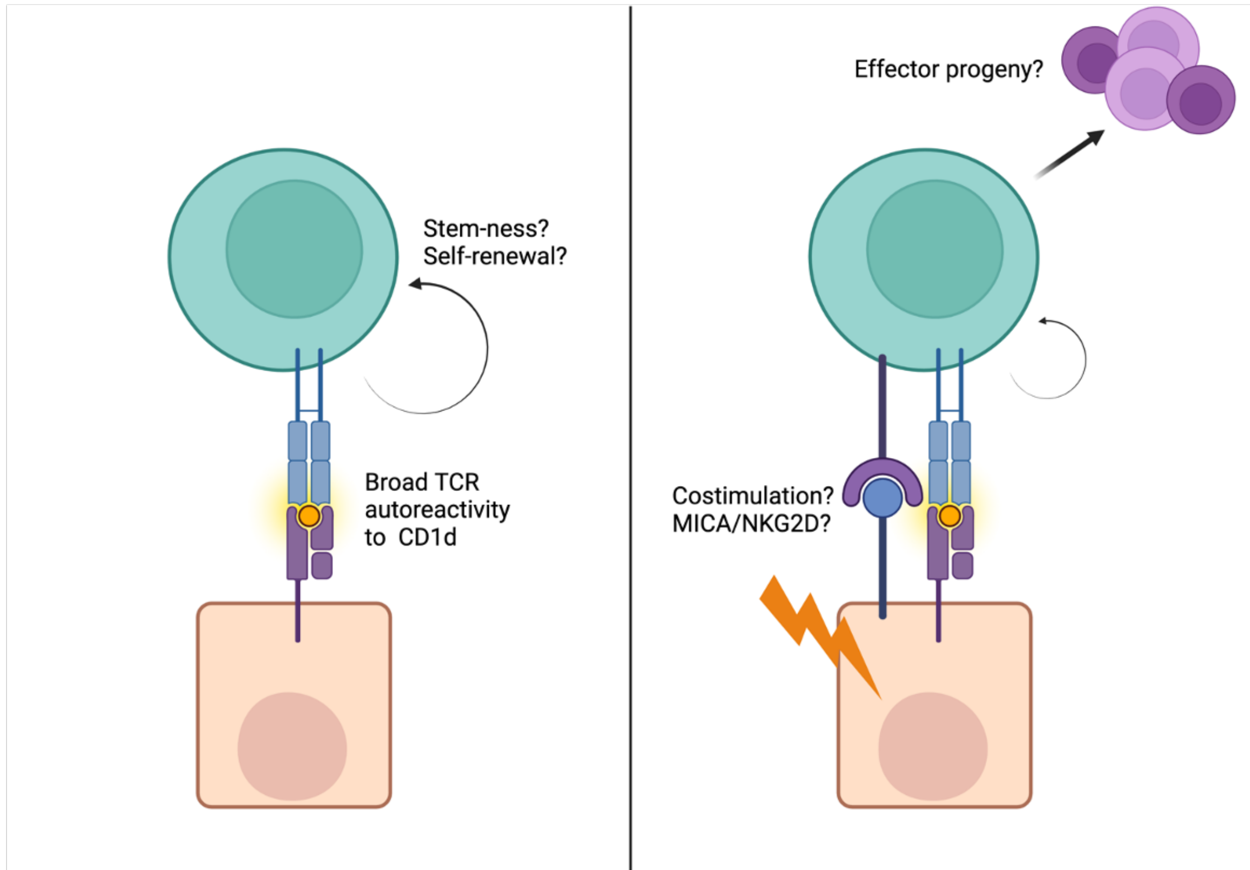


Figure 4.7: Model of CD1d-autoreactive T cell function

At steady state, encounters with CD1d serve to maintain the stem-like CD1d autoreactive T cell population. Because the TCR is broadly autoreactive to different CD1d-lipid complexes, low-level stimulation is constant as these CCR7$^+$ cells recirculate. When CD1d-expressing cells are stressed, they may upregulate factors that costimulate the TCR, and enable bona fide production of effector progeny to address the dysbiotic threat.

CHAPTER 5

RECOGNITION OF SELF-DERIVED LIPIDS SHAPES THE LUNG γδ T CELL COMPARTMENT IN ASTHMA

5.1 Introduction

γδ T cells are an important but enigmatic component of human immunity. They are named for their use of recombined TRG(γ) and TRD(δ) chains in their T cell receptor (TCR); the single marker which distinguishes this population. αβ T cells, in contrast, express a receptor composed of recombined α and β chains; this lineage of T cells is restricted to recognizing antigen presented by MHC molecules. Antigen recognition by γδ T cells is less well defined; the best-characterized activators of the γδ TCR are butyrophilin (BTN) and butyrophilin-like (Btnl) proteins that bind to germline-encoded regions of some γ chains[44–47]. There have been pivotal advances in our understanding of this innate-like recognition, but what of antigenic recognition? The adaptive molecular faces of the γδ TCR, the complementarity determining regions (CDRs) resulting from recombination, do not have a clear purpose in the BTN/Btnl system. Diverse molecules have been put forward as adaptive γδ TCR antigens[53]. However, γδ TCR recognition of MHC[54,55] and MHC-like proteins[56–59,64–66] has emerged as a major unifying paradigm. We and others have shown that CD1d, an MHC-like protein that presents lipids rather than peptides, could represent a major class of γδ TCR antigens: in structural studies, the highly variable CDR3 loops of the γδ TCR mediated the interaction with CD1d-lipid complexes[60,61].

The portion of the γδ TCR repertoire that binds CD1d (and some other CD1 isoforms) is autoreactive by definition – it is directed toward self. Biochemical studies show that this autoreactivity comes from two distinct modes of recognition: either the TCR specifically recognizes self-derived lipids in the context of CD1d[62,72,86], or the TCR binds to the surface of CD1d in a manner that is lipid-agnostic[87,88]. In conventional T cells, autoreactivity is rarely purposeful and often pathogenic. In contrast, autoreactivity is so common in γδ T cells that it is thought to be intentional. The stand-

ing model is that γδ T cells are tissue sentinels capable of detecting deviations from homeostasis through their recognition of self. However, the role of TCR engagement in the function of self-directed γδ T cells has not been resolved, and the biological relevance of their CD1d reactivity is unclear.

To dissect the biology of CD1d-reactive γδ T cells, I examined asthmatic lungs. Asthma is a complex inflammatory lung disease with allergic and non-allergic etiologies, characterized by airway hyperresponsiveness, inflammation, and remodeling. Asthma is associated with drastic alterations in lipid metabolism, indeed, one of the hallmarks of asthma is the elevated production of bioactive lipids such as eicosanoids, prostaglandins, and leukotrienes[130]. In addition, asthma is associated with changes in cholesterol biogenesis[131], ceramide synthesis[132] and pulmonary surfactants[133]. γδ T cells have been implicated previously in allergic asthma[98–101]. Morever, a role for CD1d-specific γδ T cells has been suggested: γδ T cells from the blood and bronchoalveolar lavage fluid (BALF) from patients with asthma had exaggerated responses to CD1d loaded with allergenic cypress lipids[58,102]. Similar activity was shown in other CD1d-restricted cells, such as invariant natural killer T (iNKT), though they are controversial players in asthma[134]. However, it is unclear what function γδ-CD1d autoreactivity could have in this disease setting. I hypothesize that the lipid environment in asthma could create a tissue stress signal and modulate the activation of CD1d-reactive γδ T cells. To understand the role of γδ T cells in peripheral tissues and human disease, I interrogated antigen-specific function within the lung immunological niche with flow cytometry and single-cell RNA-seq. I show that CD1d-autoreactive T cells are clonally expanded in asthmatic lungs and acquire a "natural memory" phenotype in situ. Further, I find evidence of interplay between the lipid environment of the lung and γδ T cell responses.

5.2 Results

5.2.1 CD1d endo tetramer$^+$ cells are present in the lung, and are more numerous in asthmatic lungs

To investigate whether CD1d-specific Vδ1 T cells are affected by the lung inflammatory environment, I used CD1d tetramers to assess the number of CD1d binding Vδ1 T cells in dissociated human lung tissue and lung-associated lymph nodes. Using flow cytometry, I analyzed lymphocytes derived from lung samples, designated "asthma" or "non-asthma" based on self-reported asthma diagnosis. The CD1d tetramers are made from recombinant protein purified from insect cells and are thus loaded with endogenous insect lipids, mostly PC, during the protein production process[68,135]. With this tetramer, I could assess the frequency of CD1d tetramer$^+$ γδ T cells within this tissue, and determine if there are differences in frequency between lungs experiencing stress (here though asthmatic responses) versus lungs with no history of asthma.

I observed an increase in CD1d-endo tetramer$^+$ Vδ1 T cells in lymphocytes from asthmatic lungs compared to non-asthmatics (Figure 5.1a). This trend was maintained across several quantification methods, including as a % of Vδ1, % of total T cells, and in absolute number (Figure 5.1b). In contrast, CD1d-endo tetramer$^+$ Vδ1 T cells were largely absent from the associated lymph nodes, irrespective of disease state (Figure 5.1c). Taken together, these data suggest that CD1d-endo tetramer$^+$ Vδ1 T cells are not proliferating extensively in the lymph node, where priming and expansion typically occur for conventional T cells, but instead undergo in situ expansion. Further, this expansion of the CD1d-endo tetramer$^+$ γδ compartment is enhanced in asthmatic lungs. I also assessed the number of CD1d-sulfatide tetramer$^+$ T cells, but no trends emerged.

Memory is a central function of the adaptive immune system: typically, antigen experience gives rise to durable and rapid recall responses, though antigen-independent memory has also been noted in γδ T cells. CD45RA$^+$CCR7$^-$marks the peak functional effector/memory subset of γδ T cells (this is different from αβ CD8$^+$ T cells, where these same markers indicate terminal differ-

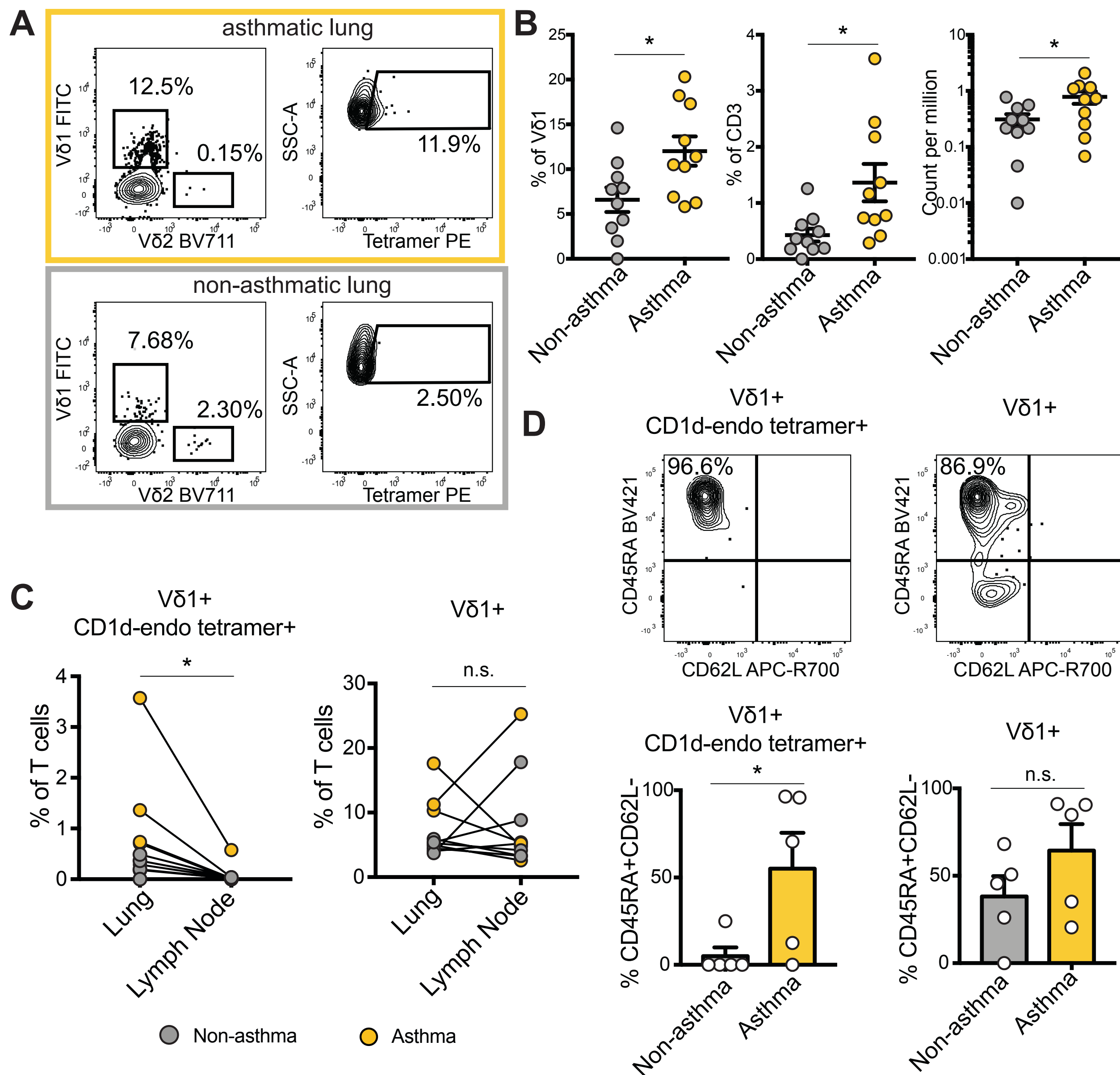


Figure 5.1: CD1d endo tetramer$^+$ cells are present in the lung and are more numerous in asthmatic lungs

A) Representative flow plots of Vδ1$^+$ and CD1d-endo tetramer$^+$ cells in asthmatic and nonasthmatic lungs. **B)** Top: CD1d-endo tetramer$^+$ cells as proportion of Vδ1$^+$ T cells, all CD3$^+$ T cells, and in absolute number, compared between asthmatic and non-asthmatic samples. **C)** Comparison between percentage of Vδ1$^+$ T cells (right) and Vδ1$^+$CD1d-endo tetramer$^+$ T cells (left) in lung and lung associated lymph node. Samples from the same individual are connected by a line, and points are colored by disease status (asthma: gold, non-asthma: grey). **D)** Comparison between % CD45RA$^+$CCR7$^-$cells of Vδ1$^+$ T cells (right) and Vδ1$^+$CD1d-endo tetramer$^+$ T cells (left) in lung, subset by disease status. * = $p < 0.05$ by t-test.

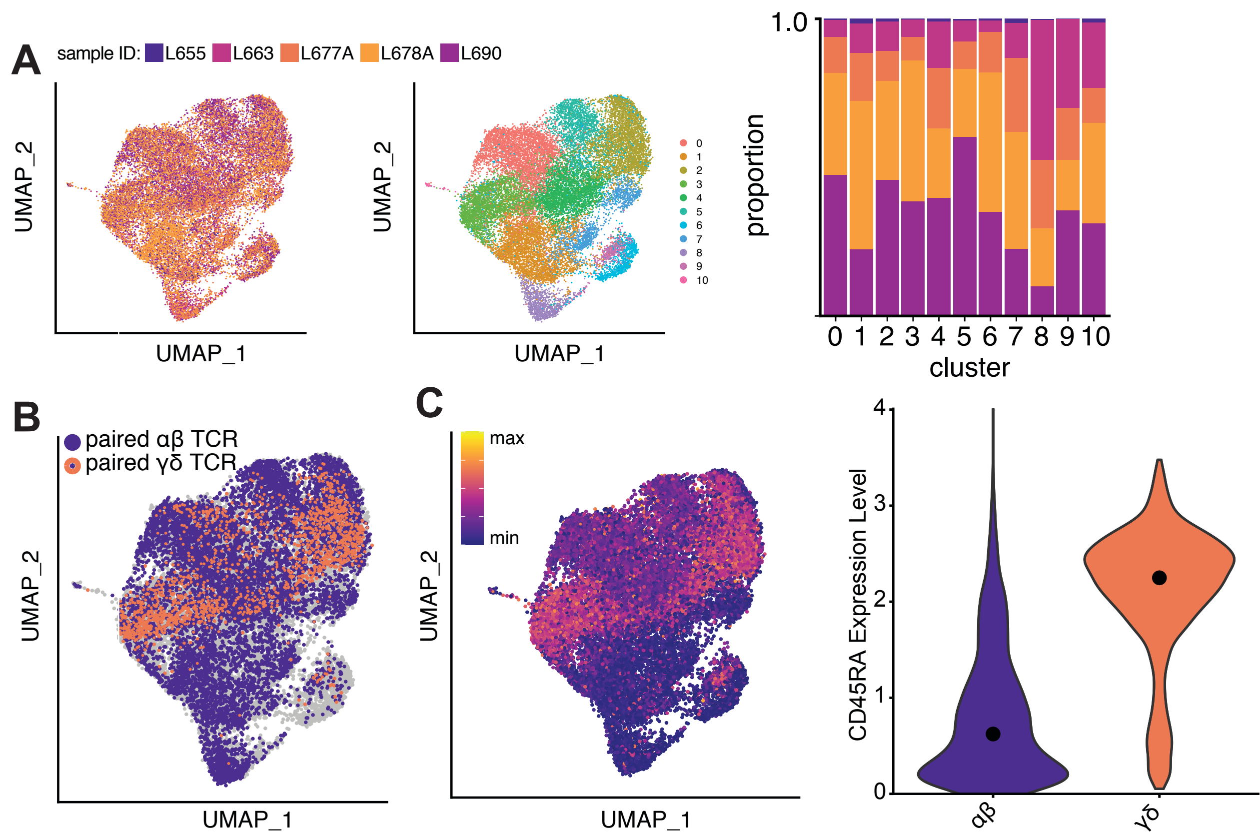


Figure 5.2: Multimodal single cell sequencing of lung T cells

A) Uniform Manifold Approximation and Projection (UMAP) representation of sequenced cells, colored by sample ID (left) and cluster designation middle). Left, cluster composition by sample. **B)** UMAP highlighting cells for which paired αβ (purple) or γδ (orange) sequences were obtained. **C)** UMAP colored by cell expression of surface CD45RA (right), comparison of CD45RA expression between αβ (purple) or γδ (orange) T cells.

entiation)[136]. I found that in total Vδ1 T cells, the % of cells in this memory pool was variable for all subjects. However, when focusing on CD1d-endo tetramer$^+$ Vδ1 T cells, I saw a difference in memory status between cells originating from non-asthmatic and asthmatic lungs (Figure 5.1d). I speculated that clonal expansion and memory development are occurring in the inflamed tissue. Further, this phenotype is likely CD1d-dependent, not antigen-independent, but because the disease/memory interaction does not extend to all Vδ1 T cells. Taken together, these data are suggestive of CD1d-driven clonal expansion in lung that is enhanced in the milieu of the asthmatic tissue.

