Human TRAV1-2 negative MR1-restricted T cells allow for broader pathogen recognition

By Erin Whitby Meermeier

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CERTIFICATE OF APPROVAL

This is to certify that the PhD dissertation of

Erin W. Meermeier

has been approved.

David M. Lewinsohn, MD PhD, Co-Mentor/Advisor

Marielle C. Gold, PhD, Co-Mentor/Advisor

Ann B. Hill, PhD, Oral Exam Committee Chair

David B. Jacoby, MD, Member

Georgiana E. Purdy, PhD, Member

Jonah B. Sacha, PhD, Member

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List of Selected Abbreviations

5-OP-RU = 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil

6-FP = 6-formyl pterin

ac6-FP = acetyl 6-formyl pterin

Ad = adenovirus

Ag = antigen

BAL = bronchoalveolar lavage

B2M = β-2-microglobulin

CDR = complementarity determining region

DC = dendritic cell

DP = double positive

DURT = donor-unrestricted T cell

ELISPOT = Enzyme-Linked ImmunoSpot

FACS = fluorescence activated cell sorting

HLA = human leukocyte antigen

ICS = Intracellular cytokine staining

IFN = interferon

ITAM = immunoreceptor tyrosine-based activation motifs

LDA = limiting dilution analysis

MAIT = mucosal associated semi-invariant T cells

MFI = geometric mean of the fluorescence intensity

MHC = major histocompatibility complex

MPXV = Monkeypox virus

MR1 = MHC class I related molecule 1

OPXV = Orthpoxviridae

PAMPs = pathogen-associated molecular patterns

PBMC = peripheral blood mononuclear cells

PHA = phytohaemagglutinin

PI3K = phosphoinositol-3 kinase

PLC = phospholipase C – gamma 1

PLZF = promyelocytic leukemia zinc finger, or ZBTB16

PMA = phorbol myristate acetate

RL = ribityllumazine

TA = transactivator

TAP = transporter associated with antigen processing

TCR = T cell receptor

TM = transmembrane

TNF = tumor necrosis factor alpha

TRAV = T cell receptor alpha variable

TRAJ = T cell receptor alpha joining

V(D)J = variable (diversity) joining

WT = wild type

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Thesis Abstract

The majority of research on T cells forms a paradigm in which classical T cells are activated by peptide antigens presented by classical MHC-molecules. Non-classical T cells do not fit into this paradigm because they generally recognize antigens presented by conserved non-classical molecules, have simplified patterns of T cell receptor (TCR) expression, and rapid effector responses – a function gained early in development. This dissertation aims to explore the functional repercussions of T cell receptors used by a subset of unconventional $CD8^+$ T cells, MR1-restricted T cells, that mediate recognition of a broad range of microbial infections. Mucosal-associated invariant T (MAIT) cells detect microbial vitamin B antigens presented by the non-classical molecule MR1 through the exclusive use of a TRAV1-2-containing TCRα. In our lab's prior evaluation of healthy individual's repertoires of anti-microbial nonclassical T cells, MAIT cells comprised a significant proportion but not all. This thesis tested the hypothesis that alternative nonclassical T cells could also recognize microbial infection through MR1 antigen presentation. By studying the *ex vivo* capacity of $CD8^+$ T cells and using a limiting dilution approach to clone $CD8^+$ T cells lacking MAIT cells, we identified a novel population of TRAV1-2 negative MR1-restricted T cells. Furthermore, we use these results to extend prior work on the role of diverse TCRs in selectively recognizing MR1-antigen from discrete microbial infections. Towards this end, we characterize an MR1-restricted clone that expresses the TRAV12-2 TCRα. In contrast to MAIT cells, this TRAV12-2^+ clone displays a distinct pattern of microbial recognition by detecting infection with the riboflavin auxotroph *Streptococcus pyogenes*. As known MAIT antigens are derived from riboflavin biosynthetic intermediates, this suggests that TRAV12-2^+ clone recognizes unique MR1-ligands. Thus, MR1-restricted T cells can discriminate between microbes in a TCR-dependent fashion. Next, because MAIT cells depend upon MR1 in the thymus for development, this thesis work was motivated to answer the question of why TRAV1-2 is dominantly used by MR1-restricted T cells. We hypothesized that in the thymus, prior to exogenous antigen contact that could cause preferential clonal expansions, we might observe a more

diverse TCR repertoire on developing MR1-restricted T cells. We conducted *ex vivo* analyses and limiting dilution T cell cloning, followed by functional assays of MR1-restricted T cells in the thymus. While we observed many different TCRs able to bind to a MR1-antigen tetramer, surprisingly, all MR1-restricted T cell clones isolated from a thymus expressed the TRAV1-2^{+} TCR; indicating a special affinity between this TCR and MR1 as early as thymic development. Overall, this work requires us to reconsider the assumption that all MR1-restricted T cells use a semi-invariant TCR and leaves open the question of whether the more rare TRAV1-2 negative cells share the innate-like qualities and unique developmental pathway of MAIT cells selected upon MR1 in the thymus. We postulate that additional MR1-restricted T cell subsets may play a unique role in defence against infection by broadening the recognition of microbial metabolites.

Chapter 1.1 T Lymphocytes in Immunity

The immune system recognizes pathogens and induces defensive responses

Heathy individuals come into contact with infectious microbes daily, however, the vast majority of these microbes never cause infection. This is because each individual has an immune system tuned to distinguish foreign entities and defend against infection. The immune system is broadly delineated into the innate versus adaptive immune system. These two categories are defined by the two different ways in which each system is programmed to discriminate between self and non-self. The innate immune system uses processes that are encoded in the germline, and thus shared across individuals, to detect general patterns of pathogens. Receptors of the innate immune system are limited in types but generally expressed by many different cells. Thus, innate immunity can act in the first lines of defense against infection because of the relatively higher frequency of mechanisms that can recognize a given pathogen instantaneously compared to the adaptive immunity. The innate immune system consists of defenses such as epithelial barriers, the complement system, and antimicrobial peptides. Additionally, phagocytic cells, such as macrophages, dendritic cells, and granulocytes, can kill pathogens opsonized by the complement cascade. Aside from directly killing the pathogen, phagocytes can also produce an inflammatory response to recruit other immune cells through cytokine and chemokine gradients. This inflammatory response depends on pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs), like cytosolic ion concentrations, lipopolysaccharide, or viral DNA. Thus, the innate immune system provides an initial discrimination between self and nonself. The inflammatory response generated by innate immune processes can activate specialized antigen presenting cells, or dendritic cells, and this is a necessary first step for the activation of adaptive immunity. In sum, innate immunity provides functional and immediate defenses against microbial infection through shared germline limited processes and receptors, and can initiate adaptive immune responses.

In stark contrast, adaptive immune processes recognize pathogens through a vast diversity of specific receptors. These receptors are generated by a complex feat of somatic cell gene rearrangements. Each cell of the adaptive immune system uniquely expresses a specific receptor from an enormous repertoire of total receptors. To provide this high diversity, the adaptive immune system relies on clonal expansion of antigen specific lymphocytes and therefore, these defense mechanisms take longer to provide protection. Another unique feature of the adaptive immune system is its ability to provide immunological memory by long-lived antigen experienced defensive cells. During an infection that overcomes the innate immune system, antigen from the pathogen accumulates and activates the adaptive immune system. Specifically, this process usually begins when an immature tissue dendritic cell recognizes and engulfs a pathogen. The activated dendritic cell then migrates to lymph nodes where it presents antigen to T cells, expresses T cell co-stimulatory markers, and secretes cytokines. Together, these signals can activate naïve helper T cells, which can then give 'help' to activate B cells and begin the adaptive immune response.

The adaptive immune response occurs over several days during which antigen specific naïve lymphocytes clonally expand and differentiate into effector T cells and antibody secreting B cells. While the humoral response, made by antibody-secreting B cells, is key for recognition of native antigens from the extracellular environment and plays a role in antigen presentation to T cells, this thesis will focus exclusively on the T cell response to infection.

Key Components of a T Cell

T cells are distinguished from other cells of the immune system by their development in the thymus and expression of a T cell receptor (TCR). TCRs are designed to engage antigen and antigen presentation molecules as depicted in Figure 1.1 below. The majority of human T cells rearrange their TCR α and β chain genes to express a $\alpha\beta$ TCR and are called $\alpha\beta$ T cells. A minority of human T cells

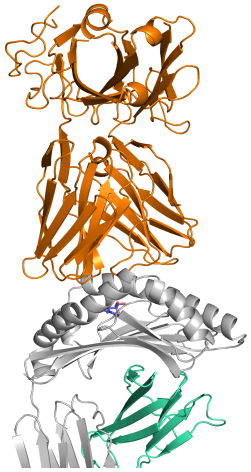


Figure 1.1 Ribbon protein structure of a TCR (orange) interacting with the MHC-related molecule MR1 (grey). Source: protein databank entry 4NQC

express a TCR expressed from γ and δ genes. γδ T cells are much less frequent in the blood (about 2% of T cells) and have not been characterized as extensively as αβ T cells and therefore, this thesis focuses mainly on αβ T cells. Each αβ TCR is a heterodimer that is generated with distinct steps that lead to a diverse TCR repertoire. Firstly, the TCRα and TCRβ gene loci undergo V(D)J rearrangement. In any given TCR there are six hypervariable complementarity determining regions (CDRs) that mediate TCR recognition of MHC-antigen; CDR1, 2, and 3 in each gene locus. The CDR1 and CDR2 regions are germline encoded within each TRAV and TRBV and are generally responsible for contacting the antigen presenting molecule. The CDR3 regions are formed at the junctional region in between V(D)J recombination and thus, are the most diverse region across TCRs. The CDR3 regions usually make contacts with the antigen bound in the groove of the MHC molecule. TCR diversity arises through different mechanisms such as gene recombination, junctional sequences and nucleotide additions, and random αβ TCR pairing. These mechanisms converge to endow the average human with $2x10^7$ different TCRs to survey the body for foreign epitopes (Arstila et al., 1999).

αβ T cells almost always express a co-receptor with the TCR: CD4 or CD8. The unique co-receptor expression on each T cell is determined during thymic development and generally indicates distinct lineages of αβ T cells. The CD4 and CD8 co-receptor function to help a T cell engage an antigen presenting cell. Specifically, CD4 and CD8 bind to a conserved site on major histocompatibility complex (MHC) class II molecules and MHC class I molecules, respectively, to help stabilize TCR recognition of antigen and MHC. In this way, the co-receptor helps the TCR with correct target recognition. T cells also express costimulatory surface proteins, like CD28 or CTLA-4, that interact with B7 family members on