5.2.2 CD1d-endo tetramer$^+$ cells are clonally expanded in asthmatic lungs

To more fully understand the relationship between CD1d specificity, clonal expansion, and function of these Vδ1 T cells in the lung I employed single-cell RNA sequencing coupled with oligonucleotide barcoded and phycoerythrin (PE) labeled CD1d and MR1 (as a control) tetramers. This approach enabled us to sort antigen-specific T cells and deconvolute which tetramer bound during downstream analysis[127,128]. I sorted both αβ and γδ T cells that bound to our tetramer pool (gating strategy Supplemental 2). 23,229 cells in total were sequenced from lung lymphocytes from 2 asthma (L677A, L678A) and 3 non-asthmatic samples (L655, L663, L690). Because non-asthmatic samples generally yielded few tetramer$^+$ cells, I sequenced an outlier non-asthmatic sample (L690) in an attempt to make the non-asthmatic cell counts in the dataset more robust. To evaluate the heterogeneity in the transcriptome data set, I implemented a graph-based clustering approach with Seurat. Cells from all five samples occupied all clusters, with no obvious partitioning based on asthma status (Figure 5.2a). I obtained 7,933 paired αβ and 1,602 paired γδ TCR sequences. Despite this, most clusters contained both αβ and γδ T cells, suggesting that tetramer$^+$ cells have globally similar transcriptional profiles in lung, regardless of αβ/γδ TCR usage (Figure 5.2b). In agreement with our previous phenotyping, the cells with a γδ TCR had high surface levels of CD45RA (measured by a CITE-Seq antibody) compared to αβ T cells (Figure 5.2c).

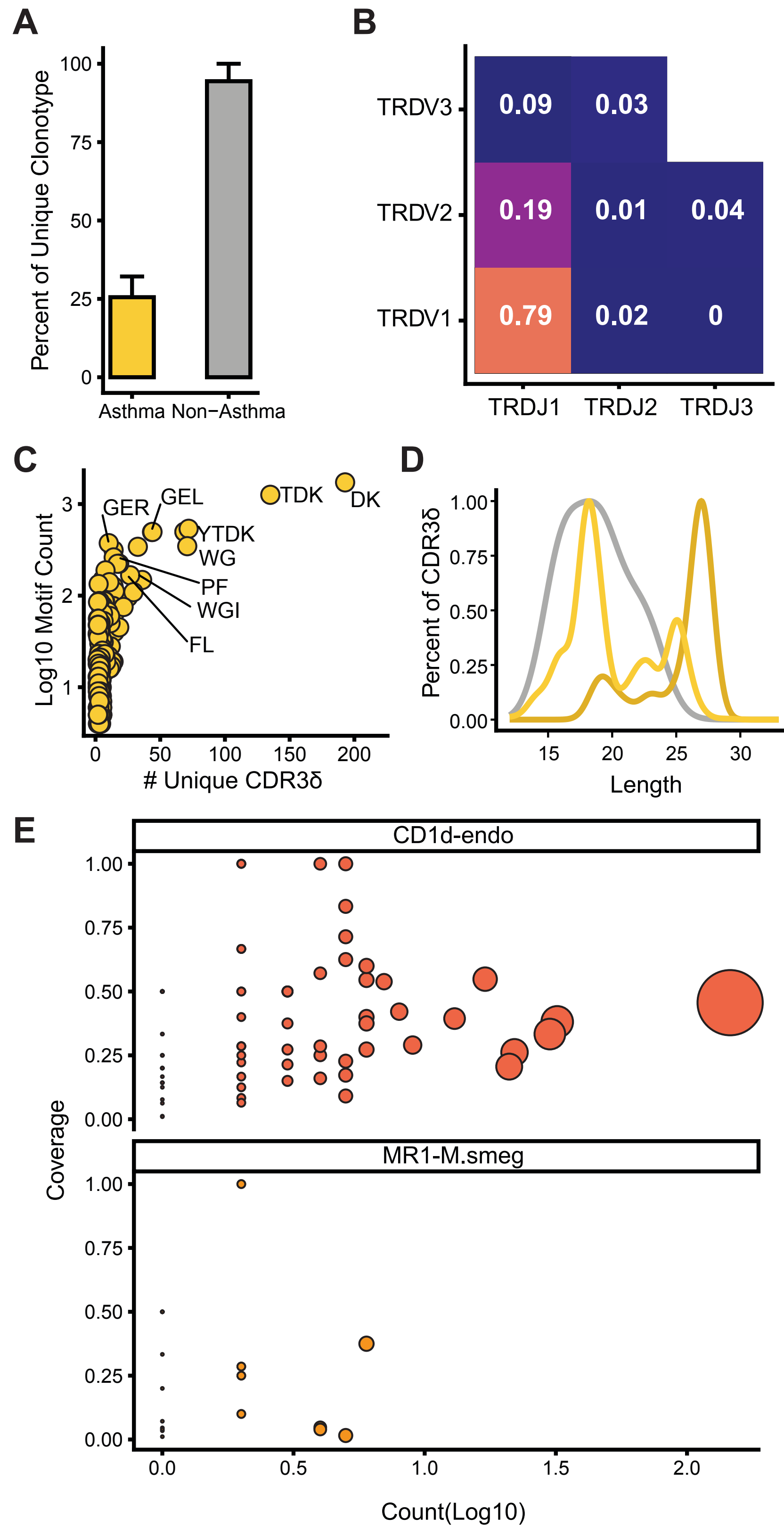


Figure 5.3: CD1d-endo tetramer⁺ cells are clonally expanded in asthmatic lungs

Figure 5.3 (continued)

A) Quantification of unique clonotypes (n=1) as a proportion of all clonotypes in asthmatic lung samples compared to non-asthmatic lungs. **B)** Frequency of TRDV-TRDJ rearrangements in the dataset colored by and labeled with scaled score. Deep purple indicates low frequency, orange indicates high frequency. **C)** Enriched CDR3δ motifs plotted by two different measures of abundance: the number of unique CDR3s containing the motif (x) and the total number of occurrences of the motif in the dataset (y). **D)** Comparison of CDR3δ length distribution between TCRs derived from non-asthmatic lungs (grey) and two separate asthmatic samples (yellow, gold). **E)** Clonal expansion within γδ clonotypes labeled with CD1d-endo tetramer (top) or MR1-M.smeg tetramer (bottom). X axis: clonotype size (count of cells with the same TCR sequence, defined by CDR3 nucleotide sequence), Y-axis: proportion of members of the clone binding to indicated tetramer. Bubble size is proportional to clonotype size/x axis.

Once I extracted γδ TCR sequence information from the dataset, I performed a clonotype-based analysis to uncover the degree of expansion of tetramer^{+} γδ cells in the lung. I defined clonotypes (or clones) as cells with identical γ and δ CDR3 nucleotide sequences and V(D)J gene usage. To ascertain if more clonal expansion had occurred in the asthmatic lungs, I quantified the percent of unique clonotypes. Most of the clonotypes derived from non-asthmatic samples were unique clonotypes, that is, un-expanded TCRs with single occurrences. In contrast, only a small proportion of clonotypes from asthmatic lungs were unique, implying that most clones had undergone clonal expansion (Figure 5.3a).

When a T cell is subjected to antigen-directed expansion, the proliferation is reflected in the focusing of the overall TCR repertoire. Thus, examining the tetramer^{+} γδ TCR sequence repertoire can provide further evidence for clonal expansion, as well as provide hints about the biochemical characteristics that enable TCR-CD1d autoreactivity. I noted skewing in γδ TCR gene usage, with the TRDV1/TRDJ1 gene pairing favored over other combinations (Figure 5.3b). I also used GLIPH2 to identify CDR3δ motifs that were significantly enriched in the TCR sequence dataset. Several motifs corresponding to TRDJ1 emerged (DK/TDK/YTDK), suggesting that TRDJ1 usage could be important for CD1d recognition. Additional germline-encoded sequences corresponding to TRDD3 (WGI) and TRDD2 (FL) were identified. Interestingly, several non-templated motifs were also identified, including GER, GEL, PF, and IL (Figure 5.3c). Because these sequences arise

rarely as a consequence of junctional diversity in TCR rearrangement, their enrichment is highly suggestive of clonal expansion.

Clonal expansion can also skew the distribution of lengths of CDR3δ. I compared the CDR3δ length of γδ TCRs from asthmatic and non-asthmatic lungs. Strikingly, the two asthmatic samples analyzed had bimodal length distributions, with many instances of long CDR3δ (≥25aa). In contrast, sequences from non-asthmatic samples were uniformly shorter (Figure 5.3d). Again, this implies CD1d-driven repertoire focusing is occurring specifically in the asthmatic lungs. I conducted parallel analyses on CDR3γ and TRGV/TRGV gene pairings but no clear trend emerged.

To provide more evidence that this expansion and repertoire skewing was a response to CD1d and not antigen-independent, I compared the sizes of CD1d tetramer$^+$ clones with MR1 tetramer$^+$ clones. As expected, the MR1 preferentially labeled αβ TCR$^+$ MAITs (data not shown). However, in the γδ T cell compartment, CD1d tetramer$^+$ clones were more prevalent and larger than MR1 tetramer$^+$ clones (Figure 5.3e). Overall, these results show extensive proliferation of CD1d-autoreactive γδ T cells in lungs with asthmatic inflammation. Beyond this, the repertoire analyses point to a critical link between δ chain characteristics and TCR recognition of CD1d.

5.2.3 CD1d-endo tetramer$^+$ cells include GPR183-expressing precursors that seed the lung niche

I noted that for expanded γδ clones, and to a lesser extent, αβ clones, occurrences of a given TCR were spread across multiple clusters. There are several explanations for this distribution: over the course of expansion, a single clone is able to generate multiple types of effector cells, or, alternatively, the clone is being captured in intermediate states along the way to terminal differentiation.

To dissect the relationship between the clusters and thereby provide an organizing principle to the transcriptional heterogeneity of the clones, I performed pseudotime trajectory analysis (Figure 5.4a). I found the inferred trajectory accurately approximates the course of clonal expansion (Figure 5.4b). I turned to our pseudotime construction of the clonal expansion process. In order to

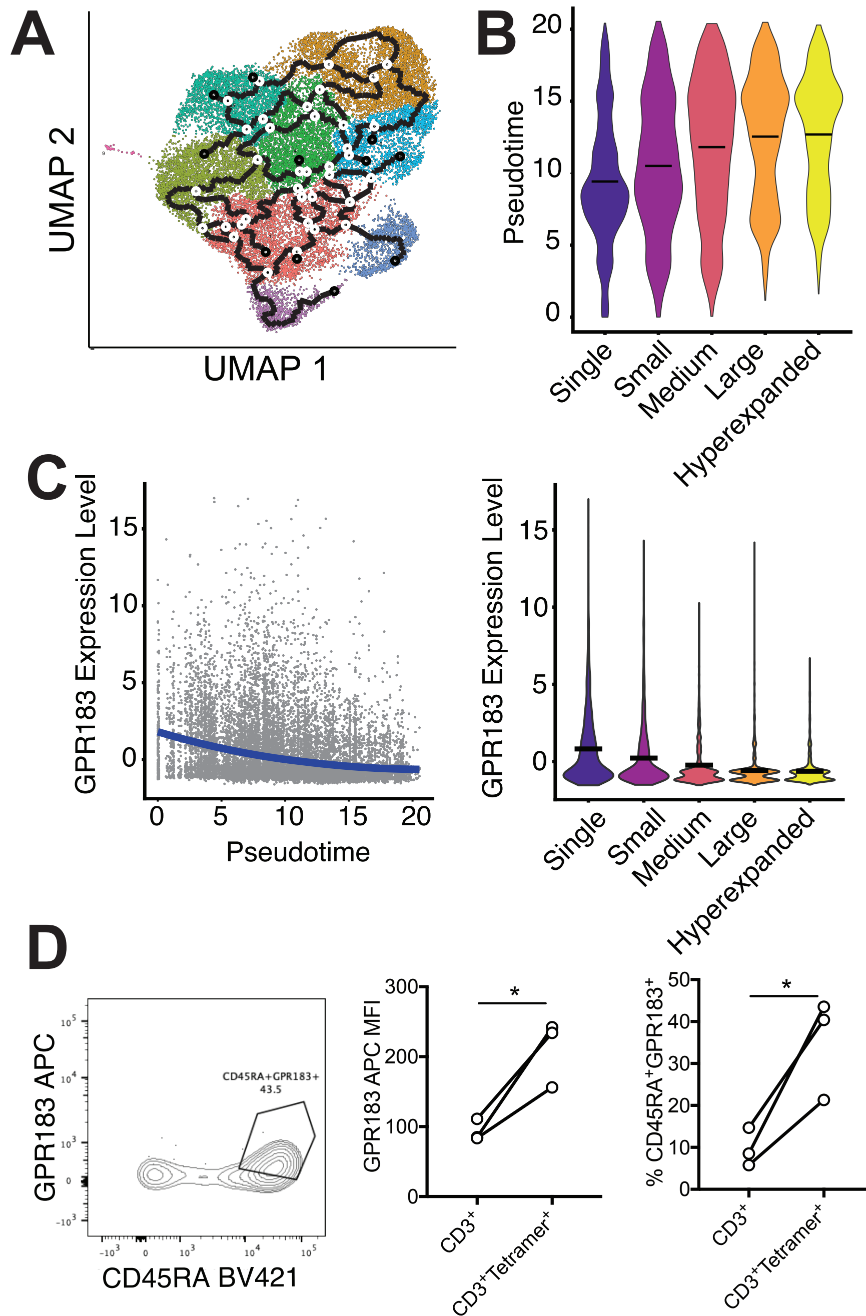


Figure 5.4: CD1d tetramer^{+} cells include GPR183-expressing precursors that seed the lung niche

A) Cells ordered along inferred pseudotime trajectory rooted at cluster 1. **B)** Pseudotime score for cells binned by clonal expansion status. Line at group mean. **C)** GPR183 expression in cells across pseudotime with line of best fit (blue), and for cells binned by clonal expansion status. Line at group mean. **D)** Representative staining for CD45RA and GPR183 from lymph node, gated on Live CD1d tetramer^{+} CD3^{+}, quantification of GPR183 fluorescence and % CD45RA^{+}GPR183^{+} cells within gate indicated on x axis. * = $p < 0.05$ by paired t-test

understand the in situ maturation process of the CD1d-specific T cells, I assessed the genes whose transcription changed the most over the trajectory.

GPR183 (also known as EBI2) emerged as a top hit among genes down regulated with clonal expansion. Similar results were obtained by analyzing differentially expressed genes for singly-occurring clones (Figure 5.4c). GPR183 is a G-protein-coupled receptor that binds oxysterols. Oxysterol production is elevated in inflamed and damaged tissues, thereby GPR183 enables cellular chemotaxis to sites of inflammation. Specifically in asthma, GPR183 expression on leukocytes is critical for their trafficking to the lung during allergic exacerbations.

With this knowledge, I hypothesized that GPR183^{+} single clones were recent arrivals to the lung niche. To investigate this possibility, I searched for their presence at a potential site of emigration, the lymph nodes. CD1d-endo tetramer^{+} γδ T cells were rare in the lung-associated lymph nodes, as previously mentioned, so I analyzed CD1d-endo tetramer^{+} CD3^{+} T cells (capturing both αβ and γδ T cells, which were both present in cluster 1) from paired lung-associated lymph nodes from samples L663, L678A, and L690. Using flow cytometry, I was able to uncover a CD1d-endo tetramer^{+} GPR183^{+} T cell population that highly expressed CD45RA in each of these lymph nodes (Figure 5.4d). Thus, I put forward a potential explanation for the observation that un-expanded clones were highly represented in asthmatic lungs: GPR183-expressing CD1d-specific T cells are likely seeded from the lymph node to the lung in an inflammation-dependent manner.

5.2.4 Clonal expansion of T cells results in natural memory differentiation

For both expanded αβ and γδ clones, occurrences of a given TCR were spread across multiple clusters. There are multiple explanations for this distribution: over the course of expansion, a single clone is able to generate multiple types of effector cells, or, alternatively, the clone is being captured in intermediate states along the way to terminal differentiation. I turned to our pseudotime construction of the clonal expansion process to understand the maturation of the CD1d-specific T cells. To interrogate this process, I assessed the genes whose transcription increased the most over

the trajectory.

5.2.5 CD1d-expressing myeloid cell composition is altered in asthma lungs

I performed single cell RNA sequencing on $CD14^+$ lung cells to understand how asthma status remodels the $CD1d^+$ myeloid cell compartment and could alter antigen presentation to γδ T cells. With this data, I identified two broad categories of cells, canonical alveolar macrophages (AM) and recruited monocytes (RM), which expressed high levels of IL1b and other inflammatory factors (Figure 5.6a). I found that in asthmatic lungs, RMs are highly represented. However, in non-asthmatic lungs, myeloid composition skews heavily toward alveolar macrophages (Figure 5.6b). Analysis of the differentially expressed genes between AMs and RMs revealed differences in enzymes that modify glycosphingolipids (Figure 5.6c), along with other programs such as NFκB signaling and general lipid metabolism (data not shown). I hypothesized that the shift toward RMs in asthmatic lungs could result in differences in endogenous lipid antigens presented by CD1d. I systematically analyzed the glycosphingolipid-modifying enzymes that differed between AMs and RMs and identified several hits converging on the neo-lacto-/globo-ceramide synthesis pathway (Figure 5.6d). Based on transcript, AMs favored production of glycosphingolipids with complex, branched head groups. On the other hand, RMs were geared toward production of lactosylceramide (many other groups have likewise reported enhanced production of lactosylceramide by immune cells under inflammatory conditions). I thought that lactosylceramide could be a more potent activator of CD1d-restricted T cells than the baseline lipid milieu. To test this, I incubated a known CD1d-autoreactive γδ TCR line with a $CD1d^+$ antigen presenting cell (APC) line. The $CD1d^+$ APCs were loaded with a molar excess of β-lactosylceramide or treated with DMSO, keeping their endogenous lipid repertoire intact. I observed significant differences in the EC50 of activation with β-lactosylceramide and DMSO treated cells, indicating that β-lactosylceramide is an especially immunogenic antigen to CD1d-autoreactive T cells (Figure 5.6e). Thus, I put forward a potential mechanism whereby alterations in the $CD1d^+$ myeloid populations could translate to

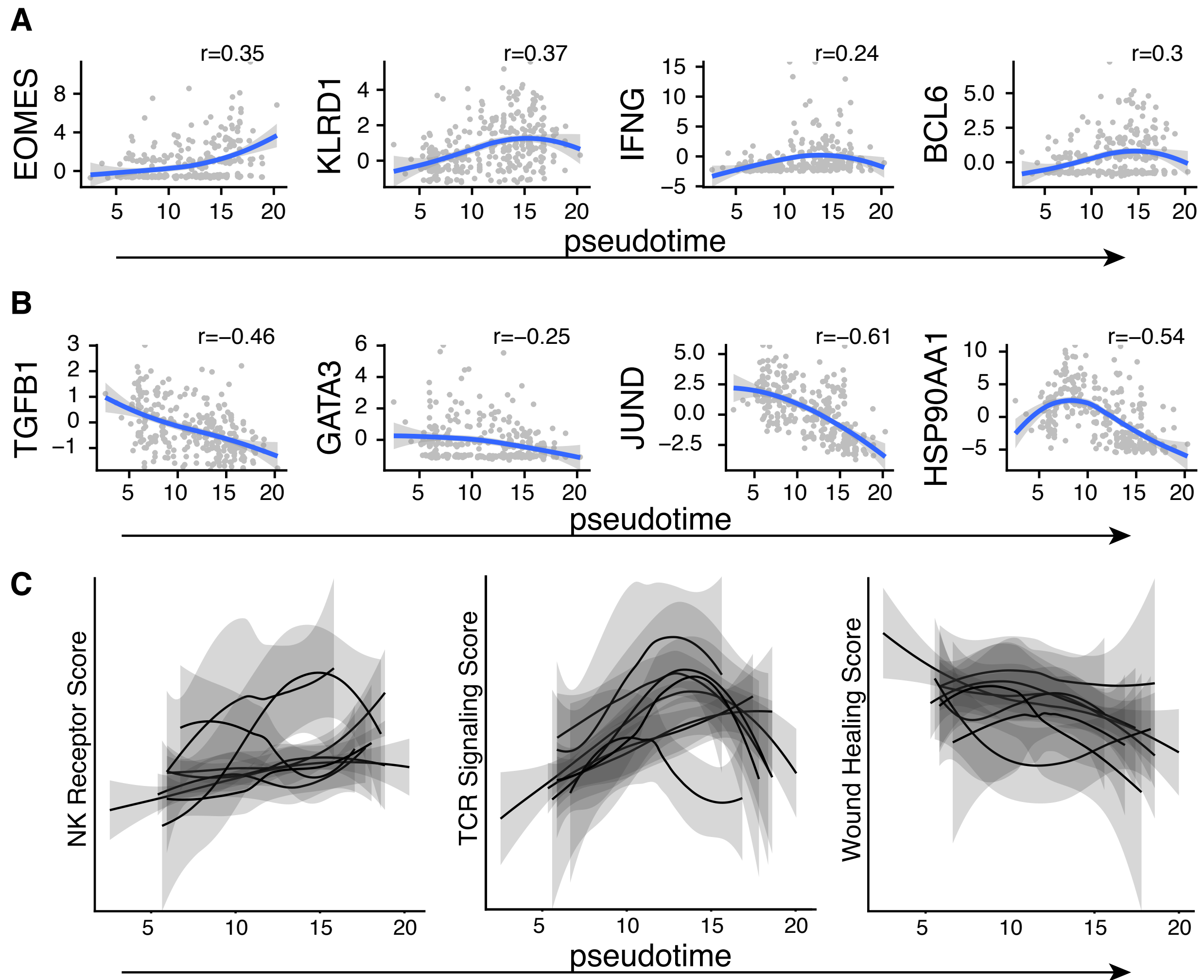


Figure 5.5: CD1d tetramer+ clones undergo a functional transition with clonal expansion in the asthmatic lung

A) Top expanded clone from Figure 3, highlighting genes with significant increase over the pseudotime trajectory. LOESS conditional means shown with blue line, points in grey represent individual cells. **B)** As in A, highlighting genes with significant decrease. **C)** Changes in gene signature score across selected expanded clones over pseudotime. For each clone, LOESS conditional means shown with black line with standard error shading

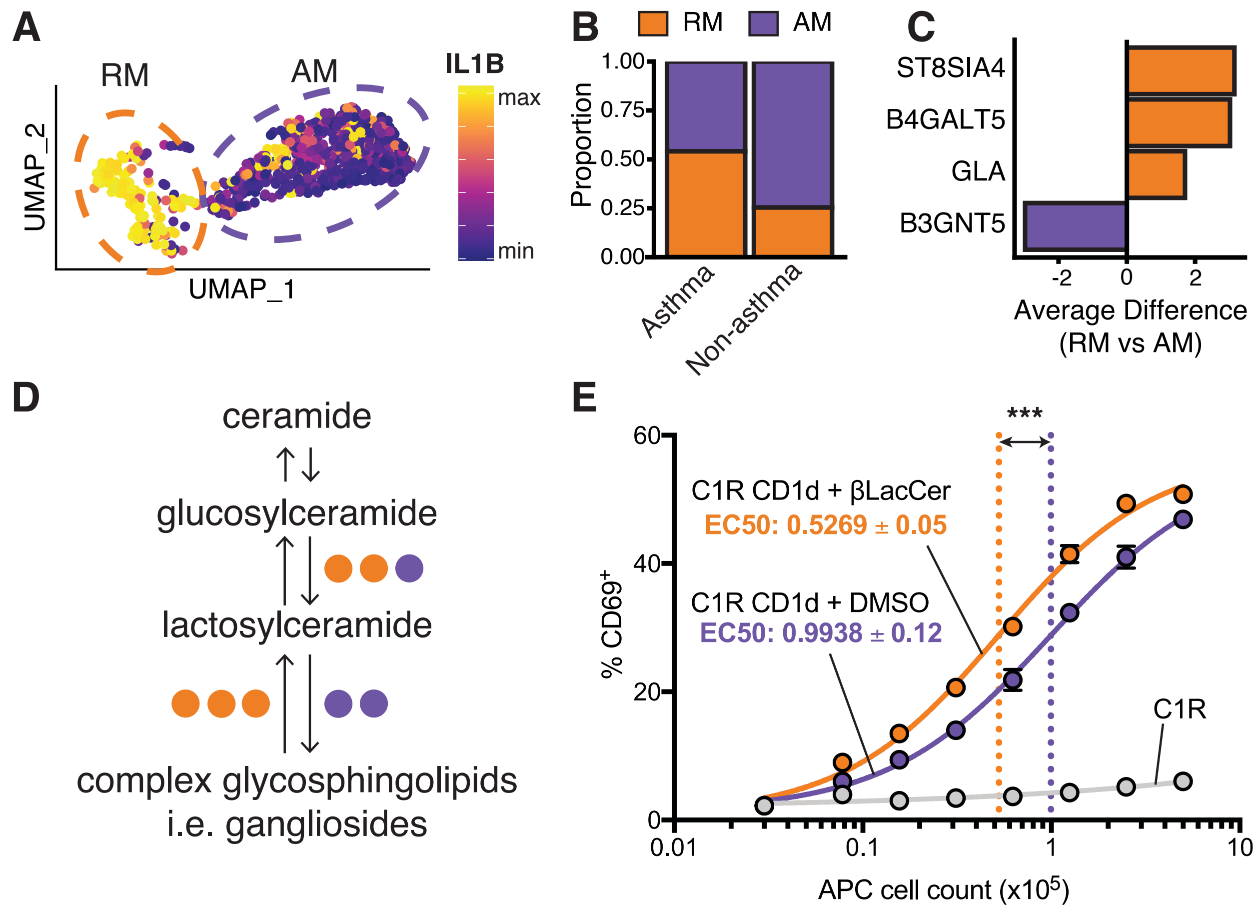


Figure 5.6: Shift in myeloid cell composition and glycosphingolipid production in asthmatic lungs

A) UMAP representation of $CD14^{+}$ myeloid cell transcriptome data, with each cell colored by expression level of IL1B (minimum=deep purple, maximum=light yellow), an example pro-inflammatory transcript. Phenotypic grouping of myeloid cells as recruited monocytic cells (RM, orange dashed circle) or alveolar macrophages (AM, purple dashed circle) is overlaid. **B)** Prevalence of alveolar macrophages (AM, purple) and recruited monocytic cells (RM, orange) by reported asthma status. **C)** Selected differentially expressed genes between AM and RM in the glycosphingolipid synthesis pathway. **D)** Overview of differentially expressed gene "hits" among lung myeloid cells mapped onto the glycosphingolipid metabolism pathway. Orange circles indicate a DEG in RM clusters, purple circles indicate a DEG in AM clusters. **E)** CD1d-restricted clone 9C2 activation in response to β-lactosylceramide (βLacCer). 9C2-expressing Jurkat line co-incubated with C1R lacking expression of CD1d (grey), or with C1R expressing CD1d loaded with endogenous cellular lipids + DMSO solvent (purple) or actively loaded with βLacCer dissolved in DMSO. *** = p value < 0.001 for difference in EC50s between DMSO and βLacCer treated cells.

changes to the lung lipid antigen pool, resulting, ultimately, in a more stimulatory environment for CD1d-autoreactive γδ T cells.

The differentially expressed genes in the recruited cells suggest two possible explanations for how monocyte-γδ T cell cross talk enables expansion and differentiation. TLR/NFkB signaling resulting pro-inflammatory cytokine production from the recruited monocytic cells could be creating TCR-independent stimuli for differentiation[137,138]. An additional consequence of TLR stimulation is an alteration in glycolipid metabolism, in which production of lactosylceramide is favored over more complex glycosphingolipids[139,140]. I hypothesized that this alteration in glycosphingolipid balance could alter repertoire of CD1d ligands, and thereby modulate the affinity of TCRs for CD1d. To test if lactosylceramide was a more potent agonist of CD1d-specific γδ TCRs, I loaded CD1d-expressing antigen presenting cells with lactosylceramide, displacing the endogenous CD1d lipid repertoire. I then used these cells to stimulate Jurkat lines expressing CD1d-autoreactive γδ TCRs. APCs loaded with lactosylceramide were more efficient at enabling activation of the TCRs, as measured by CD69 induction (Figure 5.6G). Thus, I hypothesize that these two interrelated consequences of DAMP/PAMP recognition by recruited monocytes could lead to the preferential expansion of CD1d-specific γδ T cells.

5.3 Discussion

In this report, we define the role of CD1d-reactive T cells in the lung, and their participation in asthmatic inflammation. We show that CD1d-specific γδ T cells, previously defined as a rare population in blood, undergo clonal expansion and functional specification into natural memory cells in the lung. The recruitment, expansion, and differentiation of these cells is enhanced in an asthmatic tissue. For conventional αβ T cells, TCR stimulation is required for several milestones in the "life" of a cell: thymic selection, peripheral differentiation, and effector function. In many ways, the contribution of the γδ TCR at these critical junctures is still opaque. Cytokines, particularly IL-15, have a much more clearly defined role in the maturation of γδ T cells[141] and their acquisition of

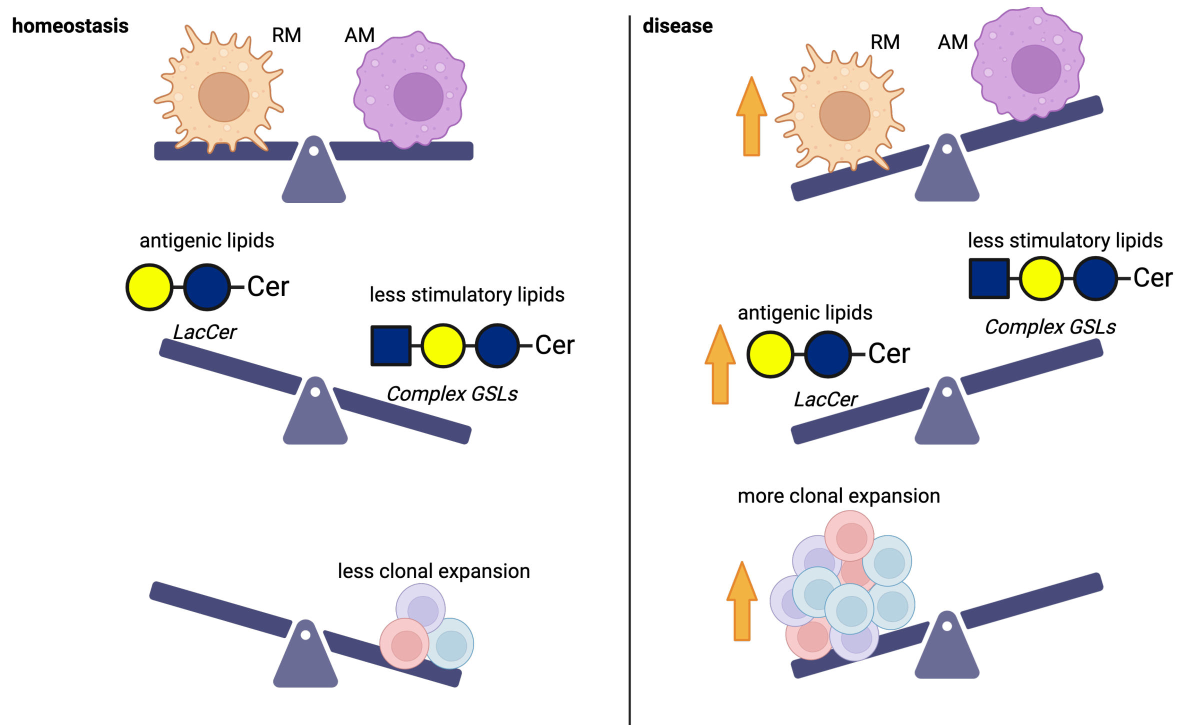


Figure 5.7: Proposed mechanism for observed clonal expansion of CD1d-autoreactive γδ T cells

NK receptor expression[142,143]. Herein, we provide observational evidence that these events can occur concomitant with activation through the γδ TCR. The CD1d-specific γδ T cells identified were more numerous and exhibit repertoire focusing in asthmatic lungs, whereas these phenomena were not reproduced by the MR-1 specific γδ T cells also analyzed. Further, we found that transcripts for NK receptors, which commonly mediate γδ T cell activation in tissues, do not appear until later in fate commitment.