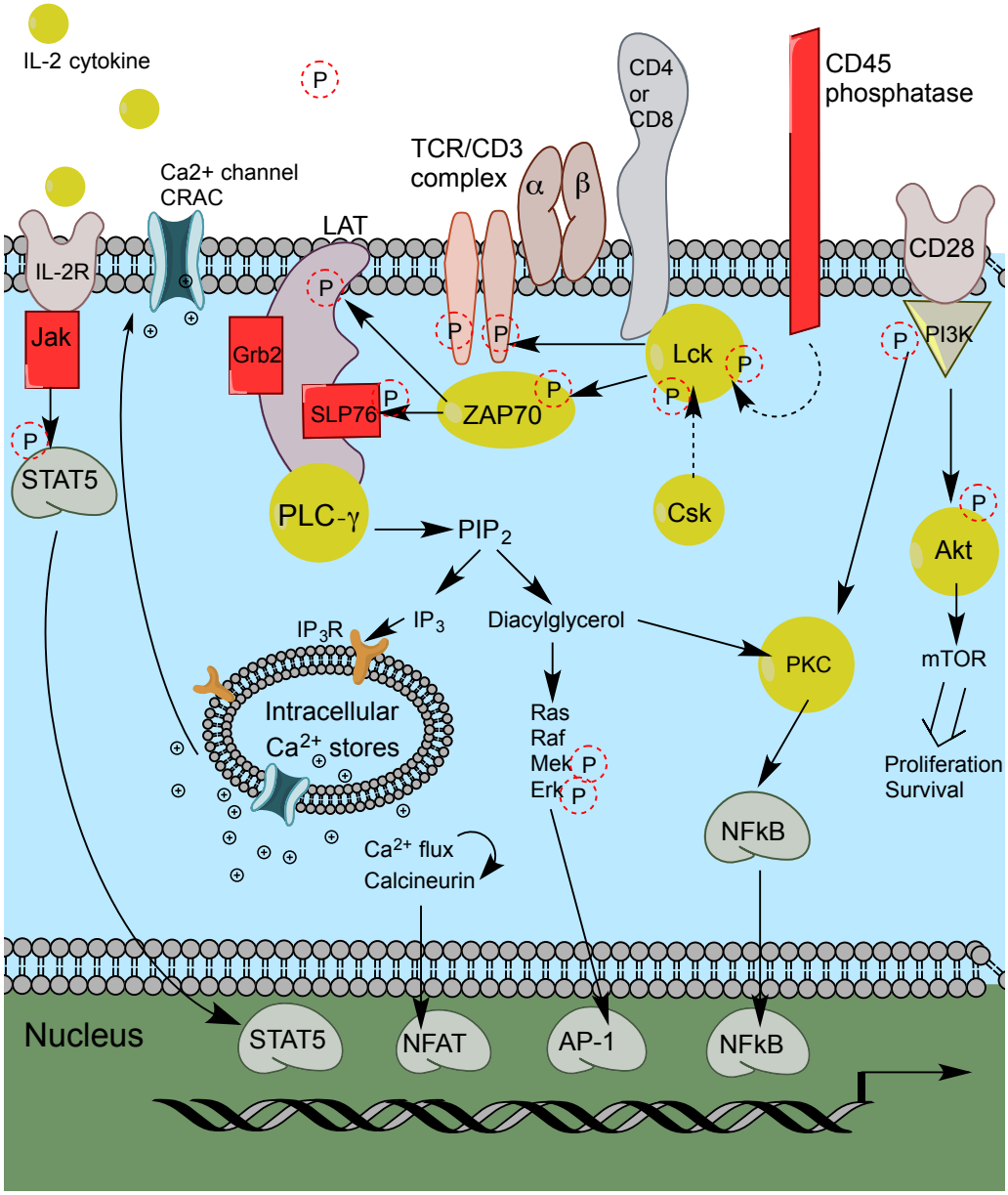
antigen presenting cells, and enhance or inhibit their activation by interacting with intracellular signaling cascades.

T Cell Activation

In addition to a TCR and co-receptor, every T cell also expresses protein machinery capable of transducing a signal from the TCR to the intracellular environment to activate the T cell response. There are many proteins involved in this signaling cascade (Figure 1.2), however, aside from the TCR, the CD3 complex is most important. The collective CD3 complex is always assembled with the TCR at the surface and is comprised of two ε chains, two ζ, one δ, and one γ. These CD3 signal-transducing chains contain cytoplasmic tails with 10 immunoreceptor tyrosine-based activation motifs (ITAMs). There are many events at the T cell surface that must coordinate in order for the cell to become activated (Chakraborty and Weiss, 2014; Weiss, 2010). Firstly, an immune synapse forms between a T cell and antigen presenting cell (APC), and its formation is required for full T cell activation (Davis et al., 2003; Norcross, 1984). A critical step in activation is the ordered phosphorylation of ITAMs intracellularly, which begins with activated Lck (a Src-family tyrosine kinase). Lck is recruited to the CD3 ITAMs by the intracellular tails of the CD8 or CD4 co-receptor (Purbhoo et al., 2001). Phosphorylated ITAMs recruit and activate the T cell specific tyrosine kinase ZAP-70 which phosphorylates transmembrane scaffold proteins to recruit phospholipase C-γ to the inner membrane. In order for the signal to progress, phospholipase C must be phosphorylated by a Tec kinase. Once activated, phospholipase C creates second messengers which act separately 1) to release intracellular calcium stores and 2) activate the small GTPase, Ras and the MAP kinase pathway. At the same time this cascade is occurring, co-stimulatory cell surface proteins, like CD28 act in a similar fashion. Specifically, when CD28 engages B7 family proteins on the APC, it activates phosphoinositol (PI)-3 kinase (PI3K) inside of the T cell. PI3K is a key signaling protein

that activates cellular pathways of proliferation and survival through mTOR. It also collaborates with the second messenger

Figure 1.2 Diagram of T Cell Signaling Cascades. The signal begins at the cell's surface and travels to the nucleus. The information for this diagram comes from Janeway, Immunobiology Textbook 7th Edition; Davis M. Annual Reviews Biochemistry 2003; and Brownlie et al., 2013 Nature Review Immunology



diacylglycerol (made by PLCγ) to activate protein kinase C. All of these pathways converge in the nucleus so that transcription factors, including NFκB, AP-1, and NFAT can induce T cell activation gene transcription.

T Cell Effector Functions

Upon activation, a T cell has a wide variety of effector functions that are usually associated with a certain lineage by convention - $CD4^+$ T cell versus $CD8^+$ T cell. However, in reality T cells can use a variety of effector functions to contain an infection. $CD8^+$ T cells are associated with cytotoxicity of an infected cell, whereas $CD4^+$ T cells help to generate an immune response tailored to the infection by secreting a distinct set of cytokines. $CD4^+$ T cells can differentiate from naïve into four main types of helper T cells (Becattini et al., 2015; Tubo et al., 2013). Helper T cells can activate B cells to make antibody (T_{FH}), promote allergic responses (T_H2), recruit granulocytes (T_H17), and create responses to deal with extracellular microbes (T_H2/T_H17). A T-helper 1 (T_H1) response involves secretion of pro-inflammatory cytokines like TNF-α and IFN-γ in order to activate an infected cell to kill intracellular microbes. $CD8^+$ T cells also secrete these two cytokines. Directly, both TNF-α and IFN-γ can induce the autophagy pathway in an infected cell. Autophagy causes a cell's cytoplasm to be cordoned off into autophagosomal vesicles and then fused with lysosomes for degradation of the enclosed contents (including intracellular pathogens). Secondly, both cytokines can induce nitric oxide synthase (NOS) production in an infected cell (Bogdan et al., 2000). NOS creates reactive nitrogen radicals that are toxic to pathogens; for example, hepatocyte NOS is crucial for control of liver stage malaria (Seguin et al., 1994). Lastly, TNF-α and IFN-γ can positively regulate the adaptive immune response by upregulating key proteins for antigen presentation like MHC. In sum, TNF-α and IFN-γ share many roles in immunity, however the main difference between them is the cell type where they are produced. While IFN-γ is a T cell and NK cell specific cytokine, TNF-α is made by T cells, monocytes, macrophages, DCs, mast cells,

epithelial cells, endothelial cells, and fibroblasts. For this reason, throughout my thesis research, I relied on T cell production of IFN-γ instead of TNF-α as a specific activation read-out. With regard to immune protection from infection, IFN-γ has been shown to be important for control of many microbes including: *Mycobacterium tuberculosis* (Condos et al., 1997; Cooper et al., 1993; Flynn et al., 1993), liver stage malaria (Doolan and Hoffman, 2000), *Francisella tularensis* (Sjostedt et al., 1996), *Salmonella* species (Mastroeni et al., 1992; Nauciel and Espinasse-Maes, 1992), *Chlamydia* infection (Rottenberg et al., 2002; Wizel et al., 2008), and many viruses. Numerous studies also address a specific role for TNF-α in control of an intracellular microbe: cutaneous leishmaniasis (Titus et al., 1989), *Salmonella* species (Mastroeni et al., 1992; Nauciel and Espinasse-Maes, 1992), *Francisella tularensis* (Cowley et al., 2007; Sjostedt et al., 1996), and *M.tuberculosis* (Flynn et al., 1995; Harris and Keane, 2010; Keane et al., 2001). In sum, secretion of pro-inflammatory cytokines by $CD4^+$ and $CD8^+$ effector T cells has a role in controlling many different intracellular infections.

There are multiple mechanisms by which $CD8^+$ T cells control intracellular infection and these can be redundant (Lefrancois and Obar, 2010; Zhang and Bevan, 2011). The effector functions of $CD8^+$ T cells are specialized to control intracellular infections and include cytolysis and secretion of cytokines. Cytolysis can occur by the granule exocytosis pathway or receptor-mediated activation of death receptors. Granule exocytosis involves the T cell directing delivery of granzymes (proteases) and granulysin (saposin-like) through perforin pores to the infected cell (Anthony et al., 2010). Granzymes activate apoptosis of the infected cell, while granulysin can induce lysis of the microbe itself through ion disruption and cell wall damage. Effector T cells can also induce cognate cell apoptosis through the FAS/FAS-ligand pathway. This pathway can be used to kill infected cells or kill lymphocytes as a way of negatively regulating the immune response, although the relative importance of the former pathway is still debated. FAS (CD95) can be triggered by FASL expressed on effector T cells following TCR activation. Mice deficient in FAS or FAS ligand have defects in lymphocyte homeostasis (Strasser et al., 2009).

Antigen Processing and Presentation

Conversion of a pathogen into antigens that can be presented on the outside of the infected cell is critical for mounting protective T cell responses. All vertebrates share a complex multigenic region called Major Histocompatibility Complex, or MHC, that contains genes responsible for antigen presentation. While the MHC region contains many conserved genes, MHC class I and class II genes exhibit immense allelic polymorphism. This genetic variation is mainly concentrated in the part of the gene encoding the structure that interacts with antigens (originally defined as a peptide binding groove), allowing different alleles to bind vastly different ranges of peptide antigens. Each molecule is composed of a constant region and an antigen binding groove. MHC class I molecules also require a soluble non MHC linked protein, beta-2-microglobulin, to fold properly.

Peptide antigens from pathogen-derived sources are generated by proteolysis. If the pathogen is extracellular, proteins are internalized by endocytosis or phagocytosis, enter vesicular pathway of the cell, and are eventually degraded by *lysosomal* proteolysis. An infected cell can also digest cytosolic pathogen-derived proteins through the autophagy pathway. Cytosolic proteins can be trafficked from an autophagosome to the lysosome for proteolysis. Lysosomal proteolysis generates peptides are loaded onto MHC class II molecules that reside in late endosomal compartments (Blum et al., 2013).

In contrast, if the pathogen resides in the cytosol, its proteins are processed primarily by the action of the *proteasome*. Proteasomal peptide antigens are transported from the cytosol to the ER by the transporter associated with antigen processing (TAP) for assembly with MHC class I molecules. In dendritic cells (professional antigen presenting cells) exogenous proteins can also be processed into this pathway by retrotranslocation from phagosomes, in a process known as cross-presentation (Blum et al., 2013). Classically restricted $CD8^+$ and $CD4^+$ T cells recognize pathogen-derived peptides presented by MHC class I and class II, respectively.

Chapter 1.2 Development of T cells in the thymus

Common lymphocyte progenitors are generated in the human bone marrow from hematopoietic stem cells (Klein et al., 2003). The cells destined to become T cells traffic through the blood to the thymus and are identified as $CD34^+$ $CD45RA^+$ $CD7^+$ by flow cytometry (Galy et al., 1993; Haddad et al., 2004). Immature thymocytes then follow a sequential pattern of T cell development in the thymus cortex and then medulla, and finally exit as naïve T cells expressing a broad repertoire of TCRs that are ideally not autoreactive (Figure 1.3). Each step in T cell development can be identified by unique expression of certain T cell surface proteins and or transcription factors. Most of our knowledge about lymphocyte development stems from studies in mice because of the ease of *in vivo* experiments and genetic modifications to elucidate mechanistic pathways. Nevertheless, human T cell development follows a similar sequential pattern but the cell surface phenotypes of human transitional cell populations can differ from those in the mouse (Blom and Spits, 2006; Dik et al., 2005).