We are unsure what additional signals might accompany TCR activation to enable expansion and differentiation in the lung. Given that binding between the γδ TCRs and CD1d was undetectable in a reconstituted system (data not shown), it is unlikely that this low-affinity interaction is solely sufficient to mediate activation. More plausible is an additive or costimulatory model of activation, where TCR signaling is integrated with many other inputs (similar to NK activation) or low level/tonic signaling through the TCR is accompanied by a stress-induced "second signal" to

trigger activation. Though we provide a reasonable TCR-centric explanation for how the asthma milieu could enhance CD1d-specific γδ T cell activation, it is still possible that fate acquisition and expansion is a wholly cytokine-driven process, but somehow cells specific for CD1d are more susceptible to the TCR-independent stimulus. We cannot formally discard this possibility, but our transcriptome data do not support it: components of the IL-12, IL-18, or IL-15 receptors were not among the differentially expressed genes for this subset.

Concluding that TCR does play a role, we propose a mechanism wherein changes in the myeloid compartment of the lung are relayed to the γδ T cells through interactions with CD1d. In our experiments, it is challenging to disentangle the effect of changes in the glycosphingolipid synthesis from the effects of myriad cellular programs altered in asthma. However, we were able to show in a reductionist activation assay that β-lactosylceramide is a potent agonist of CD1d-autoreactive γδ TCRs. This data fits well with the idea that some lipids are more "permissive" of CD1d-TCR interactions, while others, such as the extensively linked glycosphingolipids preferentially produced by alveolar macrophages, interfere with binding. Many inflammatory pathways converge upon production of lactosylceramide, so varied insults to the lung could alter the lipid repertoire to favor stronger CD1d-TCR interactions, contributing to T cell clonal expansion in the damaged or inflamed tissue.

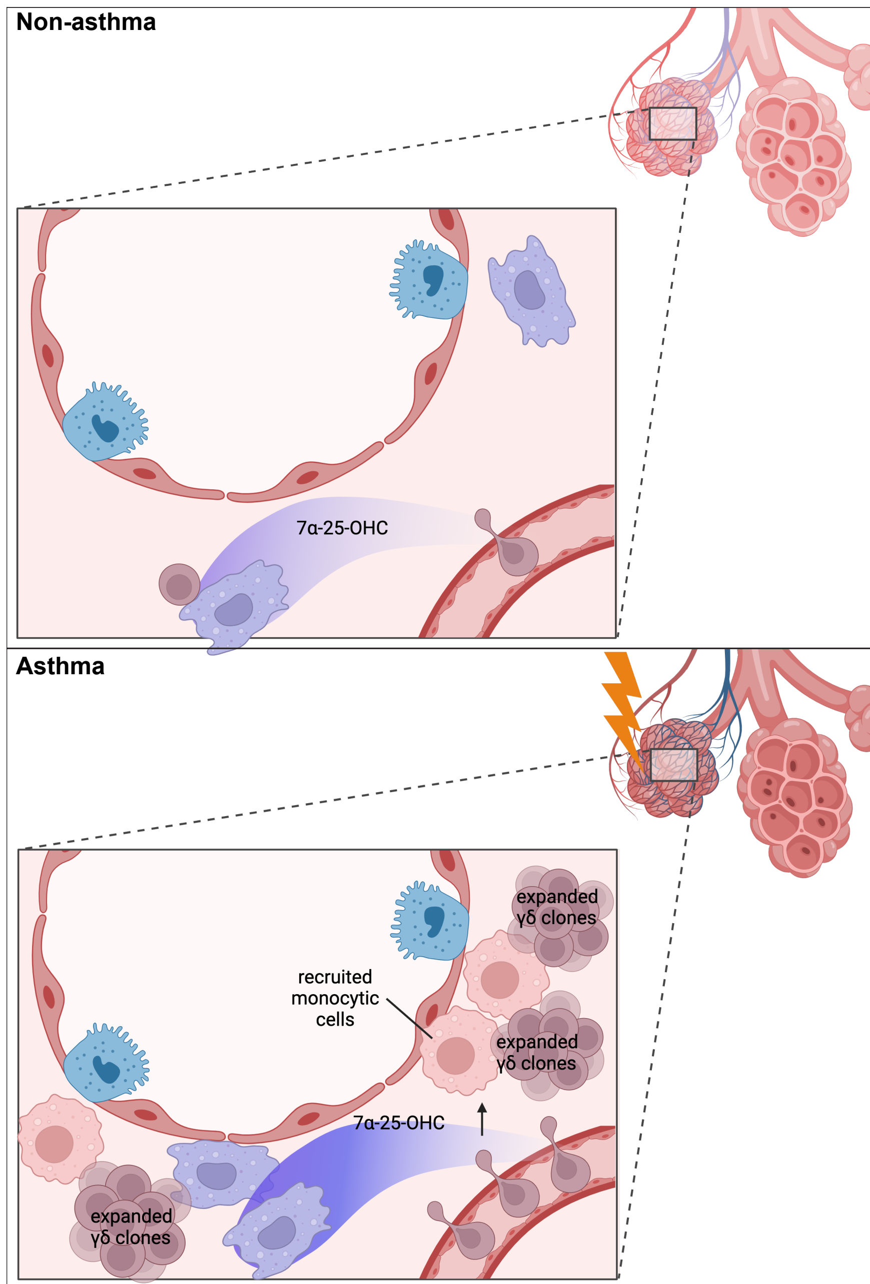


Figure 5.8: Overall model of CD1d-autoreactive γδ T cell function in lung homeostasis and disease

Top: at baseline, the lung myeloid compartment is dominant by non-immunogenic alveolar macrophages. CD1d-autoreactive γδ T cells are recruited to the lung at a very low level. Bottom: in the context of lung inflammation, inflammatory monocytes are recruited to the lung, along with many single CD1d-autoreactive γδ T cell clones. The recruited monocytes produce more antigenic lipids, which, when presented by CD1d, enable extensive clonal expansion in the diseased lung tissue.

CHAPTER 6

DISCUSSION AND FUTURE DIRECTIONS

6.1 Functional impact of self lipid specificity

Herein I describe a wide array of fates for T cells that recognize CD1d: recirculation, memory, tissue remodeling, cytotoxicity, even, strangely, quiescence. Across studies, we found partitioning of roles based on lipid specificity (or lack thereof). CD1d-endo specific cells had high CCR7, CD1d-LPC specific cells did not. Likewise, CD1d-endo specific cells had were clonally expanded in lung, CD1d-sulfatide specific cells were not; in cord blood, sulfatide specific cells expressed CD69 while CD1d-endo cells did not. Because these T cells have somewhat overlapping specificity, e.g. CD1d molecules loaded with endogenous lipids should also have some presenting LPC and sulfatide, differences in phenotype are puzzling. More puzzling still, the markers suggest that in cord blood, CD1d-endo tetramer^{+} cells tend to be relatively immature compared to their CD1d-LPC/CD1d-sulfatide specific counterparts. This turns our general understanding of T cell memory on its head. Broadly autoreactive T cells should have encountered CD1d (loaded with any lipid) and received a TCR signal many times over. Yet, this does not produce effector function. Conversely, one could hypothesize that stress-induced lipids, such as LPC and sulfatide, simply have not been encountered yet by the nascent T cell compartment in cord blood. Yet, the lipid-selective T cells are the ones that show signs of activation.

It would appear that constant encounters with endogenous antigen do not produce effector memory for CD1d-endo specific cells, but imparts them with stem-cell-like qualities instead. Assigning a stem-ness program to a broadly autoreactive T cell makes sense. Repeated TCR engagement with ubiquitous antigen could maintain cellular survival and low-level proliferation, enabling the signature self-renewal behavior of stem-like cells. Previously published results indicate that a stem-like subset of iNKT cells is maintained TCF1 dependent-manner. Future work could connect TCR signaling and TCF1 activity in diverse CD1d autoreactive T cells, providing mechanistic insight to the

observations reported here. Another key feature of stem-like T cells that they, with the right stimulus, proliferate rapidly to give rise to an assortment of functional subsets (with the same TCR). Our lung γδ clone-tracing results in the lung clearly reflect this proliferative division of labor. The presence of a CD1d-endo tetramer$^+$ stem-like progenitor would explain the bifurcated clonal products I see in the lung. Again, more experimental work is needed to close the loop between CD1d-endo specificity, stem-like T cell precursors, and the diversification of fates I have observed in this critical perinatal period.

6.2 Situating CD1d autoreactivity in models of γδ T cell function

Beyond a potential role in the perinatal period, self-reactivity is a crucial component of prevailing models of γδ T cell function. In the long-standing tissue stress hypothesis, stress-induced signals are integrated by the γδ T cell to positively regulate activation, resulting in a rapid protective immune response[144,145]. It is generally assumed that the γδ TCR participates in this process through recognition of self antigens. TCR recognition of self ligands is central to the notion of normality sensing, the other view that has emerged in the field[146]. Autoreactive T cells are maintained in tissues by expression of their ligand (canonically Btnls). When the tissue is damaged or infected, the ligand disappears, signaling a departure from homeostatic conditions. This critically remodels the γδ compartment to be more pro-inflammatory[52,147]. To summarize both of these ideas, the tissue stress hypothesis holds that a positive signal through the TCR promotes inflammation, whereas normality sensing posits that the absence of TCR signal promotes inflammation.

As an aside, use of the weaker TRGC2 constant region in γδ TCRs is congruent with the theoretical requirements of the normality sensing model. With a tissue-retention signal constantly relayed to the γδ T cell through the TCR, an attenuated signalling cascade could be advantageous. It is tempting to conjecture that TRGC1 is specialized for true antigenic specificity in keeping with the tissue stress hypothesis, and TRGC2 is adapted to normality sensing. Vγ4$^+$ TCRs, however, do not preferentially use TRGC2, despite their proven function in Btnl3/8-mediated normality sensing.

Excitingly, CD1d autoreactivity has the potential bring together both of these ideas. From a tissue surveillance perspective, the mixture of endogenous lipids in the groove of CD1d could be displaced by more immunogenic lipids that signal dysbiosis, such as β-lactosylceramide. Certainly, an analogous mechanism of T cell activation via "permissive" lipids has been convincingly shown for CD1a[86,88]. In the context of normality sensing, CD1d loaded with baseline self lipids could engage the γδ TCR and maintain the homeostatic γδ population. Much like Btnl3/8 or Skint1 expression, CD1d could convey to γδ T cells that all is well in their tissue niche. In sum, autoreactivity fits into both a comprehensive surveillance model that can be used to explain the function of CD1d-specific T cells in homeostasis and disease. Disentangling the many signals that come together to support γδ T cell survival and proliferation in the periphery is a major challenge. Because γδ T cells operate within complex, multi-cellular organ systems, this may be a case where manipulations in a mouse model could be useful. Notably, in mouse lungs, a γδ T cell population has been reported that engages endogenous lipids presented by CD1d to participate in antiviral responses[148,149]. A Nur77 reporter be used ascertain if CD1d-dependent γδ TCR signaling occurs in this population at baseline and during inflammation. Since asthma and acute viral infection have different etiologies and immunological signatures, it would be interesting to see if CD1d-restricted γδ T cell activation is a generalized response to a range of pulmonary insults.

6.3 Understanding the biochemistry of CD1d autoreactivity

This work has perhaps raised more questions about the nature of CD1d autoreactivity than it has answered. Among the most tantalizing are lines of inquiry around the biochemical nature of TCR-CD1d interaction. Most putative CD1d-specific TCRs that were identified by our 10X approach did not bind CD1d when expressed recombinantly. This was true for both lung γδ and cord blood αβ TCRs. Strangely, I affirmatively identified known MAIT and iNKT TCRs with this same method, verifying the validity of the approach. However, many methods for confirming binding in reductionist systems failed. Recombinant affinity measurements by BLI and SPR in both orienta-

tions failed to generate signal, even with analyze concentrations > 100μM. Jurkat lines and $CD3^+$ HEK293 lines expressing the TCR sequences of interest were generated, only to fail to bind to the same CD1d tetramers used to identify them. I screened potential high affinity CD1d antigens, including βLacCer, for their ability to recruit binding. I also added CD8 to the cell lines to see if coreceptor expression helped. I also tried activation assays, which are thought to be more sensitive than biochemical binding assays. None of these approaches elicited signs of TCR-CD1d binding. Even more puzzling, I followed this same workflow for a MR1 $tetramer^+$ γδ T cell clone, FWG, and were able to verify MR1 tetramer binding to a Jurkat clone reconstituted with the FWG TCR.

One interpretation is that the T cell-CD1d interaction could be mediated by a protein other than the TCR. Though a handful of protein receptors have been shown to bind to CD1d, none of them are expressed by both cord blood αβ T cells and lung γδ T cells, which are relatively transcriptionally distinct due to differences in antigen experience. However, there is also the possibility that this mysterious (and highly consistent) phenomenon could tell us something important about CD1d autoreactivity. Perhaps the early discovery and biochemical characterization of higher-affinity CD1d-specific TCRs has led us astray, the standard physiological affinity of the 1:1 CD1d-TCR interaction is extremely low. It is possible that what triggers signaling in CD1d-specific TCRs is an ensemble interaction, made up of many fleeting and weak binding events. Many of our immunological assays, biochemical and cellular, are not designed to register such events. Approaches such as such as imaging of of the immunological synapse or measurement of mechanical force could clarify the nature of this interaction.

In absence of robust biochemical binding data, computational analyses suggest that several J segments are important for imparting CD1d autoreactivity, TRDJ1 for lung γδ and TRBJ2-7 for cord blood αβ. While enriched in CD1d repertoire data sets, they are obviously present as well in other TCRs, including conventional αβ TCRs restricted to peptide MHC. Clearly, they are not sufficient for reactivity, but certainly, domain-swap type experiments, such as those undertaken for TRGC, could uncover the contribution of these J genes to TCR-CD1d reactivity.

In this vein, it is still unclear how TCR autoreactivity is generated piece-by-piece by rearrangement in the thymus. Most models of T cell selection in the thymus include TCR affinity or signal strength as a critical determinant. I put forward TRGC usage as an example of a parameter that can be tuned, compensating for reactivity to ubiquitous self antigens. How then, is TRGC usage selected? Potentially, a thymocyte with a newly recombined γδ TCR could incorporate TRGC2 after trying out TRGC1 and receiving a strong signal. In this way, TRGC2 usage could be a fail-safe mechanism to salvage a functionally rearranged but strongly autoreactive TCR. Another tempting possibility is that TRGC2 is part of a autoreactive TCR program. Previous work supports a transcription-factor-mediated temporal switch from TRGC1 to TRGC2 during human development–maybe TRGC2 could be preferentially incorporated along with TRDV1/TRDJ1 to robustly generate CD1d autoreactive T cells at specific stages in life. Careful dissection of TCR repertoires and chromatin accessibility during thymic development could clarify how and why TRGC2 is used in the TCR.

6.4 Translational implications

In this work, I have shown that a significant proportion of CD1d-restricted T cells are directed toward self (this has been previously demonstrated in mouse). Further, I suggest that many CD1d-restricted T cells are more or less antigen agnostic, and will bind indiscriminately to αGalCer, sulfatide, LPC, or just a mix of endogenous membrane lipids. αGalCer and its analogues have been investigated for their potential to invigorate an iNKT cell response against human cancers, with minimal success in the clinic. Our results would suggest that administration of αGalCer could also modulate a significant population of αβ and γδ T cells in tissues all over the body, which would undoubtedly impact clinical effectiveness and potentially lead to off target effects. Indeed, it would behoove researchers to remember that iNKT cells are not the only CD1d-restricted T cells, and, if anything, they are the anomalous CD1d-restricted subset.