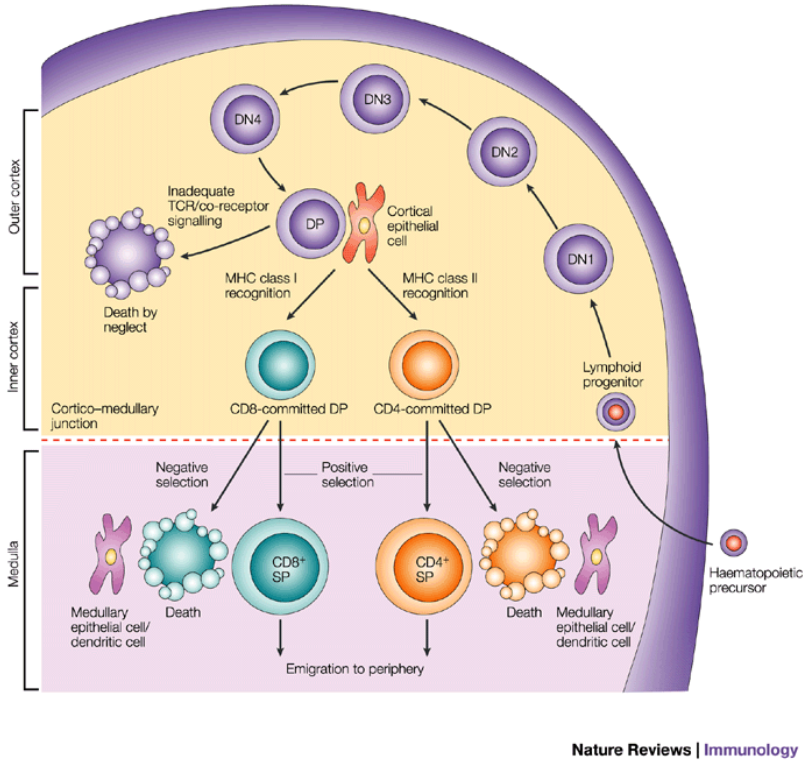


Figure 1.3 Overall Scheme of T Cell Development in the Thymus. With permission from Germain R., Nature Immunology, 2002. DN = double negative CD4-CD8-. DP= double positive CD4+CD8+, SP = single positive

Once the progenitor cell enters the thymus, it will progressively migrate from the cortex to the medulla. It commits to the T cell lineage through sequential steps of expression of regulatory transcription factors, Notch1 receptor, and homing receptors (Rothenberg et al., 2010). Firstly, Notch1 signaling switches on genes to instruct the thymocyte and cause it to solidify its T cell identity (Blom and Spits, 2006; Maillard et al., 2004; Radtke et al., 2004). All T cells at this initial phase do not express distinctive cell surface markers of mature T cell lineage, such as the TCR and CD3 complex. They are called "double negative" thymocytes because they lack CD4 or CD8 co-receptor expression.

TCR Gene Rearrangement

To prepare for recognition of diverse pathogens, the adaptive immune system has adopted a brilliant solution by recombining variable, diversity, and junctional DNA with constant chains of the T cell receptor to generate a plethora of TCR that are capable of recognizing foreign antigens (Davis and Bjorkman, 1988). Each lymphocyte expresses many copies of a single antigen receptor with a unique binding site that determines the specificity of the T cells effector functions. The receptor is made up of variable (V) and invariant constant regions of the protein chains. The amino acid sequence of the V region engages antigen-MHC and its variability allows for a wide range of antigen specificities across the TCR repertoire. The constant region provides stable structure and signaling functions. The specificities of the TCR repertoire are randomly generated by VDJ somatic DNA recombination. This process begins when the RAG-1 and RAG-2 lymphocyte specific enzymes are expressed in developing thymocytes. This enzyme complex binds at random to signal sequences at each of

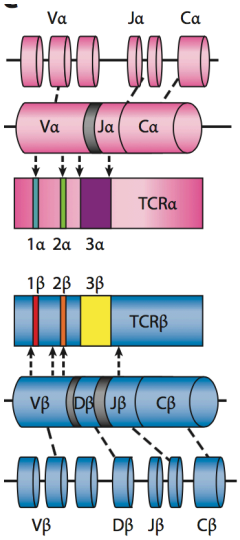


Figure 1.4. The Genetic Recombination of TCR genes from the α-chain (pink) and the β–chain (blue). The complementarity determining regions (CDRs) are highlighted in alternative colors. With permission from Rossjohn et al., Annual Reviews, 2014.

one of many V and J (or D) segments to be recombined. The RAG enzymes and chromatin proteins bring the V and J (or D) gene segments into proximity and carry out endonucleolytic cleavage with two single strand breaks. For diversity, transferase enzymes clip and add nucleotides to the ends of each segment before a DNA ligase joins the processed ends together. A simplified progression of gene recombination is depicted in Figure 1.4.

Allelic Exclusion

Monoallelic gene expression is a generally important phenomenon for normal biology; for example the inactivation of the X chromosome in females. Allelic exclusion, as it relates to T lymphocytes, is a phenomenon that increases the frequency of T cells that express a single T cell receptor and are monospecific for a certain antigen-MHC. This occurs for the majority of developing T cells (Wucherpfennig et al., 2007) . Alleles are excluded when one allele of a TCR genetic loci rearranges successfully that then causes the allele on the other chromosome to become transcriptionally silent. Multiple mechanisms have a role in TCR allelic exclusion (Jackson et al., 2005). These involve transcriptional accessibility through chromatin remodeling of the specific loci on the excluded chromosome, TCR signaling, and ubiquitination of protein from the gene (alpha chain) to be excluded (Brady et al., 2010; Jackson et al., 2005; Niederberger et al., 2003).

The Sequence and Mechanisms of TCR Gene Rearrangement

There are two types of TCRs that T cells can rearrange and express - $\gamma\delta$ or $\alpha\beta$; each resulting in a separate lineage of T cell. $\gamma\delta$ T cell lineage comprises a minority population while $\alpha\beta$ T cells are the majority and hence, their developmental pathway is more widely researched. In the cortex, TCR loci rearrange in a highly ordered and sequential fashion to generate a productive TCR (Dik et al., 2005; Joachims et al., 2006; Sherwood et al., 2011). In humans this is *TCRD-TCRG-TCRB-TCRA.* Rearrangement

of the γ and δ genes occurs earliest in development, followed soon after by the β chain if a productive γδ TCR is not made. The productive γδ TCR allows for a strong intracellular signaling downstream of the TCR, as studies have shown comparing levels of active Lck and phospho-Erk that then upregulates the γδ specific transcription factor Id3. These T cells then leave the thymus without progressing onto the double positive stage and undergoing selection. Because I have studied development of αβTCR$^+$ T cells in my thesis research, I will focus on their development for the rest of this section.

At this stage, rearrangement of the TCR β chain occurs. Cells that generate productive TCR β get signals to proliferate, whereas those that do not, receive signals to apoptose. At the same time, the TCR β chain associates at the cell surface with a pre-T cell receptor. This is a protein that mimics a generic TCR α chain and allows a functioning TCR-CD3 complex to be stabilized at the immature T cell surface. Key to the progression of the T cell into its next stage of development, this pre-TCR complex induces ligand independent intracellular signaling through Lck kinase. This induces phospho-Erk that causes the T cell to stop β chain rearrangement by degradation of RAG, undergo allelic exclusion, proliferate, become large in size, and express CD4 and CD8 co-receptors. After this brief proliferative burst, downregulation of the global transcriptional activator Myc, transitions the developing T cell into a small quiescent DP stage characterized by low metabolism (Mingueneau et al., 2013).

Once the T cell reaches the small DP stage, it begins to rearrange its α–chain by expressing RAG1 and RAG2. Each T cell can undergo successive rearrangements at each allele of the α chain loci until the cell apoptoses or succeeds positive selection. For this reason, it is more common for a T cell to be able to produce two types of α chains. However, it would be rare for a single T cell to express two α chains that are functionally specific and pass the selection stages. Once successful α chain rearrangement has occurred, the T cell can progress to the next selection stage.

Positive and Negative Selection

Thymic selection of T cells is imperative to ensure that the random receptors generated through TCR rearrangement are functional and not autoreactive. At this intermediate stage of development, T cells have successfully rearranged their $\alpha\beta$ TCR and express both the CD4 and CD8 co-receptor. In order to be saved from apoptosis, double positive thymocytes must first be able to recognize MHC and self-peptide expressed by thymic cortex stromal cells in a process called positive selection. Positive selection generally determines the lineage fate of the immature T cell. T cells with receptors that are able to interact with MHC class I, will express CD8 and become cytotoxic effectors (Germain, 2002). In contrast, T cells with receptors that interact with MHC class II, will express CD4 and become cytokine producing helper T cells. Therefore, the selecting MHC molecule contributes to lineage fate by determining the co-receptor expression of the mature T cell. Lineage fate is also determined by TCR signal strength which regulates expression of transcription factors, ThPOK or Runx3, specific to $CD4^+$ helper T cells or $CD8^+$ cytolytic T cells, respectively (Mucida et al., 2013; Reis et al., 2013). The $CD4^+$ and $CD8^+$ T cell dichotomy persists in the periphery for mature T cells, in which ThPOK continues to promote the fate of helper T cells even as they differentiate into effector subsets. Switching the expression of these dichotomous transcription factors in peripheral mature T cells correlates with appropriate switching of effector functions aka cytolytic MHC class II restricted CD4+ T cells and this is an antigen driven process (Mucida et al., 2013). Whether or not nonclassical antigen presentation molecules play a role in the lineage fate of the T cells restricted by them is unknown.

Developing lymphocytes also undergo a second selection process called negative selection in the thymus medulla. This process's purpose is to protect self-proteins from being recognized and attacked by mature T cells in the periphery. This process is also called clonal deletion because T cell clones expressing self-reactive TCRs get deleted (Lederberg, 1959). Negative selection therefore generates central tolerance to proteins expressed by "self". Tolerance can also be generated by peripheral mechanisms, such as suppression of T cell activation by regulatory T cells (Weiss et al., 2012). The

relative contributions of central and peripheral tolerance to overall tolerance is still debated; a recent study, showed that negative selection is much less efficient at clonal deletion than previously thought, suggesting an integral role for peripheral tolerance mechanisms (Yu et al., 2015). Multiple cell types, like medullary stromal cells expressing AIRE, dendritic cells, and macrophages in the thymus, can influence negative selection by expressing self-antigens. Medullary thymic stromal cells have been shown to use AIRE and Fezf2 transcription factors to express a wide array of self-antigen peptides (Takaba et al., 2015). During negative selection, if the T cell's TCR interacts too strongly with peptide-MHC, it will undergo apoptosis (initial proposition by Burnet, 1959)(Mouse models of negative selection reviewed in (Hogquist et al., 2005).

Furthermore, it is still incompletely known how a signal through the TCR during positive selection can signal for cell survival, while the opposite outcome occurs from a TCR signal during negative selection. The affinity hypothesis states that a quantitative difference in receptor affinity to peptide MHC in the thymus determines cell survival. A strong TCR signal would undergo apoptosis whereas a lower affinity interaction over a shorter duration can give the T cell signals it requires to survive. Thymocytes that pass negative selection then egress into the periphery where they constantly circulate through the blood and lymph.

These processes have been studied extensively for classically restricted $\alpha\beta$ TCR+ T cells. However, many questions remain about what is required to generate a mature nonclassically-restricted T cell that tend to express less diverse T cell receptors, or more specifically a MR1-restricted MAIT cell.

Development of Nonclassically-restricted T Cells

In 1999, Tilloy et al., proposed that "restricted TCR repertoires seem to define discrete lymphocyte subpopulations at the frontier between innate and adaptive immunity, as these selected repertoires may allow the presence of a high frequency of cells reactive against phylogenetically

conserved antigens." It is evident now that certain subpopulations of T cells with constrained TCR repertoires are restricted by MHC-I-related molecules for antigen presentation. Major histocompatibility complex (MHC) class I-related molecules including H2-M3, and CD1d in mice and HLA-E, HLA-F, HLA-G, MR1 and CD1a to CD1d in humans, all seem to differ from MHC class I molecules primarily because they have limited polymorphism. Consequently, the family of T cells restricted by these molecules have recently been termed – Donor UnRestricted T cells (DURTs) - because the nature of antigen presentation they recognize is shared across individuals, unlike classical antigen presentation that is highly divergent. Work over the past two decades has shown that these T cells undergo a unique developmental pathway from classical $CD4^+$ and $CD8^+$ T cells. It is still unknown how this unique development endows certain nonclassical T cells with innate functional reactivity. I will review the three main advancements in our understanding of the development of MR1-restricted MAIT cells.

MAIT cells develop in the thymus

An initial observation was made that human blood had high frequencies of two populations of T cells that expressed limited TCRs (Porcelli et al., 1993). Now we understand that these two families of TCRs belong to MR1-restricted MAIT cells and CD1d-restricted NKT cells. With regard to MAIT cells, Lantz and colleagues explored these findings further with genetic knockouts to find what MAIT cell development was dependent upon (Tilloy et al., 1999). One key observation they made was that nude mice (athymic, $FOXN1^{-/-}$) did not have MAIT cells, suggesting that MAIT cells require an intact thymus for development. MAIT cells can be found in the mouse and human thymus as defined by their effector function, and they show signs of being immature and naïve. They express surface markers commonly associated with naïve T cells like, CD45-RA isoform, CCR7, and CD62L (Gold et al., 2013; Martin et al., 2009a). Secondly, MAIT cells in the thymus have detectable "signal-joining TCR excision circles", or sjTRECs (Gold et al., 2013). These small pieces of excised DNA are created during TCR rearrangement

and are diluted from T cell during mitosis. Therefore, these data collectively suggest that MAIT cells develop in the thymus and are not simply recirculating peripheral T cells.

MAIT cells require thymic selection on hematopoietic cells

Conventional T cells in the thymus rely upon signals from thymic epithelial cells for their successful selection. However, the selecting cell type for certain nonclassically restricted T cells seems to be different. Using bone marrow–chimeric mice, Urdahl et al. showed that nonclassically–restricted, but not MHC class I–restricted, $CD8^+$ T cells were preferentially selected when antigen presentation was only permitted on hematopoietic cells (Urdahl et al., 2002). The authors specifically measured H2-M3-restricted T cells from the spleen by tetramer staining, cytolytic capacity, and cytokines release upon antigen stimulation. While this study focused on murine T cells reactive to *Listeria monocytogenes*, selection by hematopoietic thymocytes that express either CD1d or MR1 is necessary for NKT and MAIT cells, respectively (Bendelac, 1995; Coles and Raulet, 2000; Seach et al., 2013; Treiner et al., 2003). More specifically, the responsible thymic hematopoietic cells can be identified by co-expression of CD4 and CD8. With regard to MAIT cells, thymic development requires the presence of MR1 (Hashimoto et al., 1995; Treiner et al., 2003). In the human thymus, high expression of MR1 protein on the surface of a population of DP thymocytes has been observed (Gold et al., 2013). Definitive experiments are needed to prove that these are the cells responsible for selecting MAIT cells. In recent work that I have contributed to, we have defined a stable phenotype for the human MR1-high thymocytes (Kurtz I., Meermeier E., and Gold M., *in preparation*). We found that MR1-expressing thymocytes are a uniform small resting subset with high expression of CD3, CD8, CD4, CXCR4, and Notch 3. Furthermore, MR1-high thymocytes have the capacity to stimulate human MAIT cell clones to produce IFN-γ in the presence of *M. smegmatis* and *E. coli*. Our results outline unique and stable characteristics for a novel antigen presenting cell resident in the human thymus that may provide positive selection for MAIT cells.

Whether MAIT cells undergo negative selection in the thymus is unknown. Because many TCRs of nonclassically restricted T cells recognize non-peptide antigens, one can imagine that a unique pathway may exist for presentation of related self-antigens in the thymus.

MAIT cell effector function is acquired in the thymus

While MHC class I restricted T cells acquire effector function in the periphery following antigenic stimulation, certain nonclassical T cells, like MAIT cells, acquire effector function without prior exogenous antigenic exposure. This function is one of the reasons that these cells are called "innate-like". This was observed in the context of *M. tuberculosis* antigens, when our laboratory measured thymocyte function directly *ex vivo* in response to infected allogeneic dendritic cells. They found that the T cells with thymic effector function were non-classically restricted and upon stimulation, these cells expressed IFN-γ, granzyme, and high levels of BCL-2, the anti-apoptotic factor (Gold et al., 2008). In a follow-up study, many of these cells were found to be MR1-restricted MAIT cells (Gold et al., 2013). One possibility for the innate effector function, is that MAIT cells gain their function because they are selected on hematopoietic cells that may be able to give more complex co-stimulation during MHC recognition. Like human MAIT cells, both H2-M3 and CD1d restricted T cells can be found as thymic effectors (Bendelac et al., 2007; Urdahl et al., 2002). Although the signals contributed by the hematopoietic cell remain to be identified for these nonclassically restricted T cells, homotypic interactions have been shown to be critical for NKT cell selection. Specifically, both SAP dependent and independent SLAMR players have been shown to be important in mouse iNKT and innate T cell development (De Calisto et al., 2014). Also, whether the hematopoietic cell presents a self-ligand during the selection process is unknown. Evidence that self-derived MR1 ligands exist has been provided (Huang et al., 2009), although the identity of such ligands remains unknown. Alternatively, innate T cell function could be programmed through the use of a common transcription factor. Several studies have

implicated the transcription factor PLZF (promyelocytic leukemia zinc finger, or ZBTB16) for this role in NKT and MAIT cells (Martin et al., 2009a; Savage et al., 2008). In NKT cells, the PLZF transcription factor is essential for development (Eidson et al., 2011; Kovalovsky et al., 2008), drives their early effector phenotype, and also programs their accumulation in tissue sites like the liver (Thomas et al., 2011). Although several studies have shown that human MAIT cells express PLZF, whether it is necessary for their innate-like qualities is not known.

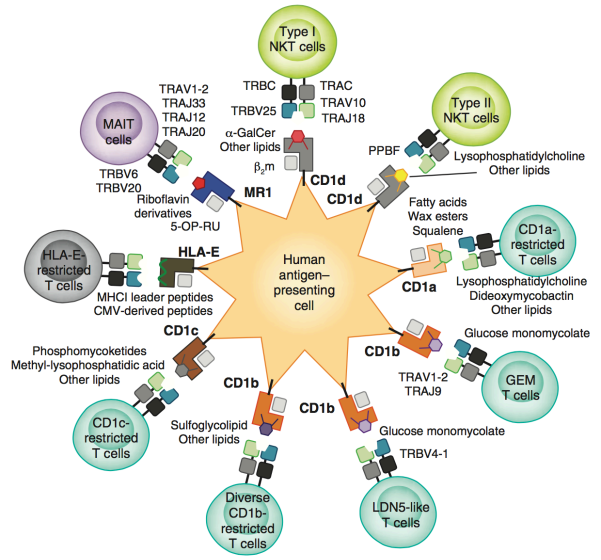


Figure 1.5. Interactions of unconventional T cells with their antigen targets via their TCRs in human cells.
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1.3 Unconventional T Cells Respond to Antigen Presented by MHC-Ib Molecules or MHC-like Molecules

Most studies of T cells have focused on those that react to major histocompatibility complex (MHC) proteins bound to peptide antigens, however many other types of T cells do not fit this 'textbook' model. Collectively, these T cells are considered 'unconventional' or 'nonclassical' and include CD1-restricted T cells, γδ T cells, MHC class Ib (including HLA-E)–reactive T cells, MR1-restricted mucosal associated invariant T cells (MAIT cells), and others. The family of unconventional MHC-like molecules and the αβTCR that can recognize them is depicted in Figure 1.5. In contrast to classically-restricted T cells, unconventional T cells generally use a less diverse repertoire of TCR, are present at higher frequencies, and have rapid effector responses. Also, unconventional T cells recognize unconventional 'antigens', such as lipids, small-molecule metabolites, broad patterns of peptides, or antigen-modified presentation molecules. The ability to rapidly activate a large amount of T cells with a single stimulus regardless of genetic background or prior antigen exposure differentiates these immunological systems from classically restricted T cells. These features represent a crossover between innate and adaptive immunity, hence, unconventional T cells are also referred to as "innate-like" T cells. The crux of the

adaptive immune system relies on the diversity of receptors to cope with a multitude of foreign entities, while evidence suggest that the opposite is true for unconventional T cell restriction. This dissymmetry can be highlighted by the utilization of conserved receptors by unconventional T cells to recognize broad patterns of infection, in a similar way to Toll-Like receptors of innate immunity. Research into the biology of unconventional T cells over the past two decades has highlighted previously unappreciated complexity within the adaptive immune system. I will review what is known about the most characterized classes of unconventional T cell types to provide evidence that these alternatives to the classical adaptive immune paradigm are a necessary component of the human immune system. Then I will review subsets of MR1-restricted T cells in more detail.

Gamma-delta T cells

Gamma delta T cells ($\gamma\delta$ T cells) are T cells that express a unique TCR composed of one γ-chain and one δ-chain instead of an $\alpha\beta$ TCR. The TCRs used by $\gamma\delta$ T cells are comprised of predisposed pairings of more limited combinations of VDJ segments. It remains enigmatic how many discrete populations exist for $\gamma\delta$ T cells; nevertheless, I will focus on key findings that relate to their role in host defense against infection. $\gamma\delta$ T cells are less prevalent than their $\alpha\beta$ T cell counterparts, at 0.5%-10% of total T cells, but the majority express a single TCR: γV9/δV2. So far the antigens discovered to be recognized by $\gamma\delta$ T cells are phosphorylated small molecules derived from the human mevalonate pathway or synthesis of microbial isoprenoids (Constant et al., 1994). While humans cells synthesize weak $\gamma\delta$ agonists in this family, certain bacterial infections create isoprenoid-based antigens that are 10,000 fold stronger agonists, and thus serve as patterns of pathogen infection (Hintz et al., 2001). γV9/δV2 require cell-cell contact but not conventional antigen presentation to react to phosphate antigens. Several lines of recent evidence indicate that 'B7' family proteins called butryophilins are necessary for the activation of γV9/δV2 T cells. Although, whether butryophilins present phosphate antigens or are simply modified by

these 'antigens' into a form recognized by the γδ TCR is still unclear (Sandstrom et al., 2014; Vavassori et al., 2013). Different subsets of γδ T cells also can recognize lipid antigens on CD1 molecules (reviewed in (Luoma et al., 2014)), and also MHC-like molecules that likely do not present antigen but are hypothesized to be indicators of cellular stress (reviewed in Vantourout and Hayday (2013); (Willcox et al., 2012)).