γδ T cells can rapidly mount cytolytic responses and have adaptations that enable tissue res-

idency. Recent reports suggest that they do not become canonically exhausted like other TILs. These traits make for a promising cancer immunotherapy target, but commercial development of γδ therapeutics has been limited by the relative dearth of identified antigens. However, many emergent modalities could unlock the potential of tissue resident γδ T cells. For instance, bispecific T cell engagers bypass the need for antigen specificity, activating the TCR by cross-linking CD3 with overexpressed tumor antigens. It is also worth noting that not all γδ T cells will make effective cancer-fighting TILs. Strategies that modify the balance of pro- and anti-inflammatory γδ in tissues could increase the effectiveness of the immune response to cancer.

REFERENCES

[1] Erin J Adams, Siyi Gu, and Adrienne M Luoma. Human gamma delta T cells: Evolution and ligand recognition. *Cellular Immunology*, 296(1):31–40, 2015. ISSN 0008-8749. doi:10.1016/j.cellimm.2015.04.008.

[2] Mark M. Davis and Pamela J. Bjorkman. T-cell antigen receptor genes and T-cell recognition. *Nature*, 334(6181):395–402, 1988. ISSN 0028-0836. doi:10.1038/334395a0.

[3] Yueh-hsiu Chien, Makio Iwashima, Kenneth B Kaplan, John F Elliott, and Mark M Davis. A new T-cell receptor gene located within the alpha locus and expressed early in T-cell differentiation. *Nature*, 327(6124):677–682, 1987. ISSN 0028-0836. doi:10.1038/327677a0.

[4] Na Xiong and David H Raulet. Development and selection of γδ T cells. *Immunological Reviews*, 215(1):15–31, 2007. ISSN 0105-2896. doi:10.1111/j.1600-065x.2006.00478.x.

[5] Ferenc Livak, Howard T. Petrie, I.Nicholas Crisps, and David G. Schatz. In-frame TCR δ gene rearrangements play a critical role in the αβ/γδ T cell lineage decision. *Immunity*, 2(6): 617–627, 1995. ISSN 1074-7613. doi:10.1016/1074-7613(95)90006-3.

[6] Kirk D.C. Jensen, Xiaoqin Su, Sunny Shin, Luke Li, Sawsan Youssef, Sho Yamasaki, Lawrence Steinman, Takashi Saito, Richard M. Locksley, Mark M. Davis, Nicole Baumgarth, and Yueh-hsiu Chien. Thymic Selection Determines γδ T Cell Effector Fate: Antigen-Naive Cells Make Interleukin-17 and Antigen-Experienced Cells Make Interferon γ. *Immunity*, 29(1):90–100, 6 2008. ISSN 1074-7613. doi:10.1016/j.immuni.2008.04.022. Mouse gd thymic development and peripheral stimulation.

[7] You Jeong Lee, Gabriel J. Starrett, Seungeun Thera Lee, Rendong Yang, Christine M. Henzler, Stephen C. Jameson, and Kristin A. Hogquist. Lineage-Specific Effector Signatures of Invariant NKT Cells Are Shared amongst γδ T, Innate Lymphoid, and Th Cells. *The Journal of Immunology*, 197(4):1460–1470, 2016. ISSN 0022-1767. doi:10.4049/jimmunol.1600643.

[8] T M Vroom, G Scholte, F Ossendorp, and J Borst. Tissue distribution of human gamma delta T cells: no evidence for general epithelial tropism. *Journal of Clinical Pathology*, 44(12): 1012–1017, 1991. ISSN 0021-9746. doi:10.1136/jcp.44.12.1012. Prevalence at different sites in human.

[9] Kai Deusch, Friedrich Lüling, Kristian Reich, Meinhard Classen, Hermann Wagner, and Klaus Pfeffer. A major fraction of human intraepithelial lymphocytes simultaneously expresses the γ/δ T cell receptor, the CD8 accessory molecule and preferentially uses the Vδ1 gene segment. *European Journal of Immunology*, 21(4):1053–1059, 1991. ISSN 0014-2980. doi:10.1002/eji.1830210429.

[10] R Shimonkevitz, C Colburn, JA Burnham, RS Murray, and BL Kotzin. Clonal expansions of activated gamma/delta T cells in recent-onset multiple sclerosis. *Proceedings of the National Academy of Sciences*, 90(3):923–927, 1993. ISSN 0027-8424. doi:10.1073/pnas.90.3.923.

[11] Jonna Hvas, Jorge R Oksenberg, Raji Fernando, Lawrence Steinman, and Claude Bernard. γδ T cell receptor repertoire in brain lesions of patients with multiple sclerosis. *Journal of Neuroimmunology*, 46(1-2):225–234, 1993. ISSN 0165-5728. doi:10.1016/0165-5728(93)90253-u.

[12] Andrei Augustin, Ralph T Kubo, and Gek-Kee Sim. Resident pulmonary lymphocytes expressing the γ/δ T-cell receptor. *Nature*, 340(6230):239–241, 1989. ISSN 0028-0836. doi:10.1038/340239a0.

[13] Stuart Hunter, Carrie R Willcox, Martin S Davey, Sofya A Kasatskaya, Hannah C Jeffery, Dmitriy M Chudakov, Ye H Oo, and Benjamin E Willcox. Human liver infiltrating γδ T cells are composed of clonally expanded circulating and tissue-resident populations. *Journal of Hepatology*, 69(3):654–665, 2018. ISSN 0168-8278. doi:10.1016/j.jhep.2018.05.007.

[14] Marie-Paule Lefranc. IMGT-ONTOLOGY and IMGT databases, tools and Web resources for immunogenetics and immunoinformatics. *Molecular Immunology*, 40(10):647–659, 2004. ISSN 0161-5890. doi:10.1016/j.molimm.2003.09.006.

[15] Yueh-hsiu Chiei, Makio Iwashima, Daniel A Wettstein, Kenneth B Kaplan, John F Elliott, Willi Born, and Mark M Davis. T-cell receptor δ gene rearrangements in early thymocytes. *Nature*, 330(6150):722–727, 1987. ISSN 0028-0836. doi:10.1038/330722a0.

[16] A Forster, S Huck, N Ghanem, M P Lefranc, and T H Rabbitts. New subgroups in the human T cell rearranging V gamma gene locus. *The EMBO Journal*, 6(7):1945–1950, 1987. ISSN 0261-4189. doi:10.1002/j.1460-2075.1987.tb02456.x.

[17] C M Parker, V Groh, H Band, S A Porcelli, C Morita, M Fabbi, D Glass, J L Strominger, and M B Brenner. Evidence for extrathymic changes in the T cell receptor gamma/delta repertoire. *The Journal of Experimental Medicine*, 171(5):1597–1612, 1990. ISSN 0022-1007. doi:10.1084/jem.171.5.1597.

[18] Paola Tieppo, Maria Papadopoulou, Deborah Gatti, Naomi McGovern, Jerry K Y Chan, Françoise Gosselin, Glenn Goetgeluk, Karin Weening, Ling Ma, Nicolas Dauby, Alexandra Cogan, Catherine Donner, Florent Ginhoux, Bart Vandekerckhove, and David Vermijlen. The human fetal thymus generates invariant effector γδ T cells. *The Journal of experimental medicine*, 217(3), 2020. ISSN 0022-1007. doi:10.1084/jem.20190580.

[19] Tanya Dimova, Margreet Brouwer, Françoise Gosselin, Joël Tassignon, Oberdan Leo, Catherine Donner, Arnaud Marchant, and David Vermijlen. Effector Vγ9Vδ2 T cells dominate the human fetal γδ T-cell repertoire. *Proceedings of the National Academy of Sciences of the United States of America*, 112(6):E556–65, 2015. ISSN 0027-8424. doi:10.1073/pnas.1412058112.

[20] Biagio Lorenzo, Sarina Ravens, and Bruno Silva-Santos. High-throughput analysis of the human thymic Vδ1+ T cell receptor repertoire. *Scientific Data*, 6(1):115, 2019. doi:10.1038/s41597-019-0118-2. Vd1 sole thymus study.

[21] Martin S Davey, Carrie R Willcox, Stephen P Joyce, Kristin Ladell, Sofya A Kasatskaya, James E McLaren, Stuart Hunter, Mahboob Salim, Fiyaz Mohammed, David A Price, Dmitriy M Chudakov, and Benjamin E Willcox. Clonal selection in the human Vδ1 T cell repertoire indicates γδ TCR-dependent adaptive immune surveillance. *Nature Communications*, 8:14760, 2017. ISSN 2041-1723. doi:10.1038/ncomms14760.

[22] Martin S Davey, Carrie R Willcox, Stuart Hunter, Sofya A Kasatskaya, Ester B M Remmerswaal, Mahboob Salim, Fiyaz Mohammed, Frederike J Bemelman, Dmitriy M Chudakov, Ye H Oo, and Benjamin E Willcox. The human Vδ2+ T-cell compartment comprises distinct innate-like Vγ9+ and adaptive Vγ9- subsets. *Nature Communications*, 9(1):1760, 2018. doi:10.1038/s41467-018-04076-0.

[23] Jack L. McMurray, Anouk von Borstel, Taher E. Taher, Eleni Syrimi, Graham S. Taylor, Maria Sharif, Jamie Rossjohn, Ester B.M. Remmerswaal, Frederike J. Bemelman, Felipe A. Vieira Braga, Xi Chen, Sarah A. Teichmann, Fiyaz Mohammed, Andrea A. Berry, Kirsten E. Lyke, Kim C. Williamson, Michael J.T. Stubbington, Martin S. Davey, Carrie R. Willcox, and Benjamin E. Willcox. Transcriptional profiling of human Vδ1 T cells reveals a pathogen-driven adaptive differentiation program. *Cell Reports*, 39(8):110858, 2022. ISSN 2211-1247. doi:10.1016/j.celrep.2022.110858.

[24] Hong Wang, Zhimei Fang, and Craig T Morita. Vγ2Vδ2 T Cell Receptor Recognition of Prenyl Pyrophosphates Is Dependent on All CDRs. *The Journal of Immunology*, 184(11): 6209–6222, 2010. ISSN 0022-1767. doi:10.4049/jimmunol.1000231.

[25] Anna Vyborova, Dennis X Beringer, Domenico Fasci, Froso Karaiskaki, Eline van Diest, Lovro Kramer, Aram de Haas, Jasper Sanders, Anke Janssen, Trudy Straetemans, Daniel Olive, Jeanette Leusen, Lola Boutin, Steven Nedellec, Samantha L Schwartz, Michael J Wester, Keith A Lidke, Emmanuel Scotet, Diane S Lidke, Albert J R Heck, Zsolt Sebestyen, and Jürgen Kuball. γ9δ2T cell diversity and the receptor interface with tumor cells. *Journal of Clinical Investigation*, 2020. ISSN 0021-9738. doi:10.1172/jci132489.

[26] Kelly Hudspeth, Bruno Silva-Santos, and Domenico Mavilio. Natural cytotoxicity receptors: broader expression patterns and functions in innate and adaptive immune cells. *Frontiers in immunology*, 4:69, 2013. doi:10.3389/fimmu.2013.00069.

[27] Franck Halary, Marie□Alix Peyrat, Eric Champagne, Miguel Lopez□Botet, Alessandro Moretta, Lorenzo Moretta, Henri Vié, Jean□Jacques Fournié, and Marc Bonneville. Control of self□reactive cytotoxic T lymphocytes expressing γδ T cell receptors by natural killer inhibitory receptors. *European Journal of Immunology*, 27(11):2812–2821, 1997. ISSN 1521-4141. doi:10.1002/eji.1830271111.

[28] Stefan Bauer, Veronika Groh, Jun Wu, Alexander Steinle, Joseph H. Phillips, Lewis L. Lanier, and Thomas Spies. Activation of NK Cells and T Cells by NKG2D, a Receptor for Stress-Inducible MICA. *Science*, 285(5428):727–729, 1999. ISSN 0036-8075. doi:10.1126/science.285.5428.727.

[29] Bladimiro Rincon-Orozco, Volker Kunzmann, Philine Wrobel, Dieter Kabelitz, Alexander Steinle, and Thomas Herrmann. Activation of Vγ9Vδ2 T Cells by NKG2D. *The Journal of Immunology*, 175(4):2144–2151, 2005. ISSN 0022-1767. doi:10.4049/jimmunol.175.4.2144.

[30] M Brandes, K Willimann, G Bioley, N Levy, M Eberl, M Luo, R Tampe, F Levy, P Romero, and B Moser. Cross-presenting human T cells induce robust CD8+ T cell responses. *Proceedings of the National Academy of Sciences*, 106(7):2307–2312, 2009. ISSN 0027-8424. doi:10.1073/pnas.0810059106.

[31] Hans-Heinrich Oberg, Matthias Juricke, Dieter Kabelitz, and Daniela Wesch. Regulation of T cell activation by TLR ligands. *European Journal of Cell Biology*, 90(6-7):582–592, 2011. ISSN 0171-9335. doi:10.1016/j.ejcb.2010.11.012.

[32] Daniela Wesch, Christian Peters, Hans-Heinrich Oberg, Kathrin Pietschmann, and Dieter Kabelitz. Modulation of γδ T cell responses by TLR ligands. *Cellular and Molecular Life Sciences*, 68(14):2357–2370, 2011. ISSN 1420-682X. doi:10.1007/s00018-011-0699-1.

[33] Daniela Wesch, Susann Beetz, Hans-Heinrich Oberg, Matthias Marget, Kirsten Krengel, and Dieter Kabelitz. Direct Costimulatory Effect of TLR3 Ligand Poly(I:C) on Human γδ T Lymphocytes. *The Journal of Immunology*, 176(3):1348–1354, 2006. ISSN 0022-1767. doi:10.4049/jimmunol.176.3.1348.

[34] M S Krangel, B E Bierer, P Devlin, M Clabby, J L Strominger, J McLean, and M B Brenner. T3 glycoprotein is functional although structurally distinct on human T-cell receptor gamma T lymphocytes. *Proceedings of the National Academy of Sciences*, 84(11):3817–3821, 1987. ISSN 0027-8424. doi:10.1073/pnas.84.11.3817.

[35] De Dong, Lvqin Zheng, Jianquan Lin, Bailing Zhang, Yuwei Zhu, Ningning Li, Shuangyu Xie, Yuhang Wang, Ning Gao, and Zhiwei Huang. Structural basis of assembly of the human T cell receptor–CD3 complex. *Nature*, 573(7775):546–552, 2019. ISSN 0028-0836. doi:10.1038/s41586-019-1537-0.

[36] Sandra M Hayes and Paul E Love. Distinct Structure and Signaling Potential of the γδTCR Complex. *Immunity*, 16(6):827–838, 2002. ISSN 1074-7613. doi:10.1016/s1074-7613(02)00320-5.

[37] Hans Heiken, Ralf-Joachim Schulz, Jeffrey V Ravetch, Ellis L Reinherz, and Shigeo Koyasu. T lymphocyte development in the absence of Fcε receptor Iγ subunit: analysis of thymic-dependent and independent αβ and γδ pathways. *European Journal of Immunology*, 26(8): 1935–1943, 1996. ISSN 0014-2980. doi:10.1002/eji.1830260839.

[38] R Khattri, A I Sperling, D Qian, F W Fitch, E W Shores, P E Love, and J A Bluestone. TCR-gamma delta cells in CD3 zeta-deficient mice contain Fc epsilon RI gamma in the receptor complex but are specifically unresponsive to antigen. *Journal of immunology (Baltimore, Md. : 1950)*, 157(6):2320–7, 1996. ISSN 0022-1767.

[39] H Arase, S Ono, N Arase, S Y Park, K Wakizaka, H Watanabe, H Ohno, and T Saito. Developmental arrest of NK1.1+ T cell antigen receptor (TCR)-alpha/beta+ T cells and expansion of NK1.1+ TCR-gamma/delta+ T cell development in CD3 zeta-deficient mice. *The Journal of Experimental Medicine*, 182(3):891–895, 1995. ISSN 0022-1007. doi:10.1084/jem.182.3.891.

[40] Steven P Balk, John E Polischuk, Christopher Probert, Christopher Stevens, Ellen Ebert, Jian She, Cox Terhorst, and Richard S Blumberg. Composition of TCR-CD3 complex in human intestinal intraepithelial lymphocytes: lack of FcεRIγ chain. *International Immunology*, 7 (8):1237–1241, 1995. ISSN 0953-8178. doi:10.1093/intimm/7.8.1237.

[41] D Qian, A I Sperling, D W Lancki, Y Tatsumi, T A Barrett, J A Bluestone, and F W Fitch. The gamma chain of the high-affinity receptor for IgE is a major functional subunit of the T-cell antigen receptor complex in gamma delta T lymphocytes. *Proceedings of the National Academy of Sciences*, 90(24):11875–11879, 1993. ISSN 0027-8424. doi:10.1073/pnas.90.24.11875.

[42] Beatriz Garcillán, Ana V M Marin, Anaïs Jiménez-Reinoso, Alejandro C Briones, Miguel Muñoz-Ruiz, María J García-León, Juana Gil, Luis M Allende, Eduardo Martínez-Naves, María L Toribio, and José R Regueiro. γδ T Lymphocytes in the Diagnosis of Human T Cell Receptor Immunodeficiencies. *Frontiers in immunology*, 6:20, 2015. doi:10.3389/fimmu.2015.00020.

[43] Gabrielle M. Siegers, Mahima Swamy, Edgar Fernández-Malavé, Susana Minguet, Sylvia Rathmann, Alberto C. Guardo, Verónica Pérez-Flores, Jose R. Regueiro, Balbino Alarcón, Paul Fisch, and Wolfgang W.A. Schamel. Different composition of the human and the mouse γδ T cell receptor explains different phenotypes of CD3γ and CD3δ immunodeficiencies. *The Journal of Experimental Medicine*, 204(11):2537–2544, 2007. ISSN 0022-1007. doi:10.1084/jem.20070782.

[44] Andrew Sandstrom, Cassie-Marie Peigné, Alexandra Léger, James E Crooks, Fabienne Konczak, Marie-Claude Gesnel, Richard Breathnach, Marc Bonneville, Emmanuel Scotet, and Erin J Adams. The Intracellular B30.2 Domain of Butyrophilin 3A1 Binds Phosphoantigens to Mediate Activation of Human Vγ9Vδ2 T Cells. *Immunity*, 40(4):490–500, 2014. ISSN 1074-7613. doi:10.1016/j.immuni.2014.03.003.

[45] Siyi Gu, Joseph R. Sachleben, Christopher T. Boughter, Wioletta I. Nawrocka, Marta T. Borowska, Jeffrey T. Tarrasch, Georgios Skiniotis, Benoît Roux, and Erin J. Adams. Phosphoantigen-induced conformational change of butyrophilin 3A1 (BTN3A1) and its implication on Vγ9Vδ2 T cell activation. *Proceedings of the National Academy of Sciences*, 114(35):E7311–E7320, 2017. ISSN 0027-8424. doi:10.1073/pnas.1707547114.

[46] Yunyun Yang, Liping Li, Linjie Yuan, Xiaoying Zhou, Jianxin Duan, Hongying Xiao, Ningning Cai, Shuai Han, Xianqiang Ma, Weidong Liu, Chun-Chi Chen, Lingle Wang, Xin Li, Jiahuan Chen, Ning Kang, Jing Chen, Zhixun Shen, Satish R. Malwal, Wanli Liu, Yan Shi, Eric Oldfield, Rey-Ting Guo, and Yonghui Zhang. A Structural

Change in Butyrophilin upon Phosphoantigen Binding Underlies Phosphoantigen-Mediated Vγ9Vδ2 T Cell Activation. *Immunity*, 50(4):1043–1053.e5, 2019. ISSN 1074-7613. doi:10.1016/j.immuni.2019.02.016.

[47] Marc Rigau, Simone Ostrouska, Thomas S. Fulford, Darryl N. Johnson, Katherine Woods, Zheng Ruan, Hamish E.G. McWilliam, Christopher Hudson, Candani Tutuka, Adam K. Wheatley, Stephen J. Kent, Jose A. Villadangos, Bhupinder Pal, Christian Kurts, Jason Simmonds, Matthias Pelzing, Andrew D. Nash, Andrew Hammet, Anne M. Verhagen, Gino Vairo, Eugene Maraskovsky, Con Panousis, Nicholas A. Gherardin, Jonathan Cebon, Dale I. Godfrey, Andreas Behren, and Adam P. Uldrich. Butyrophilin 2A1 is essential for phosphoantigen reactivity by γδ T cells. *Science*, page eaay5516, 2020. ISSN 0036-8075. doi:10.1126/science.aay5516.

[48] Yoshimasa Tanaka, Craig T. Morita, Yoko Tanaka, Edward Nieves, Michael B. Brenner, and Barry R. Bloom. Natural and synthetic non-peptide antigens recognized by human γδ T cells. *Nature*, 375(6527):155–158, 1995. ISSN 0028-0836. doi:10.1038/375155a0.

[49] Jandke Anett, Melandri Daisy, Monin Leticia, Dmitry S, Ushakov, Adam G, Laing, Vantourout Pierre, East Philip, Nitta Takeshi, Narita Tomoya, Takayanagi Hiroshi, Feederle Regina, and Hayday Adrian. Butyrophilin-like proteins display combinatorial diversity in selecting and maintaining signature intraepithelial γδ T cell compartments. *Nature Communications*, 11(1):3769, 2020. doi:10.1038/s41467-020-17557-y.

[50] Carrie R Willcox, Pierre Vantourout, Mahboob Salim, Iva Zlatareva, Daisy Melandri, Leonor Zanardo, Roger George, Svend Kjaer, Mark Jeeves, Fiyaz Mohammed, Adrian C Hayday, and Benjamin E Willcox. Butyrophilin-like 3 Directly Binds a Human Vγ4+ T Cell Receptor Using a Modality Distinct from Clonally-Restricted Antigen. *Immunity*, 2019. ISSN 1074-7613. doi:10.1016/j.immuni.2019.09.006.

[51] Daisy Melandri, Iva Zlatareva, Raphaël A. G. Chaleil, Robin J. Dart, Andrew Chancellor, Oliver Nussbaumer, Oxana Polyakova, Natalie A. Roberts, Daniela Wesch, Dieter Kabelitz, Peter M. Irving, Susan John, Salah Mansour, Paul A. Bates, Pierre Vantourout, and Adrian C. Hayday. The γδTCR combines innate immunity with adaptive immunity by utilizing spatially distinct regions for agonist selection and antigen responsiveness. *Nature Immunology*, 19(12):1352–1365, 2018. ISSN 1529-2908. doi:10.1038/s41590-018-0253-5.

[52] Mayassi Toufic, Ladell Kristin, Gudjonson Herman, James E, McLaren, Dustin G, Shaw, Mai T, Tran, Jagoda J, Rokicka, Lawrence Ian, Grenier Jean-Christophe, Vincent van, Unen, Ciszewski Cezary, Dimaano Matthew, Hoda E, Sayegh, Kumar Vinod, Wijmenga Cisca, Green Peter, Gokhale Ranjana, Jericho Hilary, Carol E, Semrad, Guandalini Stefano, Aaron R, Dinner, Sonia S, Kupfer, Hugh H, Reid, Luis B, Barreiro, Rossjohn Jamie, David A, Price, and Jabri Bana. Chronic Inflammation Permanently Reshapes Tissue-Resident Immunity in Celiac Disease. *Cell*, 176(5):967–981.e19, 2019. ISSN 0092-8674. doi:10.1016/j.cell.2018.12.039.

[53] Benjamin E Willcox and Carrie R Willcox. γδ TCR ligands: the quest to solve a 500-million-year-old mystery. *Nature Immunology*, 20(2):121–128, 2019. ISSN 1529-2908. doi:10.1038/s41590-018-0304-y.

[54] Malte Deseke, Francesca Rampoldi, Inga Sandrock, Eva Borst, Heike Böning, George Liam Ssebyatika, Carina Jürgens, Nina Plückebaum, Maleen Beck, Ahmed Hassan, Likai Tan, Abdi Demera, Anika Janssen, Peter Steinberger, Christian Koenecke, Abel Viejo-Borbolla, Martin Messerle, Thomas Krey, and Immo Prinz. A CMV-induced adaptive human Vδ1+ γδ T cell clone recognizes HLA-DR. *Journal of Experimental Medicine*, 219(9):e20212525, 2022. ISSN 0022-1007. doi:10.1084/jem.20212525.

[55] Patricia M Benveniste, Sobhan Roy, Munehide Nakatsugawa, Edward LY Chen, Linh Nguyen, Douglas G Millar, Pamela S Ohashi, Naoto Hirano, Erin J Adams, and Juan Zúñiga-Pflücker. Generation and molecular recognition of melanoma-associated antigen-specific human γδ T cells. *Science Immunology*, 3(30):eaav4036, 2018. ISSN 2470-9468. doi:10.1126/sciimmunol.aav4036.

[56] Franca M Spada, Ethan P Grant, Peter J Peters, Masahiko Sugita, Augustín Melián, David S Leslie, Hoi K Lee, Elly van Donselaar, Dennis A Hanson, Alan M Krensky, Otto Majdic, Steven A Porcelli, Craig T Morita, and Michael B Brenner. Self-Recognition of Cd1 by γ/δ T Cells. *The Journal of Experimental Medicine*, 191(6):937–948, 2000. ISSN 0022-1007. doi:10.1084/jem.191.6.937.

[57] Anna M Russano, Gabrio Bassotti, Elisabetta Agea, Onelia Bistoni, Alessandro Mazzocchi, Antonio Morelli, Steven A Porcelli, and Fabrizio Spinozzi. CD1-Restricted Recognition of Exogenous and Self-Lipid Antigens by Duodenal γδ + T Lymphocytes. *The Journal of Immunology*, 178(6):3620–3626, 2007. ISSN 0022-1767. doi:10.4049/jimmunol.178.6.3620.

[58] Elisabetta Agea, Anna Russano, Onelia Bistoni, Roberta Mannucci, Ildo Nicoletti, Lanfranco Corazzi, Anthony D Postle, Gennaro De Libero, Steven A Porcelli, and Fabrizio Spinozzi. Human CD1-restricted T cell recognition of lipids from pollens. *The Journal of Experimental Medicine*, 202(2):295–308, 2005. ISSN 0022-1007. doi:10.1084/jem.20050773.

[59] Jérôme Le Nours, Nicholas A Gherardin, Sri H Ramarathinam, Wael Awad, Florian Wiede, Benjamin S Gully, Yogesh Khandokar, T Praveena, Jacinta M Wubben, Jarrod J Sandow, Andrew I Webb, Anouk von Borstel, Michael T Rice, Samuel J Redmond, Rebecca Seneviratna, Maria L Sandoval-Romero, Shihan Li, Michael N T Souter, Sidonia B G Eckle, Alexandra J Corbett, Hugh H Reid, Ligong Liu, David P Fairlie, Edward M Giles, Glen P Westall, Richard W Tothill, Martin S Davey, Richard Berry, Tony Tiganis, James McCluskey, Daniel G Pellicci, Anthony W Purcell, Adam P Uldrich, Dale I Godfrey, and Jamie Rossjohn. A class of γδ T cell receptors recognize the underside of the antigen-presenting molecule MR1. *Science (New York, N.Y.)*, 366(6472):1522–1527, 2019. doi:10.1126/science.aav3900.

[60] Adrienne M Luoma, Caitlin D Castro, and Erin J Adams. γδ T cell surveillance via

CD1 molecules. *Trends in immunology*, 35(12):613–621, 2014. ISSN 1471-4906. doi:10.1016/j.it.2014.09.003.

[61] Li Bai, Damien Picard, Brian Anderson, Vinod Chaudhary, Adrienne Luoma, Bana Jabri, Erin J Adams, Paul B Savage, and Albert Bendelac. The majority of CD1d-sulfatide-specific T cells in human blood use a semiinvariant Vδ1 TCR: Clinical immunology. *European Journal of Immunology*, 42(9):2505–2510, 2012. ISSN 0014-2980. doi:10.1002/eji.201242531.

[62] Luoma Adrienne M., Castro Caitlin D., Mayassi Toufic, Bembinster Leslie A., Bai Li, Picard Damien, Anderson Brian, Scharf Louise, Kung Jennifer E., Sibener Leah V., Savage Paul B., Jabri Bana, Bendelac Albert, and Adams Erin J. 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*, 39(6):1032–1042, 2013. ISSN 1074-7613. doi:10.1016/j.immuni.2013.11.001.

[63] Adam P Uldrich, Jérôme Le Nours, Daniel G Pellicci, Nicholas A Gherardin, Kirsty G McPherson, Ricky T Lim, Onisha Patel, Travis Beddoe, Stephanie Gras, Jamie Rossjohn, and Dale I Godfrey. CD1d-lipid antigen recognition by the γδ TCR. *Nature Immunology*, 14(11):1137–1145, 2013. ISSN 1529-2908. doi:10.1038/ni.2713. Gd CD1d sulf structure.

[64] Sobhan Roy, Dalam Ly, Caitlin D Castro, Nan-Sheng Li, Andrew J Hawk, John D Altman, Stephen C Meredith, Joseph A Piccirilli, D Branch Moody, and Erin J Adams. Molecular Analysis of Lipid-Reactive Vδ1 γδ T Cells Identified by CD1c Tetramers. *Journal of immunology (Baltimore, Md. : 1950)*, 196(4):1933–42, 2016. ISSN 0022-1767. doi:10.4049/jimmunol.1502202.

[65] Josephine F. Reijneveld, Tonatiuh A. Ocampo, Adam Shahine, Benjamin S. Gully, Pierre Vantourout, Adrian C. Hayday, Jamie Rossjohn, D. Branch Moody, and Ildiko Van Rhijn. Human γδ T cells recognize CD1b by two distinct mechanisms. *Proceedings of the National Academy of Sciences*, page 202010545, 2020. ISSN 0027-8424. doi:10.1073/pnas.2010545117.

[66] Marcin Wegrecki, Tonatiuh A. Ocampo, Sachith D. Gunasinghe, Anouk von Borstel, Shin Yi Tin, Josephine F. Reijneveld, Thinh-Phat Cao, Benjamin S. Gully, Jérôme Le Nours, D. Branch Moody, Ildiko Van Rhijn, and Jamie Rossjohn. Atypical sideways recognition of CD1a by autoreactive γδ T cell receptors. *Nature Communications*, 13(1):3872, 2022. doi:10.1038/s41467-022-31443-9.

[67] A Shamshiev, H J Gober, A Donda, Z Mazorra, L Mori, and G De Libero. Presentation of the Same Glycolipid by Different CD1 Molecules. *Journal of Experimental Medicine*, 195 (8):1013–1021, 2002. ISSN 0022-1007. doi:10.1084/jem.20011963.

[68] Shouxiong Huang, Adam Shahine, Tan-Yun Cheng, Yi-Ling Chen, Soo Weei Ng, Gautham R. Balaji, Rachel Farquhar, Stephanie Gras, Clare S. Hardman, John D. Altman, Nabil Tahiri, Adriaan J. Minnaard, Graham S. Ogg, Jacob A. Mayfield, Jamie Rossjohn, and D. Branch Moody. CD1 lipidomes reveal lipid-binding motifs and size-based antigen-display mechanisms. *Cell*, 2023. ISSN 0092-8674. doi:10.1016/j.cell.2023.08.022.

[69] Duarte C Barral and Michael B Brenner. CD1 antigen presentation: how it works. *Nature Reviews Immunology*, 7(12):929–941, 2007. ISSN 1474-1733. doi:10.1038/nri2191.

[70] Erin J Adams and Adrienne M Luoma. The Adaptable Major Histocompatibility Complex (MHC) Fold: Structure and Function of Nonclassical and MHC Class I–Like Molecules. *Annual Review of Immunology*, 31(1):529–561, 2013. ISSN 0732-0582. doi:10.1146/annurev-immunol-032712-095912.

[71] Dirk M Zajonc. The CD1 family: serving lipid antigens to T cells since the Mesozoic era. *Immunogenetics*, 68(8):561–576, 2016. ISSN 0093-7711. doi:10.1007/s00251-016-0931-0.

[72] Ya-Hui Chiu, Jayanthi Jayawardena, Angela Weiss, Daniel Lee, Se-Ho Park, Alice Dautry-Varsat, and Albert Bendelac. Distinct Subsets of CD1d-restricted T Cells Recognize Self-antigens Loaded in Different Cellular Compartments. *The Journal of Experimental Medicine*, 189(1):103–110, 1999. ISSN 0022-1007. doi:10.1084/jem.189.1.103.

[73] Albert Bendelac, Olivier Lantz, Mary E. Quimby, Jonathan W. Yewdell, Jack R. Bennink, and Randy R. Brutkiewicz. CD1 Recognition by Mouse NK1+ T Lymphocytes. *Science*, 268(5212):863–865, 1995. ISSN 0036-8075. doi:10.1126/science.7538697.

[74] Albert Bendelac, Paul B. Savage, and Luc Teyton. The Biology of NKT Cells. *Annual Review of Immunology*, 25(1):297–336, 2007. ISSN 0732-0582. doi:10.1146/annurev.immunol.25.022106.141711.