Several studies in mice without γδ T cells provide convincing evidence that these cells contribute to defense against infection and are crucial sources of initial IL-17 and IFN-γ cytokines. Mice without γδ T cells are more susceptible to several infections, including *Nocardia* spp., *Klebsiella* spp., *Listeria* spp., *Escherichia coli*, *Salmonella* spp., *Mycobacterium* spp., *Pseudomonas* spp., and *Plasmodium* spp. (Vantourout and Hayday, 2013). From this comparison, γδ T cell activation to patterns of infection and cellular stress represent unique alternatives to canonical αβ T cell antigen recognition. Thus, while there are many unanswered questions about γδ T cells, it provides an interesting model whereby subsets of T cells can react to patterns of infection by unique pathways that may allow for rapid recognition of microbes and play a role in early immune responses.

MHC-Ib-Restricted T Cells

The human molecules HLA-E, F, and G are considered to be MHC- 'Ib' molecules because they are ancestrally-related to MHC class Ia molecules, yet evolutionarily divergent and far less polymorphic. HLA-F and HLA-G may play a role in immunity, as suggested by evidence that they bind peptides to present to T cells for maternal-fetal tolerance (Ishitani et al., 2003). Meanwhile, studies of HLA-E are particularly intriguing as this molecule seems to have evolved dual roles in regulating innate and adaptive immune responses. In an innate fashion, HLA-E presents peptides derived from leader sequences of other classical class I molecules as an inhibitory signal which binds CD94/NKG2 on NK cells in order to combat immune evasion tactics of microbes (Braud et al., 1998). In this way, HLA-E acts as

remote sensor of the intracellular antigen processing machinery. In parallel, HLA-E has the capability to elicit a $CD8^+$ αβ T cell response to a variety of pathogens such as *Salmonella enterica, Mycobacterium tuberculosis*, LCMV, HIV, HBV, EBV, HCV and *cytomegalovirus* ((Heinzel et al., 2002; Joosten et al., 2010), reviewed in (Sullivan et al., 2006)). These HLA-E restricted T cells are cytolytic and secrete a broad range of cytokines but their relative contribution to adaptive control of infection is still unknown.

With regard to HLA-E ligands, when a particular strain of CMV was used as a vaccine vector for SIV antigens, rhesus macaques were able to mount a robust $CD8^+$ T cell immune response to SIV by presenting a very broad array of SIV peptides via MHC-E (Hansen et al., 2016). Interestingly, the peptides that HLA-E presents from these microbes can be similar or highly disparate patterns of amino acids from the canonical leader sequences bound by HLA-E. For example, the CMV protein UL-40 encodes an MHC class I leader peptide mimic (Ulbrecht et al., 2000). Structural simulation studies have shown that HLA-E provides an open peptide binding groove with sufficient plasticity to present widely different antigens due to both deep and shallow binding pockets with heterogeneous binding sites (Hansen et al., 2016). Thus HLA-E provides a model where a nearly monomorphic antigen presentation system can present diverse antigens to $CD8^+$ T cells.

CD1 Restricted T Cells

Humans encode a locus of genes collectively called CD1 that express proteins which present antigen, but are unlinked to the MHC and display very limited polymorphism. However, CD1 genes have similar genetic structure and protein domains to those encoded by MHC class I. There are five CD1 molecules (CD1a, b, c, d, and e) that all bind lipid antigens in deep hydrophobic binding grooves and all but CD1e are recognized by the TCR of CD1-restricted T cells. Each CD1 molecule binds a different repertoire of self and foreign antigens that is hypothesized to be regulated by slight variations in the shape of each binding groove and the sampling of antigens via different intracellular trafficking patterns

unique to each CD1 molecule (Brigl and Brenner, 2004; Moody and Porcelli, 2003). With regard to known microbial ligands, studies have focused on mainly on mycobacterial antigens and include lipids from the following classes: mycobactins, insect venoms (CD1a), Mycolic acids, sulfolipids, lipomannan, phosphatidylinositol mannosides (CD1b), Mycoketides (CD1c), and glycolipids (CD1d) (as reviewed in (Willcox et al., 2007)). CD1-restricted T cells can use CD4 or CD8 co-receptor, or neither, indicating that these T cells can differentiate into subsets of the major phenotypic T cell groups. Upon activation, CD1-restricted T cells can secrete Th1 or Th2 or Th17 cytokines and are strongly cytolytic; it has been hypothesized that tissue distribution regulates their cytokine profile (Lee et al., 2015; Rosat et al., 1999). T cells that recognize antigen presented on CD1a, b, c, and d use a variety of TCRs comprised of $\alpha\beta$ or $\gamma\delta$ heterodimers, and are collectively known as Group II CD1 restricted T cells in the literature. Group II CD1 restricted T cells do not have an apparent bias for unique V or J genes of the TCR.

There also exists a subset of CD1d-restricted T cells that use an invariant TCRα that seems to be highly conserved in mice and to a lesser extent in humans. These T cells are known in the literature as Group I CD1d-restricted T cells or invariant Natural Killer T cells (iNKT cells). There is abundant evidence that Group I CD1d-restricted T cells can act immediately as effector cells upon stimulation. It has been suggested that this is due to their unique developmental pathway in the thymus and expression of the PLZF transcription factor which leads to an effector program prior to T cell activation (Bendelac, 1995; Savage et al., 2011). Current evidence suggest that CD1 restricted T cells participate in the immune response to the clinically important intracellular pathogen, *M.tuberculosis*, and might form the basis for improved immune therapies to prevent tuberculosis (Cerundolo et al., 2009). With regard to CD1d-restricted T cells, this pathway of immune activation provides us with a paradigm where a conserved, semi-invariant TCR can be used to respond immediately to microbial infection via surveillance of lipids. In sum, CD1 antigen presentation of lipids to T cells gives a broader perspective of the immune system's capacity to sense and response to a diverse array of macromolecules.

Chapter 1.4: Introduction to MR1-restricted T Cell Biology

MR1 Genetics and Tissue Expression

MR1 is a non-polymorphic MHC-like molecule encoded in the genome outside of the MHC locus on the human chromosome 1. Based on primary structure, *MR1* appears to be distantly related to classical MHC molecules and may represent a separate lineage that split from a common ancestor of MHC proteins early in evolution (Stroynowski and Forman, 1995). This suggests an evolutionary conservation of the gene products and a selective pressure for its unique functional roles.

In the late 1980s, MHC-like molecules CD1, ZAG, and FcNR were first described using targeted approaches with antibodies (Hashimoto, 2016). Because these proteins were discovered using non-reductionist approaches, logically, the field thought it was reasonable that the human genome contained other *MHC* related genes and a search began. In 1995, the Kurosawa lab discovered MHC class I-related molecule 1, or MR1 (Hashimoto, 2016; Hashimoto et al., 1995). To survey the human genome for similar genes outside of the MHC locus, they took a cross-hybridization PCR approach with a generic primers designed to the conserved regions of *MHC* (Hashimoto et al., 1992). They found a region of high similarity on chromosome 1, distinct from the MHC locus on chromosome 6, and named this gene MHC-related protein 1 or MR1 (Hashimoto et al., 1995). Comparison of the amino acid sequence of the functionally important α1 and α2 domains revealed that MR1 exhibits 40-50% identity with MHC class I of human, mouse, and chicken species. This is higher than any other MHC-like molecule outside of the MHC locus. Subsequent studies found that *MR1* is highly conserved across mammalian evolution, and *MR1* transcript is expressed across many human cell lines and all healthy tissues tested (Riegert et al., 1998; Yamaguchi et al., 2014). MR1 also shares the same gene organization as MHC class I, where each exon encodes a single structural domain, specifically, a leader peptide, α1 domain, α2 domain, α3 domain, a trans-membrane domain, and a cytoplasmic domain. MR1 protein associates with β-2-microglobulin, suggesting a molecular form typical of the MHC class I heterodimer (Yamaguchi and

Hashimoto, 2002). At this time, CD1, another MHC-like protein encoded on chromosome 1, was described to bind lipid and not peptide antigens; lending to the hypothesis that MR1 might also present antigen.

Despite sharing a common MHC protein fold, MR1 has some unique qualities. With regard to DNA sequence, it was under control of a unique promoter from other MHC class I genes and was located outside of the MHC locus on chromosome 6 (Riegert et al., 1998). Intriguingly, analysis of MR1 transcripts revealed the presence of three additional isoforms resulting from alternative splicing following transcription (Lion et al., 2013; Riegert et al., 1998; Yamaguchi et al., 2014). All predicted alternative isoforms lacked certain exons of the full length MR1 gene. While it remains unclear if the isoforms serve as functional proteins *in vivo*, there is evidence that one of the isoforms called MR1-B can be expressed at the cell surface and can activate MAIT cells (Lion et al., 2013).

Lastly, with regard to MR1 protein sequence, MR1 lacked many of the key residues of the antigen binding groove that have been shown to be necessary for peptide antigen presentation in MHC class I. Evidence strongly suggested that MAIT cell activation was still ligand-dependent (Huang et al., 2005) but likely non-peptidic. There is currently disagreement with respect to whether or not MR1 has a CD8 binding site on the a3 domain based on alignment with MHC class I (Riegert et al., 1998; Walter and Gunther, 1998). However, we have shown that *M. tuberculosis*-reactive MAIT T cell clones require CD8 for activation (Gold et al., 2013). The final unique element of MR1 protein structure is its high degree of conservation across mammalian evolution. It is also conserved across individuals and therefore non-polymorphic (Parra-Cuadrado et al., 2000) Taken together, these observations suggest that MR1 has a functional deviation from the peptide binding classical MHC class I molecules.

MR1 Ligands and Presentation of Microbial products

MR1 ligands were recently identified by the Rossjohn and McCluskey labs as small vitamin B synthetic intermediates that bind and stabilize MR1 on the cell surface (Figure 1.6). These ligands commonly share an aromatic ring structure and are structurally distinct to any previously described MHC-ligands (Eckle et al., 2015). MR1 ligands derived from vitamin B9/folic acid (6-formylpterin (6-FP and acetyl-6-FP)) are antagonistic to MAIT cells, whereas the riboflavin metabolites known as ribityllumazines (RL-6,7-diMe, RL-6-Me-7-OH, and reduced RL-6-CH_2OH) are agonists (Kjer-Nielsen et al., 2012). In this initial finding, Kjer-Nielsen and colleagues identified the folic acid based antagonists from RPMI medium, while the riboflavin agonists were derived from *Salmonella*. Another class of ligands, the pyrimidine intermediates of riboflavin (5-OE-RU, 5-OP-RU), were more recently identified as potent activators of MAIT cells (Corbett et al., 2014). These ligands are universally recognized by MAIT cells as evidenced by an MR1 tetramer reagent bound to 5-OP-RU that labels all human MAIT cells (Reantragoon et al., 2013). MR1 tetramer loaded with 6FP bound to MAIT TCRs weakly. The pyrimidine antigens are generated in a chemical reaction between bacterially-derived synthetic intermediates and small host or pathogen chemicals. Riboflavin and folic acid are essential for metabolic functions and are synthesized by many bacteria, fungi, and plants, but not by mammals, other animals, or parasites, which acquire it from their environment or do not require it.

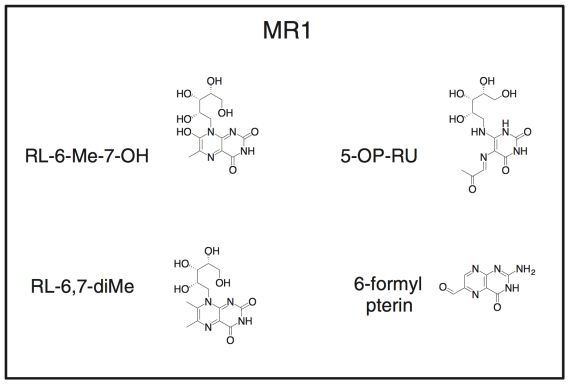


Figure 1.6. Chemical Structures of the MR1-ligands described to date. Reprinted with permission from Godfrey et al. Nature Reviews Immunology 2015.