[75] Tetsu Kawano, Junqing Cui, Yasuhiko Koezuka, Isao Toura, Yoshikatsu Kaneko, Kazuhiro Motoki, Hitomi Ueno, Ryusuke Nakagawa, Hiroshi Sato, Eisuke Kondo, Haruhiko Koseki, and Masaru Taniguchi. CD1d-Restricted and TCR-Mediated Activation of Vα14 NKT Cells by Glycosylceramides. *Science*, 278(5343):1626–1629, 1997. ISSN 0036-8075. doi:10.1126/science.278.5343.1626.

[76] Melissa Bedard, Dilip Shrestha, David A Priestman, Yuting Wang, Falk Schneider, Juan D Matute, Shankar S Iyer, Uzi Gileadi, Gennaro Prota, Matheswaran Kandasamy, Natacha Veerapen, Gurdyal Besra, Marco Fritzsche, Sebastian Zeissig, Andrej Shevchenko, John C Christianson, Frances M Platt, Christian Eggeling, Richard S Blumberg, Mariolina Salio, and Vincenzo Cerundolo. Sterile activation of invariant natural killer T cells by ER-stressed antigen-presenting cells. *Proceedings of the National Academy of Sciences*, page 201910097, 2019. ISSN 0027-8424. doi:10.1073/pnas.1910097116.

[77] Suryasarathi Dasgupta and Vipin Kumar. Type II NKT cells: a distinct CD1d-restricted immune regulatory NKT cell subset. *Immunogenetics*, 68(8):665–76, 2016. ISSN 0093-7711. doi:10.1007/s00251-016-0930-1.

[78] Lisa M. Fox, Daryl G. Cox, Jennifer L. Lockridge, Xiaohua Wang, Xiuxu Chen, Louise Scharf, David L. Trott, Rachel M. Ndonye, Natacha Veerapen, Gurdyal S. Besra, Amy R. Howell, Mark E. Cook, Erin J. Adams, William H. Hildebrand, and Jenny E. Gumperz. Recognition of Lyso-Phospholipids by Human Natural Killer T Lymphocytes. *PLoS Biology*, 7(10):e1000228, 2009. ISSN 1544-9173. doi:10.1371/journal.pbio.1000228.

[79] Catarina F. Almeida, Srinivasan Sundararaj, Jérôme Le Nours, T. Praveena, Benjamin Cao, Satvika Burugupalli, Dylan G. M. Smith, Onisha Patel, Manfred Brigl, Daniel G. Pellicci, Spencer J. Williams, Adam P. Uldrich, Dale I. Godfrey, and Jamie Rossjohn. Distinct CD1d docking strategies exhibited by diverse Type II NKT cell receptors. *Nature Communications*, 10(1):5242, 2019. doi:10.1038/s41467-019-12941-9.

[80] Laurent Gapin, Dale I Godfrey, and Jamie Rossjohn. Natural Killer T cell obsession with self-antigens. *Current Opinion in Immunology*, 25(2):168–173, 2013. ISSN 0952-7915. doi:10.1016/j.coi.2013.01.002.

[81] López-Sagaseta Jacinto, Leah V, Sibener, Jennifer E, Kung, Gumperz Jenny, and Erin J, Adams. Lysophospholipid presentation by CD1d and recognition by a human Natural Killer T-cell receptor: Lysophospholipid recognition by iNKT cells. *The EMBO Journal*, 31(8): 2047–2059, 2012. ISSN 0261-4189. doi:10.1038/emboj.2012.54.

[82] Esther Dawen Yu, Enrico Girardi, Jing Wang, and Dirk M. Zajonc. Cutting Edge: Structural Basis for the Recognition of β-Linked Glycolipid Antigens by Invariant NKT Cells. *The Journal of Immunology*, 187(5):2079–2083, 2011. ISSN 0022-1767. doi:10.4049/jimmunol.1101636.

[83] Daniel G Pellicci, Andrew J Clarke, Onisha Patel, Thierry Mallevaey, Travis Beddoe, Jérôme Le Nours, Adam P Uldrich, James McCluskey, Gurdyal S Besra, Steven A Porcelli, Laurent Gapin, Dale I Godfrey, and Jamie Rossjohn. Recognition of β-linked self glycolipids mediated by natural killer T cell antigen receptors. *Nature Immunology*, 12(9): 827–833, 2011. ISSN 1529-2908. doi:10.1038/ni.2076.

[84] Thierry Mallevaey, Andrew J. Clarke, James P. Scott-Browne, Mary H. Young, Laila C. Roisman, Daniel G. Pellicci, Onisha Patel, Julian P. Vivian, Jennifer L. Matsuda, James McCluskey, Dale I. Godfrey, Philippa Marrack, Jamie Rossjohn, and Laurent Gapin. A Molecular Basis for NKT Cell Recognition of CD1d-Self-Antigen. *Immunity*, 34(3):315–326, 2011. ISSN 1074-7613. doi:10.1016/j.immuni.2011.01.013.

[85] Se-Ho Park, Angela Weiss, Kamel Benlagha, Tim Kyin, Luc Teyton, and Albert Bendelac. The Mouse Cd1d-Restricted Repertoire Is Dominated by a Few Autoreactive T Cell Receptor Families. *The Journal of Experimental Medicine*, 193(8):893–904, 2001. ISSN 0022-1007. doi:10.1084/jem.193.8.893.

[86] Annemieke de Jong, Tan-Yun Cheng, Shouxiong Huang, Stephanie Gras, Richard W Birkinshaw, Anne G Kasmar, Ildiko Van Rhijn, Victor Peña-Cruz, Daniel T Ruan, John D Altman, Jamie Rossjohn, and D Branch Moody. CD1a-autoreactive T cells recognize natural skin oils that function as headless antigens. *Nature Immunology*, 15(2):177–185, 2013. ISSN 1529-2908. doi:10.1038/ni.2790.

[87] Kwok S Wun, Josephine F Reijneveld, Tan-Yun Cheng, Kristin Ladell, Adam P Uldrich, Jérôme Le Nours, Kelly L Miners, James E McLaren, Emma J Grant, Oscar L Haigh, Thomas S Watkins, Sara Suliman, Sarah Iwany, Judith Jimenez, Roger Calderon, Kattya L

Tamara, Segundo R Leon, Megan B Murray, Jacob A Mayfield, John D Altman, Anthony W Purcell, John J Miles, Dale I Godfrey, Stephanie Gras, David A Price, Ildiko Van Rhijn, D Branch Moody, and Jamie Rossjohn. T cell autoreactivity directed toward CD1c itself rather than toward carried self lipids. *Nature immunology*, 19(4):397–406, 2018. ISSN 1529-2908. doi:10.1038/s41590-018-0065-7.

[88] Richard W Birkinshaw, Daniel G Pellicci, Tan-Yun Cheng, Andrew N Keller, Maria Sandoval-Romero, Stephanie Gras, Annemieke de Jong, Adam P Uldrich, D Branch Moody, Dale I Godfrey, and Jamie Rossjohn. αβ T cell antigen receptor recognition of CD1a presenting self lipid ligands. *Nature Immunology*, 16(3):258–266, 2015. ISSN 1529-2908. doi:10.1038/ni.3098.

[89] Vincent Pitard, David Roumanes, Xavier Lafarge, Lionel Couzi, Isabelle Garrigue, Marie-Edith Lafon, Pierre Merville, Jean-François Moreau, and Julie Déchanet-Merville. Long-term expansion of effector/memory Vdelta2-gammadelta T cells is a specific blood signature of CMV infection. *Blood*, 112(4):1317–24, 2008. ISSN 0006-4971. doi:10.1182/blood-2008-01-136713.

[90] Lionel Couzi, Vincent Pitard, Sonia Netzer, Isabelle Garrigue, Marie□Edith Lafon, Jean□François Moreau, Jean□Luc Taupin, Pierre Merville, and Julie Déchanet□Merville. Common Features of γδ T Cells and CD8 + αβ T Cells Responding to Human Cytomegalovirus Infection in Kidney Transplant Recipients. *The Journal of Infectious Diseases*, 200(9):1415–1424, 2009. ISSN 0022-1899. doi:10.1086/644509.

[91] Julie Déchanet, Pierre Merville, Annick Lim, Christelle Retière, Vincent Pitard, Xavier Lafarge, Susan Michelson, Claude Méric, Marie-Martine Hallet, Philippe Kourilsky, Luc Potaux, Marc Bonneville, and Jean-François Moreau. Implication of γδ T cells in the human immune response to cytomegalovirus. *Journal of Clinical Investigation*, 103(10):1437–1449, 1999. ISSN 0021-9738. doi:10.1172/jci5409.

[92] Carrie R Willcox, Vincent Pitard, Sonia Netzer, Lionel Couzi, Mahboob Salim, Tobias Silberzahn, Jean-François Moreau, Adrian C Hayday, Benjamin E Willcox, and Julie Déchanet-Merville. Cytomegalovirus and tumor stress surveillance by binding of a human γδ T cell antigen receptor to endothelial protein C receptor. *Nature Immunology*, 13(9):872–879, 2012. ISSN 1529-2908. doi:10.1038/ni.2394.

[93] Xavier Lafarge, Pierre Merville, Marie□Cécile Cazin, Frank Bergé, Luc Potaux, Jean□François Moreau, and Julie Déchanet□Merville. Cytomegalovirus Infection in Transplant Recipients Resolves When Circulating γδ T Lymphocytes Expand, Suggesting a Protective Antiviral Role. *The Journal of Infectious Diseases*, 184(5):533–541, 2001. ISSN 0022-1899. doi:10.1086/322843.

[94] Gregory S Olson, Sarah W Moore, James M Richter, John J Garber, Brittany A Bowman, Crystal A Rawlings, Meaghan Flagg, Björn Corleis, and Douglas S Kwon. Increased frequency of systemic pro-inflammatory Vδ1+ γδ T cells in HIV elite controllers correlates with gut viral load. *Scientific Reports*, 8(1):16471, 2018. doi:10.1038/s41598-018-34576-4.

[95] Eleonora Cimini, Chiara Agrati, Gianpiero D'Offizi, Chrysoula Vlassi, Rita Casetti, Alessandra Sacchi, Raffaella Lionetti, Veronica Bordoni, Nicola Tumino, Paola Scognamiglio, and Federico Martini. Primary and Chronic HIV Infection Differently Modulates Mucosal Vδ1 and Vδ2 T-Cells Differentiation Profile and Effector Functions. *PLOS ONE*, 10(6):e0129771, 2015. doi:10.1371/journal.pone.0129771.

[96] Paul Ogongo, James Zachary Porterfield, and Alasdair Leslie. Lung Tissue Resident Memory T-Cells in the Immune Response to Mycobacterium tuberculosis. *Frontiers in Immunology*, 10:992, 2019. doi:10.3389/fimmu.2019.00992. Nice background on lung Trm.

[97] I Tsuyuguchi, H Kawasumi, C Ueta, I Yano, and S Kishimoto. Increase of T-cell receptor gamma/delta-bearing T cells in cord blood of newborn babies obtained by in vitro stimulation with mycobacterial cord factor. *Infection and immunity*, 59(9):3053–9, 1991. ISSN 0019-9567.

[98] Y Bai, Y Lin, W He, Y Chen, and Y Ma. Analysis of the T cell receptor V delta region gene repertoire in bronchoalveolar lavage fluid (BALF) and peripheral blood of asthmatics. *Chinese medical journal*, 114(12):1252–7, 2001. ISSN 0366-6999.

[99] F Spinozzi, E Agea, O Bistoni, N Forenza, A Monaco, G Bassotti, I Nicoletti, C Riccardi, F Grignani, and A Bertotto. Increased Allergen-Specific, Steroid-Sensitive γδ T Cells in Bronchoalveolar Lavage Fluid from Patients with Asthma. *Annals of Internal Medicine*, 124(2):223, 1996. ISSN 0003-4819. doi:10.7326/0003-4819-124-2-199601150-00005.

[100] J. Krejsek, B. Král, D. Vokurková, V. Derner, M. Toušková, Z. Paráková, and O. Kopecký. Decreased peripheral blood γδ T cells in patients with bronchial asthma. *Allergy*, 53(1): 73–77, 1998. ISSN 1398-9995. doi:10.1111/j.1398-9995.1998.tb03776.x.

[101] Fabrizio Spinozzi, Elisabetta Agea, Onelia Bistoni, Nicola Forenza, Alessandro Monaco, Brunangelo Falini, Gabrio Bassotti, Fernando De Benedictis, Fausto Grignani, and Alberto Bertotto. Local Expansion of Allergen-Specific CD30+Th2-Type γδ T Cells in Bronchial Asthma. *Molecular Medicine*, 1(7):821–6, 1995. ISSN 1076-1551. doi:10.1007/bf03401896.

[102] Anna M Russano, Elisabetta Agea, Lanfranco Corazzi, Antyony D Postle, Gennaro De Libero, Steven Porcelli, Fernando M de Benedictis, and Fabrizio Spinozzi. Recognition of pollen-derived phosphatidyl-ethanolamine by human CD1d-restricted γδ T cells. *Journal of Allergy and Clinical Immunology*, 117(5):1178–1184, 2006. ISSN 0091-6749. doi:10.1016/j.jaci.2006.01.001. Potential for lung cd1d reactive cells.

[103] Susumu Isogai, Aravind Athiviraham, Russell S. Fraser, Rame Taha, Qutayba Hamid, and James G. Martin. Interferon□γ□dependent inhibition of late allergic airway responses and eosinophilia by CD8+γδ T cells. *Immunology*, 122(2):230–238, 10 2007. ISSN 1365-2567. doi:10.1111/j.1365-2567.2007.02632.x.

[104] Pin Wu, Dang Wu, Chao Ni, Jun Ye, Wuzhen Chen, Guoming Hu, Zhen Wang, Changrong Wang, Zhigang Zhang, Wenjie Xia, Zhigang Chen, Ke Wang, Tao Zhang, Jinghong Xu,

Yuehua Han, Ting Zhang, Xianguo Wu, Jianwei Wang, Weihua Gong, Shu Zheng, Fuming Qiu, Jun Yan, and Jian Huang. γδT17 Cells Promote the Accumulation and Expansion of Myeloid-Derived Suppressor Cells in Human Colorectal Cancer. *Immunity*, 40(5):785–800, 2014. ISSN 1074-7613. doi:10.1016/j.immuni.2014.03.013.

[105] Bernardo S. Reis, Patrick W. Darcy, Iasha Z. Khan, Christine S. Moon, Adam E. Kornberg, Vanessa S. Schneider, Yelina Alvarez, Olawale Eleso, Caixia Zhu, Marina Schernthanner, Ainsley Lockhart, Aubrey Reed, Juliana Bortolatto, Tiago B. R. Castro, Angelina M. Bilate, Sergei Grivennikov, Arnold S. Han, and Daniel Mucida. TCR-Vγδ usage distinguishes protumor from antitumor intestinal γδ T cell subsets. *Science*, 377(6603):276–284, 2022. ISSN 0036-8075. doi:10.1126/science.abj8695.

[106] Daniel V Correia, Manuela Fogli, Kelly Hudspeth, Maria Gomes da Silva, Domenico Mavilio, and Bruno Silva-Santos. Differentiation of human peripheral blood Vδ1+ T cells expressing the natural cytotoxicity receptor NKp30 for recognition of lymphoid leukemia cells. *Blood*, 118(4):992–1001, 2011. ISSN 0006-4971. doi:10.1182/blood-2011-02-339135.

[107] Cathal Harmon, Alexandra Zaborowski, Haim Moore, Pamela St. Louis, Karen Slattery, Danielle Duquette, John Scanlan, Harry Kane, Britta Kunkemoeller, Claire L. McIntyre, Aine Ni Scannail, Bruce Moran, Ana C. Anderson, Des Winter, Donal Brennan, Michael A. Brehm, and Lydia Lynch. γδ T cell dichotomy with opposing cytotoxic and wound healing functions in human solid tumors. *Nature Cancer*, 4(8):1122–1137, 2023. doi:10.1038/s43018-023-00589-w.

[108] Virginie Lafont, Françoise Sanchez, Emilie Laprevotte, Henri-Alexandre Michaud, Laurent Gros, Jean-François Eliaou, and Nathalie Bonnefoy. Plasticity of γδ T Cells: Impact on the Anti-Tumor Response. *Frontiers in Immunology*, 5:622, 2014. ISSN 1664-3224. doi:10.3389/fimmu.2014.00622. URL `https://www.frontiersin.org/articles/10.3389/fimmu.2014.00622/full`.

[109] Lianne Kok, David Masopust, and Ton N. Schumacher. The precursors of CD8+ tissue resident memory T cells: from lymphoid organs to infected tissues. *Nature Reviews Immunology*, 22(5):283–293, 2022. ISSN 1474-1733. doi:10.1038/s41577-021-00590-3.

[110] Scott N Mueller, Thomas Gebhardt, Francis R Carbone, and William R Heath. Memory T Cell Subsets, Migration Patterns, and Tissue Residence. *Annual Review of Immunology*, 31 (1):137–161, 2013. ISSN 0732-0582. doi:10.1146/annurev-immunol-032712-095954.

[111] Kriti Verma, Justyna Ogonek, Pavankumar Reddy Varanasi, Susanne Luther, Ivonne Bünting, Katrin Thomay, Yvonne Lisa Behrens, Eva Mischak-Weissinger, and Lothar Hambach. Human CD8+ CD57- TEMRA cells: Too young to be called ”old”. *PLoS ONE*, 12(5): e0177405, 2017. doi:10.1371/journal.pone.0177405.

[112] Andreia Monteiro, Catarina Cruto, Pedro Rosado, António Martinho, Luiza Rosado, Mafalda Fonseca, and Artur Paiva. Characterization of circulating gamma-delta T cells in relapsing vs remission multiple sclerosis. *Journal of Neuroimmunology*, (J. Neurol. 2017), 2018. ISSN 0165-5728. doi:10.1016/j.jneuroim.2018.02.009.

[113] John Jumper, Richard Evans, Alexander Pritzel, Tim Green, Michael Figurnov, Olaf Ronneberger, Kathryn Tunyasuvunakool, Russ Bates, Augustin Žídek, Anna Potapenko, Alex Bridgland, Clemens Meyer, Simon A. A. Kohl, Andrew J. Ballard, Andrew Cowie, Bernardino Romera-Paredes, Stanislav Nikolov, Rishub Jain, Jonas Adler, Trevor Back, Stig Petersen, David Reiman, Ellen Clancy, Michal Zielinski, Martin Steinegger, Michalina Pacholska, Tamas Berghammer, Sebastian Bodenstein, David Silver, Oriol Vinyals, Andrew W. Senior, Koray Kavukcuoglu, Pushmeet Kohli, and Demis Hassabis. Highly accurate protein structure prediction with AlphaFold. *Nature*, 596(7873):583–589, 2021. ISSN 0028-0836. doi:10.1038/s41586-021-03819-2.

[114] Marie-Paule Lefranc and T H Rabbitts. The human T-cell receptor γ (TRG) genes. *Trends in Biochemical Sciences*, 14(6):214–218, 1989. ISSN 0968-0004. doi:10.1016/0968-0004(89)90029-7.

[115] PG Pelicci, M Subar, A Weiss, R Dalla-Favera, and DR Littman. Molecular diversity of the human T-gamma constant region genes. *Science*, 237(4818):1051–1055, 1987. ISSN 0036-8075. doi:10.1126/science.3112943.

[116] Catherine Buresi, Nada Ghanem, Sylvie Huck, G□rard Lefranc, and Marie-Paule Lefranc. Exon duplication and triplication in the human T-cell receptor gamma constant region genes and RFLP in French, Lebanese, Tunisian, and Black African populations. *Immunogenetics*, 29(3):161–172, 1989. ISSN 0093-7711. doi:10.1007/bf00373641.

[117] F Hochstenbach, C Parker, J McLean, V Gieselmann, H Band, I Bank, L Chess, H Spits, J L Strominger, and J G Seidman. Characterization of a third form of the human T cell receptor gamma/delta. *The Journal of Experimental Medicine*, 168(2):761–776, 1988. ISSN 0022-1007. doi:10.1084/jem.168.2.761.

[118] F. Triebel and T. Hercend. Subpopulations of human peripheral T gamma delta lymphocytes. *Immunology Today*, 10(6):186–188, 1989. ISSN 0167-5699. doi:10.1016/0167-5699(89)90321-6.

[119] Juliette Roels, Jolien Van Hulle, Marieke Lavaert, Anna Kuchmiy, Steven Strubbe, Tom Putteman, Bart Vandekerckhove, Georges Leclercq, Filip Van Nieuwerburgh, Lena Boehme, and Tom Taghon. Transcriptional dynamics and epigenetic regulation of E and ID protein encoding genes during human T cell development. *Frontiers in Immunology*, 13:960918, 2022. doi:10.3389/fimmu.2022.960918.

[120] Timothy J. Allison, Christine C. Winter, Jean-Jacques Fournié, Marc Bonneville, and David N. Garboczi. Structure of a human γδ T-cell antigen receptor. *Nature*, 411(6839): 820–824, 2001. ISSN 0028-0836. doi:10.1038/35081115.

[121] Carter J. Wilson, Wing-Yiu Choy, and Mikko Karttunen. AlphaFold2: A Role for Disordered Protein/Region Prediction? *International Journal of Molecular Sciences*, 23(9):4591, 2022. doi:10.3390/ijms23094591.

[122] Peter H. Sudmant, Swapan Mallick, Bradley J. Nelson, Fereydoun Hormozdiari, Niklas Krumm, John Huddleston, Bradley P. Coe, Carl Baker, Susanne Nordenfelt, Michael Bamshad, Lynn B. Jorde, Olga L. Posukh, Hovhannes Sahakyan, W. Scott Watkins, Levon Yepiskoposyan, M. Syafiq Abdullah, Claudio M. Bravi, Cristian Capelli, Tor Hervig, Joseph T. S. Wee, Chris Tyler-Smith, George van Driem, Irene Gallego Romero, Aashish R. Jha, Sena Karachanak-Yankova, Draga Toncheva, David Comas, Brenna Henn, Toomas Kivisild, Andres Ruiz-Linares, Antti Sajantila, Ene Metspalu, Jüri Parik, Richard Villems, Elena B. Starikovskaya, George Ayodo, Cynthia M. Beall, Anna Di Rienzo, Michael F. Hammer, Rita Khusainova, Elza Khusnutdinova, William Klitz, Cheryl Winkler, Damian Labuda, Mait Metspalu, Sarah A. Tishkoff, Stanislav Dryomov, Rem Sukernik, Nick Patterson, David Reich, and Evan E. Eichler. Global diversity, population stratification, and selection of human copy-number variation. *Science*, 349(6253):aab3761, 2015. ISSN 0036-8075. doi:10.1126/science.aab3761.

[123] Luis B. Barreiro and Lluís Quintana-Murci. From evolutionary genetics to human immunology: how selection shapes host defence genes. *Nature Reviews Genetics*, 11(1):17–30, 2010. ISSN 1471-0056. doi:10.1038/nrg2698.

[124] Robert J. Mallis, Jonathan S. Duke-Cohan, Dibyendu Kumar Das, Aoi Akitsu, Adrienne M. Luoma, Debasis Banik, Hannah M. Stephens, Paul W. Tetteh, Caitlin D. Castro, Sophie Krahnke, Rebecca E. Hussey, Brian Lawney, Kristine N. Brazin, Pedro A. Reche, Wonmuk Hwang, Erin J. Adams, Matthew J. Lang, and Ellis L. Reinherz. Molecular design of the γδT cell receptor ectodomain encodes biologically fit ligand recognition in the absence of mechanosensing. *Proceedings of the National Academy of Sciences*, 118(26):e2023050118, 2021. ISSN 0027-8424. doi:10.1073/pnas.2023050118.

[125] Kamel Benlagha, Datsen G. Wei, Joel Veiga, Luc Teyton, and Albert Bendelac. Characterization of the early stages of thymic NKT cell development. *The Journal of Experimental Medicine*, 202(4):485–492, 2005. ISSN 0022-1007. doi:10.1084/jem.20050456.

[126] Jie Zhao, Xiufang Weng, Sreya Bagchi, and Chyung-Ru Wang. Polyclonal type II natural killer T cells require PLZF and SAP for their development and contribute to CpG-mediated antitumor response. *Proceedings of the National Academy of Sciences*, 111(7):2674–2679, 2014. ISSN 0027-8424. doi:10.1073/pnas.1323845111.

[127] Marlon Stoeckius, Christoph Hafemeister, William Stephenson, Brian Houck-Loomis, Pratip K Chattopadhyay, Harold Swerdlow, Rahul Satija, and Peter Smibert. Simultaneous epitope and transcriptome measurement in single cells. *Nature Methods*, 14(9):865–868, 2017. ISSN 1548-7091. doi:10.1038/nmeth.4380.

[128] Shu-Qi Zhang, Ke-Yue Ma, Alexandra A Schonnesen, Mingliang Zhang, Chenfeng He, Eric Sun, Chad M Williams, Weiping Jia, and Ning Jiang. High-throughput determination of the antigen specificities of T cell receptors in single cells. *Nature Biotechnology*, 36(12):1156, 2018. ISSN 1546-1696. doi:10.1038/nbt.4282.

[129] Xudong Zhao, Qiang Shan, and Hai-Hui Xue. TCF1 in T cell immunity: a broadened frontier. *Nature Reviews Immunology*, 22(3):147–157, 2022. ISSN 1474-1733. doi:10.1038/s41577-021-00563-6.

[130] R. Stokes Peebles. Prostaglandins in asthma and allergic diseases. *Pharmacology & Therapeutics*, 193:1–19, 2019. ISSN 0163-7258. doi:10.1016/j.pharmthera.2018.08.001.

[131] Rodney D. Britt, Ned Porter, Mitchell H. Grayson, Kymberly M. Gowdy, Megan Ballinger, Kara Wada, Hye-Young Kim, and Mireia Guerau-de Arellano. Sterols and immune mechanisms in asthma. *Journal of Allergy and Clinical Immunology*, 151(1):47–59, 2023. ISSN 0091-6749. doi:10.1016/j.jaci.2022.09.025.

[132] Tianci Jiang, Lingling Dai, Pengfei Li, Junwei Zhao, Xi Wang, Lin An, Meng Liu, Shujun Wu, Yu Wang, Youmei Peng, Di Sun, Caopei Zheng, Tingting Wang, Xuejun Wen, and Zhe Cheng. Lipid metabolism and identification of biomarkers in asthma by lipidomic analysis. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 1866(2): 158853, 2021. ISSN 1388-1981. doi:10.1016/j.bbalip.2020.158853.

[133] Bárbara Olmeda, Marta Martínez-Calle, and Jesus Pérez-Gil. Pulmonary surfactant metabolism in the alveolar airspace: Biogenesis, extracellular conversions, recycling. *Annals of Anatomy - Anatomischer Anzeiger*, 209:78–92, 2017. ISSN 0940-9602. doi:10.1016/j.aanat.2016.09.008.

[134] Chiaki Iwamura and Toshinori Nakayama. Role of CD1d- and MR1-Restricted T Cells in Asthma. *Frontiers in Immunology*, 9:1942, 2018. doi:10.3389/fimmu.2018.01942.

[135] Daryl Cox, Lisa Fox, Runying Tian, Wilfried Bardet, Matthew Skaley, Danijela Mojsilovic, Jenny Gumperz, and William Hildebrand. Determination of cellular lipids bound to human CD1d molecules. *PloS one*, 4(5):e5325, 2009. doi:10.1371/journal.pone.0005325.

[136] Francesco Dieli, Fabrizio Poccia, Martin Lipp, Guido Sireci, Nadia Caccamo, Caterina Di Sano, and Alfredo Salerno. Differentiation of Effector/Memory Vδ2 T Cells and Migratory Routes in Lymph Nodes or Inflammatory Sites. *The Journal of Experimental Medicine*, 198 (3):391–397, 2003. ISSN 0022-1007. doi:10.1084/jem.20030235.

[137] J Balhara and A S Gounni. The alveolar macrophages in asthma: a double-edged sword. *Mucosal Immunology*, 5(6):605–609, 2012. ISSN 1933-0219. doi:10.1038/mi.2012.74.

[138] T. Anienke van der Veen, Linsey E.S. de Groot, and Barbro N. Melgert. The different faces of the macrophage in asthma. *Current Opinion in Pulmonary Medicine*, 26(1):62–68, 2020. ISSN 1070-5287. doi:10.1097/mcp.0000000000000647.

[139] Subroto Chatterjee, Amrita Balram, and Wendy Li. Convergence: Lactosylceramide-Centric Signaling Pathways Induce Inflammation, Oxidative Stress, and Other Phenotypic Outcomes. *International Journal of Molecular Sciences*, 22(4):1816, 2021. doi:10.3390/ijms22041816.

[140] Karen Muindi, Manuela Cernadas, Gerald F. M. Watts, Louise Royle, David C. A. Neville, Raymond A. Dwek, Gurdyal S. Besra, Pauline M. Rudd, Terry D. Butters, and Michael B. Brenner. Activation state and intracellular trafficking contribute to the repertoire of endogenous glycosphingolipids presented by CD1d. *Proceedings of the National Academy of Sciences*, 107(7):3052–3057, 2010. ISSN 0027-8424. doi:10.1073/pnas.0915056107.

[141] Julie C. Ribot, Sérgio T. Ribeiro, Daniel V. Correia, Ana E. Sousa, and Bruno Silva-Santos. Human γδ Thymocytes Are Functionally Immature and Differentiate into Cytotoxic Type 1 Effector T Cells upon IL-2/IL-15 Signaling. *The Journal of Immunology*, 192(5):2237–2243, 2014. ISSN 0022-1767. doi:10.4049/jimmunol.1303119. OP9 differentiation here: cytokine pathway.

[142] Arthur I. Roberts, Leanne Lee, Eliezer Schwarz, Veronika Groh, Thomas Spies, Ellen C. Ebert, and Bana Jabri. Cutting Edge: NKG2D Receptors Induced by IL-15 Costimulate CD28-Negative Effector CTL in the Tissue Microenvironment. *The Journal of Immunology*, 167(10):5527–5530, 2001. ISSN 0022-1767. doi:10.4049/jimmunol.167.10.5527.

[143] Bertrand Meresse, Zhangguo Chen, Cezary Ciszewski, Maria Tretiakova, Govind Bhagat, Thomas N. Krausz, David H. Raulet, Lewis L. Lanier, Veronika Groh, Thomas Spies, Ellen C. Ebert, Peter H. Green, and Bana Jabri. Coordinated Induction by IL15 of a TCR-Independent NKG2D Signaling Pathway Converts CTL into Lymphokine-Activated Killer Cells in Celiac Disease. *Immunity*, 21(3):357–366, 2004. ISSN 1074-7613. doi:10.1016/j.immuni.2004.06.020.

[144] Julie C. Ribot, Noëlla Lopes, and Bruno Silva-Santos. γδ T cells in tissue physiology and surveillance. *Nature Reviews Immunology*, pages 1–12, 2020. ISSN 1474-1733. doi:10.1038/s41577-020-00452-4.

[145] Malte Deseke and Immo Prinz. Ligand recognition by the γδ TCR and discrimination between homeostasis and stress conditions. *Cellular & Molecular Immunology*, 17(9):914–924, 2020. ISSN 1672-7681. doi:10.1038/s41423-020-0503-y.

[146] Duncan R. McKenzie, Rosie Hart, Nourdine Bah, Dmitry S. Ushakov, Miguel Muñoz-Ruiz, Regina Feederle, and Adrian C. Hayday. Normality sensing licenses local T cells for innate-like tissue surveillance. *Nature Immunology*, 23(3):411–422, 2022. ISSN 1529-2908. doi:10.1038/s41590-021-01124-8.

[147] Susannah D. Barbee, Martin J. Woodward, Gleb Turchinovich, Jean-Jacques Mention, Julia M. Lewis, Lynn M. Boyden, Richard P. Lifton, Robert Tigelaar, and Adrian C. Hayday. Skint-1 is a highly specific, unique selecting component for epidermal T cells. *Proceedings of the National Academy of Sciences*, 108(8):3330–3335, 2011. ISSN 0027-8424. doi:10.1073/pnas.1010890108.

[148] Mélanie Dieudé, Harald Striegl, Aaron J Tyznik, Jing Wang, Samuel M Behar, Ciriaco A Piccirillo, Jerrold S Levine, Dirk M Zajonc, and Joyce Rauch. Cardiolipin binds to

CD1d and stimulates CD1d-restricted γδ T cells in the normal murine repertoire. *Journal of immunology (Baltimore, Md. : 1950)*, 186(8):4771–81, 2011. ISSN 0022-1767. doi:10.4049/jimmunol.1000921.

[149] Xiaohui Wang, Xiang Lin, Zihan Zheng, Bingtai Lu, Jun Wang, Andy Hee-Meng Tan, Meng Zhao, Jia Tong Loh, Sze Wai Ng, Qian Chen, Fan Xiao, Enyu Huang, King-Hung Ko, Zhong Huang, Jingyi Li, Kin-Hang Kok, Gen Lu, Xiaohui Liu, Kong-Peng Lam, Wanli Liu, Yuxia Zhang, Kwok-Yung Yuen, Tak Wah Mak, and Liwei Lu. Host-derived lipids orchestrate pulmonary γδ T cell response to provide early protection against influenza virus infection. *Nature Communications*, 12(1):1914, 2021. doi:10.1038/s41467-021-22242-9.