MAIT cells, through MR1 antigen presentation, recognize a broad range of microbial infections. By studying MAIT cell recognition of infection through MR1, parallel studies have found that those microbes with intact riboflavin synthetic pathways stimulate MAIT cells (Gold et al., 2013; Le Bourhis et al., 2013b) and reviewed in (Napier et al., 2015). However, it is not known whether each microbe

synthesizes the same repertoire of riboflavin metabolites, maybe at varying proportions, or whether there are ligands unique to certain species. Nevertheless, not all microorganisms are recognized by MAIT cells. In consensus with this pattern, bacteria that did not activate MAIT cells, *Streptococcus pyogenes, Listeria spp.* or *Enterococcus spp*., do not use the riboflavin synthesis pathway and instead rely upon scavenging riboflavin from their environment (Chapter 3). Thus, only microbes with an intact riboflavin synthesis pathway can be recognized by and activate MAIT cells, suggesting that other microbial metabolic pathways do not generate MAIT cell antigens. However, whether alternative MR1 ligands, recognized by TCRs not previously associated with MAIT cells, exist, is a question that has not been addressed. In this thesis, I directly address the hypothesis that MR1-restricted T cells can use atypical TCRs to recognize MR1 and more diverse antigens outside of the riboflavin pathway.

MR1 Antigen Processing and Presentation

Antigen presenting molecules, comprising MHC class I and II, use distinct intracellular trafficking pathways to bind and present peptides to CD8+ and CD4+ T cells respectively. How does MR1 survey its environment to cooperate with MAIT cells for detection of microbial infection? Research over the past decade has uncovered unique qualities of the pathway that MR1 takes to capture small molecule antigens and present them to MAIT cells.

In contrast with classical MHC molecules that are constantly expressed at high levels on the cell surface, MR1 protein is found at barely detectable levels, even during an infection (Gold et al., 2010b; Harriff et al., 2016; Huang et al., 2008; Riegert et al., 1998). Instead, MR1 resides in the endoplasmic reticulum (ER) and endosomal vesicles (Georgel et al., 2011; Harriff et al., 2016; Huang et al., 2008; McWilliam et al., 2016). There have been two models proposed of which cellular compartment is responsible for the ligand loading onto MR1. In one model, exogenous addition of vitamin B derived antigens to the cell resulted in MR1 loading in the ER and then translocation to the cell surface. While it

was not addressed how the antigen gets into the ER, this represents a way that MR1 could survey the extracellular environment. In a second model, MR1 and intracellular pathogens, like *M.tuberculosis*, co-localized in late endosomal compartments (Harriff et al., 2014; Harriff et al., 2016; Huang et al., 2008). MR1 could survey and load intracellular microbial products, and then translocate to the cell surface. Interestingly, purified vitamin B derived antigen (6-FP) - typically loaded through the ER pathway on MR1 - did not interfere with antigens derived from intracellular *M.tuberculosis*; indicating the existence of two distinct loading pathways for MR1 antigen loading (Harriff et al., 2016). Although this could also imply that the pathway used to load antigens from intracellular infection supersedes those that might occupy MR1 from the ER. The ER might provide MR1 with a source of ligands to fold properly before the molecule is trafficked toward the cell surface and given the opportunity to bind more high affinity ligands from intracellular infection. More experiments are needed to determine whether antigens can be exchanged in the MR1 antigen groove.

While the chaperones and self-ligands involved in the regulation of classical antigen presentation have been well characterized, those required for MR1 antigen presentation are less defined. MR1 antigen presentation does not require TAP, the proteasome, or any of the associated MHC class I machinery (Huang et al., 2008). However, MR1 antigen presentation does involve the MHC class II-associated invariant chain and HLA-DM; these proteins usually chaperone MHC class II to endosomes and facilitate its loading with endosomal ligands (Blum et al., 2013). This finding supports the notion of MR1 antigen loading in an endosomal compartment, in a similar fashion to MHC class II or MHC class I endosomal cross presentation (Neefjes et al., 2011). Although several lines of evidence indicate that this pathway may differ by cell type. Harriff and colleagues have established that lung epithelial cells are efficient at activating MAIT cells in an MR1 dependent way (Harriff et al., 2014). Epithelial cells do not express the aforementioned MHC-class II chaperones, but instead might use syntaxin 18 (ER), VAMP 4 (endosome), and Rab 6 (endosome) to support MR1 antigen presentation (Harriff et al., 2016). How the

different pathways that MR1 might use to survey microbial ligands of its environment affect T cell activation is still unclear.

After MR1 encounters its ligand and folds properly, the MR1-ligand complex is translocated to the cell surface for a short amount of time before being internalized and degraded (McWilliam et al., 2016). MR1 might act like a `quick sensor' of microbial products to MAIT cells in this way, while minimizing the opportunity to exchange ligands with ligands of microbiota or self that could activate autoreactive T cells.

MR1 Protein Structure and Molecular Basis for Interaction with TCRs

Structural analysis of MR1 has led to an understanding of the nature of ligands it can present to TCRs. The first crystal structure of MR1 displayed its striking similarity to HLA-A, with regard to its structural backbone (Kjer-Nielsen et al., 2012). However, the antigen binding groove of MR1 displayed novel qualities. The Rossjohn and Adams labs found, in parallel, that MR1 has a much smaller groove that is comprised of two pockets in comparison to MHC class I's long continuous groove (Kjer-Nielsen et al., 2012; Lopez-Sagaseta et al., 2013a). The 'A' pocket is deep within the MR1 antigen binding groove and provides a charged and hydrophobic environment, while the 'F' pocket is shallow and flexible (Lopez-Sagaseta et al., 2013b). An in depth structural comparison of an unliganded MR1 compared to MR1 bound to 6-FP in the A pocket showed significant movement and malleability in the antigen binding cleft, suggesting a potential degree of plasticity for structures it could accommodate (Patel et al., 2013). The unique features of the antigen presenting groove may be important for accommodating small molecule antigens with different properties, or loading of ligand into place, or providing some tuning flexibility for engagement by MAIT TCRs. One can imagine a scenario whereby a nonpolymorphic molecule like MR1 might rely upon flexibility to be able to bind and present molecules with different chemotypic qualities to semi-invariant TCRs.

Transitory chemical intermediates of microbial vitamin B metabolism, as described in the previous section, are captured and stabilized by MR1 for MAIT-cell recognition. The common ring structures of the MR1 ligands described so far are all accommodated in the A pocket of MR1 by covalent and/or hydrogen bonds (Corbett et al., 2014; Kjer-Nielsen et al., 2012; Patel et al., 2013). Despite each having unique conformations while bound to MR1, the ligands that were strongly stimulating to MAIT cells share a ribityl tail that is exposed outwards from this binding pocket. The diversity in ligand conformations also supports the notion that the MR1-binding cavity could accommodate a range of structures. Nevertheless, whether there are other ligands for MR1, potentially being presented in the other half of the groove, remains unknown.

Introduction to MAIT Cells

Human mucosal-associated invariant T (MAIT) cells have been described as an abundant population of αβ-TCR T cells that display anti-microbial Th1-like cytotoxic capacity upon detection of a range of microbial infections. By definition, MAIT cells express a semi-invariant T-cell antigen receptor (TCR) that engages antigenic ligands presented by the HLA-Ib major histocompatibility complex (MHC)-related protein I (MR1). MAIT cells were first identified in 1993 as an overrepresented T cell population in the human double-negative T cell subset that used a semi-invariant *TRAV/TRAJ* pairing of the TCRα, called *TRAV1-2* (Porcelli et al., 1993). These T cells were further defined in knockout mice and humans as requiring an intact immune system and the MHC class I light chain (beta-2-microglobulin) but not TAP, CD1, MHC class I, or class II; indicating a requirement for a novel class of antigen presentation (Tilloy et al., 1999). Also, both of these studies defined MAIT cells as enriched in the `double-negative' $CD4^-CD8^-$ or $CD8\alpha\alpha^+$ T cell subset of the blood. The TCR co-receptor CD8 can be assembled as a homodimer or heterodimer (α and β). The co-receptor, CD4 or CD8, increases the avidity of the interaction between a T cell and the antigen-presenting cell upon activation and can be downregulated

soon thereafter. Peripheral blood CD8αα or CD8β-low T cells can be derived from clonally expanded CD8β high T cells, by an antigen-driven mechanism and exhibit an effector memory phenotype (Konno et al., 2002; Walker et al., 2011). Therefore, this pattern of co-receptor expression is in line with the united finding that MAIT cells in human blood also express immunological proteins associated with effector memory T cells (Gold et al., 2010a; Martin et al., 2009a; Tilloy et al., 1999).

A pivotal study published in 2003 demonstrated that expression of a β2M-dependent MHC-like molecule expressed on thymic hematopoietic cells was necessary and sufficient for MAIT cell selection and development. Specifically, MAIT cells have a developmental and functional dependence upon the MHC-related molecule MR1 and the host's microbiota (Treiner et al., 2003). Aside from being highly abundant in the blood, MAIT cells were enriched in the gut lamina propria and lung, as compared to the skin, and hence the name Mucosal-Associated Invariant T (MAIT) cells. This study gave the first indication of an MR1-MAIT cell axis with a role in antimicrobial immunity.

The Role of MAIT cells in Antimicrobial Immunity

MR1-dependent Responses to Microbes

To address a physiological role for MAIT cells, two studies definitively showed that MAIT cells were reactive to antigen produced by bacteria and fungi and presented by MR1 (Gold et al., 2010a; Le Bourhis et al., 2010). Le Bourhis et al., based their study on a previous finding that MAIT cells were undetectable in germ-free mice (Treiner et al., 2003). Additionally, they purified human TRAV1-2$^+$ CD161$^+$ T cells and showed that they could be activated by monocytes infected with *E. coli* or *Mycobacterium abscessus* in an MR1-dependent fashion. Due to the low frequency of MAIT cells in mice, they also used transgenic mice expressing TRAV1/TRBV6 TCRs to show that MAIT cells were activated by a wide array of bacterial and fungal species. In these experiments the following microbes activated MAIT cells: *Candida albicans, Candida glabrata, E. coli, Klebsiella pneumonia, Lactobacillus*

acidophilius, Pseudomonas aeruginosa, Saccharomyces cerevisiae, Staphylococcus aureus, and *Staphylococcus epidermitis*. However, five unrelated viruses, *Enterococcus faecalis* and *Streptococcus pyogenes* were unable to elicit a response by MAIT cells.

In parallel, our laboratory was trying to understand the human immune response to *M. tuberculosis* infection. They initially observed that individuals who had never been exposed to *M. tuberculosis* had a large population of T cells that could recognize *M. tuberculosis* infection *ex vivo*, were cytolytic, and made pro-inflammatory cytokines like TNF-α and IFN-γ (Lewinsohn et al., 2000). They hypothesized that these were not classically restricted T cells, as they could respond to *M. tuberculosis* – infection without a requirement for classical antigen presentation. This finding was supported by evidence that the human thymus contained a population of 'innate-like' *M. tuberculosis* -reactive $CD8^+$ T cells whose pro-inflammatory function was also not dependent upon classical antigen presentation (Gold et al., 2008). In an effort to describe these T cells in more detail, our laboratory performed limiting dilution analysis T cell cloning on $CD8^+$ T cells from the blood of healthy individuals, and patients infected with *M. tuberculosis*. They analyzed the anti-mycobacterial function of each T cell clone to determine what each clone recognized upon infection (Gold et al., 2010a). They observed that the T cell clones universally depended upon MR1 and were broadly antimicrobial to pathogen infections including *Candida albicans, E. coli, M.tb, M. smegmatis, M. bovis BCG, Salmonella typhimurium,* and *Staphylococcus aureus* but not viruses or *Listeria monocytogenes* (Gold et al., 2010a).

Importantly, the data from these two studies suggested that viruses did not activate MAIT cells but that a wide range, but not all, of bacteria and fungi did. Taken together, these studies provided substantial evidence that MAIT cells detect an antigen common to certain microbial infections through MR1 antigen presentation.

MAIT cells can be activated in the absence of MR1 stimulation

The pro-inflammatory cytokines IL-12 and IL-18 are implicated in MR1-independent activation of MAIT cells. In an initial study, murine MAIT cells lost their *in vitro* anti-mycobacterial activity in the presence of a blocking antibody to IL-12 (Chua et al., 2012). Furthermore, MAIT cells are activated during acute human viral infections (dengue fever, hepatitis A, influenza A) and this was replicated *in vitro* to study the mechanism for activation (Ussher et al., 2014; van Wilgenburg et al., 2016). Instead of relying upon MR1-antigen presentation to become activated, MAIT cell activation was driven by IL-12 and IL-18 cytokines made by virally infected cells. In an *in vitro* culture, purified and activated MAIT cell supernatant could limit Hepatitis C viral replication in hepatocytes (in a dose dependent manner). This control was mediated by MAIT cell production of IFN-γ as shown by experimental blockade with antibody to IFN-γ (van Wilgenburg et al., 2016). It remains to be determined whether MAIT cells play a necessary role in anti-viral immunity *in vivo*. Further support for the ability for MAIT cells to be activated by IL-12 and IL-18 cytokines was given by a recent study from Jo and colleagues (Jo et al., 2014). Production of IL-12/18 by cells infected with *E. coli* was sufficient to induce cytokine production by purified human liver MAIT cells after an overnight co-culture.

Virus specific $CD8^+$ T cells can be induced to produce IFN-γ in an antigen independent manner by sensing the cytokines IL-12 and IL-18 (Beadling and Slifka, 2005). IL-12 can induce $CD8^+$ T cell transcription of IFN-γ through STAT4 mediated activation and this is independent of MHC antigen presentation and the TCR (Coccia et al., 1999; Schindler et al., 2001). IFN-γ can also promote IL-12 production in a positive feedback mechanism (Yoshida et al., 1994). The IL-12/STAT4/IFN-γ axis is important for immunity to certain intracellular bacterial infections, such as *M. tuberculosis* (Sabri et al., 2014). Overall, the mechanism for MR1-independent MAIT cell activation is most likely not unique to MAIT cells. Nevertheless, given MAIT cell's prevalence at common sites of infection, cytokine-driven MAIT cell activity may play a role in defense against certain infections *in vivo*. *In vivo* challenge experiments will be needed to address this possible anti-microbial function for MAIT cells.

A Protective Role for MAIT cells in Immunity to Microbial Infection

The hypothesis that MAIT cells are innate like T cells with a protective role in the antimicrobial immune response has been supported by mouse models where the deletion of MR1, and hence MAIT cells, rendered mice more susceptible to bacterial infections (Chua et al., 2012; Georgel et al., 2011; Le Bourhis et al., 2010; Meierovics et al., 2013). The first indication of a role for MAIT cells *in vivo* came from Le Bourhis and colleagues who showed in mice, expressing a transgenic MAIT TCR, that those without MR1 ($MR1^{-/-}$) were significantly less able to control growth of *E. coli* after intraperitoneal injection (Le Bourhis et al., 2010). Three other groups have subsequently challenged C57/BL6 WT or $MR1^{-/-}$ mice with a range of microbial infections and found MAIT cells to influence control of bacterial infection. The role for MAIT cells in control of gram-negative Enterobacteria septicemia was tested by giving WT or $MR1^{-/-}$ mice intraperitoneal injections of *Klebsiella pneumoniae, E.coli, Yersinia enterolitica,* or *Shigella dysentariae* (Georgel et al., 2011). Upon injection of *Klebsiella pneumoniae*, $MR1^{-/-}$ mice sustained higher bacterial loads, lower temperatures, and survived less well in comparison to WT mice. This key finding suggested that MAIT cells play a role in immunity to *K. pneumoniae* infection. However, WT and $MR1^{-\backslash-}$ mice handled infection by the other three bacteria equivalently, suggesting that MAIT cells do not play a significant role in protection from these pathogens. In this regard, it is important to note that these results contradict the finding from Le Bourhis et al. that MAIT cells play a role in protection from *E. coli*. However, Le Bourhis et al., tested their model in mice made transgenically to uniformly express the MAIT TCR, where up to 80% of the T cell repertoire express a MAIT TCR. Therefore, protection might depend upon high frequencies of circulating MAIT cells. The role for MAIT cells in control of mycobacteria was tested using an established respiratory infection model of *M. bovis* BCG in WT or $MR1^{-/-}$ mice. Mice deficient in MR1 sustained a 10-fold higher bacterial load in the lungs at 10 days post infection, but an equivalent bacterial load at 30 days post infection (Chua et al., 2012)

Lastly, MAIT cells have also been implicated in the induction of immunity to *Francisella tularensis* LVS (Meierovics et al., 2013). The Cowley lab first observed that MAIT cells expanded in the lungs of mice that were given an intranasal challenge with *F. tularensis* LVS. Using knockout mice, they also showed that MR1 and IL-12 were required for MAIT cell expansion and bacterial growth control. MR1 deficient mice had defects in early mucosal cytokine production, recruitment of classically-restricted $CD4^+$ and $CD8^+$ IFNγ producing T cells, and control of bacterial growth. This was the first time that MAIT cells were implicated in direct or indirect recruitment of adaptive immune cells to the site of infection.

In sum, these murine studies implicate a role for MAIT cells in the early stages of bacterial containment during infection. While all bacteria tested were strains capable of generating riboflavin antigens for MAIT cells, not all infection models seemed to have a protective role for MAIT cells in immunity (*E.coli, Yersinia enterolitica,* or *Shigella dysentariae*). This may be because the bacteria were not anatomically located at the site they would naturally infect.

MAIT TCR Repertoire

The human MAIT TCR typically contains a semi-invariant TCR α-chain comprised of *TRAV1-2* joined to *TRAJ33*, or less commonly *TRAJ12 and TRAJ20*, with a limited range of TCR β-chains commonly *TRBV20 or TRBV6* in which the CDR3β loop is hypervariable (Birkinshaw et al., 2014). This generality is derived from characterizing the TCR repertoire of MAIT cells in the blood of healthy individuals. However, the full repertoire of TCRs that can respond to MR1-Ag is unknown. Also, the TCR repertoire outside of the peripheral blood is also unknown. In this thesis, I addressed the hypothesis that the MR1-restricted T cell TCR repertoire is comprised of TRAV1-2^+ and TRAV1-2^- T cells.

MAIT TCR Structure

How do MAIT TCRs engage MR1-ligand and why are only certain ligands activating? Ternary structures of MR1 bound to different ligands and engaged by different MAIT TCRs revealed a highly conserved docking and recognition pattern (Reantragoon et al., 2012). It is clear that the TRAV1-2 alpha chain and limited set of beta chains used by MAIT TCRs make direct contacts with MR1 and activating ligands but not non-activating ligands (Lopez-Sagaseta et al., 2013a; Patel et al., 2013). With regard to the TCRα, one residue of the CDR3α sequence, Tyrosine 95 is the anchor of this interaction and conserved across MAIT TCRs. In contrast, mutagenesis screens have not found any necessary residues in the different MAIT TCRβ chains (Reantragoon et al., 2012); but instead have observed a compensatory TCRβ mechanism that converges on a consensus docking mode for the entire TCR on MR1 on every ternary structure solved to date (Eckle et al., 2014; Gherardin et al., 2016; Patel et al., 2013). This mechanism highlights how differential TCRβ chain usage could be accommodated within the highly conserved MR1 docking mode that forms the basis for MAIT cell activation by diverse microbes. In sum, the semi-invariant MAIT TCR recognizes riboflavin and folic acid synthetic intermediates stabilized by MR1 in a conserved docking mode.

MAIT TCR Heterogeneity leads to Discrimination between Antigens and Microbes

While MAIT cells are defined by their expression of a TCR including TRAV1-2, recent studies show accumulating evidence of unanticipated MAIT TCR heterogeneity through more diverse TRAJ gene usage, and TRBV gene usage, in addition to random nucleotide additions in the CDR3 regions used to detect MR1-ligand (Gold et al., 2010a; Gold et al., 2014; Greenaway et al., 2013; Lepore et al., 2014b; Reantragoon et al., 2013; van Schaik et al., 2014). Specifically, this was first recognized through genotypic characterization of several *M. tuberculosis*-reactive MR1-restricted T cell clones (Gold et al., 2010a). More recently, Reantragoon et al., used an MR1 tetramer loaded with potent stimulatory ligand

rRL-6-CH_2OH to define the underlying TCR diversity of MAIT cells able to interact with MR1 in the blood (Reantragoon et al., 2013).

How does TCR variability contribute to MAIT cell reactivity to different MR1-ligands? MAIT cells might have the capacity to respond to antigen selectively within the confines of an innate-like system, or merely require different thresholds of affinity for activation or for alternate effector functions. A recent study evaluated the functional relevance of the semi-invariant nature of the MAIT TCRs. Analysis of the MAIT TCR repertoire of individuals based on functional reactivity to infection by different microbes and expression of TRAV1-2, revealed pathogen selectivity through oligoclonal MAIT TCR usage (Gold et al., 2014). The *ex vivo* $CD8^+$ T cell cytokine response to *Mycobacterium smegmatis, Salmonella typhimurium,* and *Candida albicans*, from 4 individuals was analyzed. The functional TRAV1-2^+ T cells were sorted and the TCR α and β chains used by them were determined. Although no 'microbe-specific' MAIT TCRs were found, interestingly, each individual preferentially used slightly different TCRs to respond to each microbe. For the first time, this study shows the ability of individual MAIT TCRs to contribute to recognition of certain microbial infections more than others. In support of MAIT TCR ligand selectivity, it has been shown that MAIT cell clones with distinct TCRs can discriminate between synthetic riboflavin-derived MR1 ligands (Gold et al., 2014).

Gherardin et al., also addressed how MAIT TCR heterogeneity would impact MR1-restricted recognition of the previously described riboflavin- and folate-derived ligands (Gherardin et al., 2016). They found that certain MAIT cells expressing atypical TCRβ chains used this beta chain to be autoreactive to MR1 or to respond selectively to folate derivative antigens (MR1 ligands previously described to be antagonistic). Furthermore, they discovered T cells that used TCR chains never associated with MAIT

Table 1: A summary of contrasting characteristics between classically-restricted CD8$^+$ T cells and MAIT cells.

T Cell Feature	Classically-restricted CD8$^+$ T cells	MAIT Cells
T Cell Receptor Repertoire	Diverse TCRs	Semi-invariant TCRs
Antigen Presentation Restriction	Polymorphic MHC class I	Non-polymorphic MR1
Antigen Specificity	Peptides	small vitamin B2 synthetic intermediates
Thymic Selection	On thymic stromal cells	On hematopoietic cells
Abundance	Low frequencies	High frequencies
Effector Timeline	Slow – requires clonal expansion over 1 to 2 weeks	Rapid – pre-armed without division, response time in hours
Tissue Localization	In circulation, then traffic	Present at specific mucosal sites
Memory Responses	Memory – long lasting protection	Innate-like: no evidence for memory responses

cells previously, that displayed MR1-restricted reactivity to folate derivative antigens. These folate derivative antigens had been previously thought to be MAIT cell antagonists. The affinity of these atypical TCRs to MR1 alone or MR1-folate derivative antigens was significantly weaker than that seen with MAIT cells. This study highlighted how different TCRs can selectively react to folate or riboflavin derived MR1 ligands, and showed that the pool of MR1 restricted TCRs is more diverse than previously recognized. Nevertheless, the prevalence of non-TRAV1-2 TCRs that recognize MR1-antigen, or their role in detection of microbial infection, has not been addressed. Table 1, above, summarizes the most contrasting differences between MAIT cells and classically-restricted CD8$^+$ T cells.

My thesis project can be divided into three parts with a theme of T cell receptors: 1) MR1-restricted TCRs used in the thymus, 2) the effects of TCR diversity on MR1-restricted T cell microbial recognition, and 3) direct paralysis of TCR signal transduction by a family of viral immune evasion genes. In Chapter 2 I will first describe two approaches I used to ask whether the use of the semi-invariant TCR by MAIT cells, is pre-disposed during thymic development. Studies presented in Chapter 3 address the prevalence of alternative (TRAV1-2 negative) MR1-restricted TCRs across individuals. T cell cloning of

TRAV1-2 negative MR1-restricted T cells allowed me to address whether alternate MR1 ligands outside of riboflavin-derivatives might exist. Since we have access to a library of microbes at the VA hospital microbiology laboratory, we also had the opportunity to determine whether different MR1-restricted TCRs had the ability to recognize discrete microbial infections. Finally, Chapter 4 diverges from MR1-restricted T cells in a collaborative project with the Fruh Laboratory (VGTI). In this project we addressed the hypothesis that virulent orthopoxviruses like Variola, use an evasion gene product to cause direct functional paralysis of human T cells and moved towards uncovering the mechanism.

Chapter 1.5 Technical Advances for the Isolation and Enumeration of MR1-restricted T cells

The field of MAIT cell biology has grown steadily from the emergence of methods to phenotypically and functionally analyze these unique T cells. In the following section, I will describe and compare the most common methods I used to explore MR1-restricted T cells in this thesis.

T cell Lines for Analysis of Oligoclonal T cells

The generation of T cell lines is most widely used when the frequency of an antigen specific cell of interest is below the limit of detection. Antigen specific T cell lines established from patients in healthy or diseased states are useful in determining the presence or absence of antigen specific T cell reactivity. T cell lines are *in vitro* cultures of T cells grown by repeated cycles of stimulation. This method is primarily used to enhance the proportion of antigen-specific T cells from a polyclonal population in order to study the properties of an oligoclonal population. It is much less time-, labor-, and resource-intensive than generating T cell clones, as described next. In Chapter 3 of this thesis, I depleted MAIT cells from healthy individual's blood and then used the T cell line method to enrich for other *Mycobacterium smegmatis*-reactive $CD8^+$ T cells. T cell lines are usually not a terminal experiment, but instead used to isolate T cells of interest for further characterization. This method should not be confused with immortalized lymphoma T cell lines, like Jurkat cells.

Limiting Dilution Analysis (LDA) for Isolation of Monoclonal T cells

To get a detailed characterization of clearly defined features of T cells, such as their paired $\alpha\beta$ TCR usage, or antigen specificity, the generation of individual T cell clones is necessary. The LDA was originally designed to measure the frequency of lymphocytes able to respond to a given antigen. Because it is set-up to isolate clonal T cells, I have used it in this thesis solely for that purpose. In this

assay T cells are cultured under limiting dilution conditions and must survive and proliferate to stimulation *in vitro*. In this thesis, I used polyclonal stimulation instead of a specific antigen to expand all T cells with the capacity to survive from my input population, in Chapters 2 and 3. Limiting dilution conditions entail that T cells are diluted at very small numbers per well across multiple plates (usually 30, 3, and 0.3 cells per well) and cultured under perfect survival conditions, ideally so that every T cell has a chance to proliferate. A single T cell is allowed to clonally expand into ~$2x10^5$ identical cells over the course of two weeks. The LDA makes use of the Poisson distribution to calculate whether the resulting T cell populations were actually derived from single T cells. This equation defines a relationship between the proportion of positive culture wells and the amount of cells plated per well. From the Poisson distribution, it is predicted that one T cell was added per well if 67% of wells did not have growth. While the LDA method is one of few ways to study a T cell in isolation, it is labor-, resource-, and graduate student coffee-intake -intensive. However, there are many benefits to having T cell clones to study antigen specific responses. Aside from their uniformity, T cell clones' phenotypes and function are stable over time and need only to be rapidly expanded with anti-CD3 in preparation for experiments. In the case of MAIT cells and maybe other T cells, variations of this protocol can be used to generate immortalized T cell clones as I will discuss in Chapter 2.

ELISPOT

The ELISPOT assay is used to enumerate antigen-specific T cell responses by their secretion of cytokines – specifically in this thesis, the cytokine IFN-γ. This method was first described in the late 1990s, and is a deviation from the ELISA assay. An ELISPOT makes use of an antibody-coated membrane inside of a 96-well plate to capture IFN-γ secreted by activated T cells. Each well of the plate can contain different conditions to test the activation parameters of the T cell, such as dendritic cells infected with different microbes in Chapters 2 and 3 of this thesis. The cytokine is detected as colored spots on the

membrane and counted with a spot-enumeration camera. The primary advantage of the ELISPOT assay is that many variables can be tested simultaneously while requiring only a small number of T cells.

Flow Cytometry

Surface Protein Expression

Flow cytometry is used to analyze protein expression at the single cell level through the use of a staining procedure with monoclonal antibodies conjugated to different fluorophores. Flow cytometers can also `sort' cells based on protein expression through a method called fluorescent-activated-cell-sorting or FACS. MAIT cells are most routinely identified by their surface marker expression, including their expression of the semi-invariant TCR, TRAV1-2, by using antibodies recently generated that are specific to this TCRα (Gold et al., 2010a; Le Bourhis et al., 2010). However, given that classically restricted- and CD1b-restricted T cells can also use TRAV1-2, expression of this TCR alone is not sufficient for the identification of MAIT cells. MAIT cells also express CD161 (Dusseaux et al., 2011), or KLRB1, a C-type lectin with unknown function that has been associated with NK and NKT cells (Martin et al., 2009a). TRAV1-2^{+}CD161^{+} cells are routinely identified as MAIT cells in our field. However, upon activation or when a T cell is localized to tissue, CD161 can be downregulated from the surface of the cell making this an unstable phenotype. To work around this limitation, Sharma et al., recently found that CD26, also known as dipeptidyl peptidase-4, specifically identifies MAIT cells. They demonstrated that all CD8^{+}TRAV1-2^{+}CD26^{+} but not all CD8^{+}TRAV1-2^{+}CD161^{+} cells could produce TNFα cytokine in response to bacterially infected cells in an MR1 dependent manner (Sharma et al., 2015b). Other surface markers that are associated with MAIT cells include the IL-18 receptor and the chemokine receptors CCR6, CCR5, and CXCR6 which are associated with the ability of lymphocytes to traffic to tissues (Gold et al., 2010a; Martin et al., 2009a; Treiner et al., 2005). PLZF has been proposed as a key transcription factor for MAIT cells, although curiously, it is absent in the rare murine MAIT cells (Fergusson et al.,

2014; Martin et al., 2009a). This transcription factor is essential for the development of another unconventional T cell subset, NKT cells (Savage et al., 2008), and is responsible for enabling an effector memory program in T cells, prior to agonist TCR signaling, an aspect of `innate T cells' (Savage et al., 2011; Thomas et al., 2011). It remains to be determined whether PLZF is essential for MAIT cell biology.

The Intracellular Cytokine Staining assay (ICS)

The ICS assay is used to measure the propensity of a T cell to become activated through its secretion of cytokines, similar to the ELISPOT. However, the ICS assay is more powerful because of it is based on flow cytometry, and therefore measures phenotypic characteristics and multiple cytokines on a single cell basis. T cells are usually incubated overnight in culture conditions containing antigen presenting cells and antigen. Brefeldin-A is added as well, which blocks the secretion of cytokines, which makes them accumulate intracellularly, rendering detectable after the cell is permeabilized. In this thesis, I routinely measured the cytokines TNFα and IFNγ, produced by non-classically restricted T cells through a variation of the ICS assay developed by Marielle Gold (Gold et al., 2010a; Gold et al., 2013; Gold et al., 2014; Meermeier et al., 2016; Sharma et al., 2015b). This assay uses the human alveolar epithelial-derived A549 cell line that expresses unique MHC class I alleles that are mismatched to alleles expressed by our PBMC donors and that does not express MHC class II. Therefore, this cell line does not permit antigen specific activation of classically restricted T cells. Nevertheless, the A549 cell line can be infected with mycobacteria (Harriff et al., 2014) and loaded with MR1-ligands readily.

Tetramers

In the late 1990s, tetramers were developed for direct enumeration of antigen-specific T cells in a fashion unbiased by phenotype or function (Altman et al., 1996). Tetramer staining has become the new `gold standard' and mostly replaced the LDA for measuring frequencies of antigen specific T cells.

This is because tetramer staining could detect 10-100 times more cells than the LDA, as first exemplified with LCMV-specific T cells (Murali-Krishna et al., 1998). Structurally, this tool is comprised of four recombinant antigen presenting molecules, loaded with an antigen, and bound together by a biotin-streptavidin bond, and then conjugated to a fluorophore. Recently, tetramers were developed to label MAIT cells using an MR1 tetramer bound to the riboflavin intermediate 5-OP-RU (Reantragoon et al., 2013). One of the limitations of using tetramers to study T cells is that the antigen must be known. However, a second generation of the MR1 tetramer has just been developed to circumvent this issue (*manuscript in preparation,* Harriff et al*.,* Lewinsohn and Erin Adams laboratories). When recombinant MR1 is folded in the presence of a bacterial species, such as *E. coli*, MR1 is loaded with a myriad of putative antigens. Another limitation is that one cannot assume that a T cell that stains with a tetramer is functional. The tetramer method must be coupled with a functional T cell readout, such as cytokine production, to determine its role in the immune response. I use both of these MR1 tetramers in Chapters 2 and 3 of this thesis to identify MR1-restricted T cells.

Chapter 2: MR1-restricted T Cell Clones Display a Focused TCR Repertoire in the Thymus

Chapter 2 Abstract

Human MAIT TCRs are semi-invariant and typically comprise a *TRAV1-2/TRAJ33* gene-encoded α-chain paired with a limited array of TCR β-chains. Whether or not this limited usage of TCRs is due to their developmental ontology or focused due to exogenous antigen exposure is unclear. MAIT cells develop in the thymus but are rare in frequency compared to the peripheral blood and tissues. To evaluate the thymic MAIT TCR repertoire, the TCRs that were able to interact with MR1/Ag by tetramer staining were defined and their TCRs were sequenced. A wide variety of TCRs recognized MR1/Ag in the thymus. Then we compared this repertoire to the tetramer^{+} thymocytes that survived in a limiting dilution-based cloning culture using polyclonal anti-CD3 stimulation. This provided an overall assessment of TCRs used by MR1-restricted T cells in the thymus that were destined to survive, prior to exposure to peripheral antigen that could skew the repertoire. Using functional analysis, we found that the thymic MAIT TCR repertoire mirrored the semi-invariant TCRs used in the periphery and all had innate anti-microbial responses. These data suggest that the MR1-restricted TCR repertoire in the periphery, which is mainly TRAV1-2^{+}, is probably generated by their development in the thymus, and not merely due to peripheral antigen-specific expansions. In sum, MAIT thymocyte T cell clones display a TCR repertoire and antigen specificity similarly to MAIT cells in the periphery.

Data from this chapter are unique to this thesis and have not been published.

Effort Statement

Data in Figure 1 and 2 were the result of experiments performed by Irina Kurtz with Dr. Marielle Gold. TCR$\alpha\beta$ sequencing was performed by James McLaren, Cardiff University. The rest of the experiments were performed by Erin Meermeier, with technical assistance from Irina Kurtz.

Chapter 2 Introduction

A unifying feature of $CD8^+$ T cells is their ability to identify and lyse cells harboring intracellular infection (Zhang and Bevan, 2011). $CD8^+$ T cells recognize infection through classical antigen presentation (on MHC-Ia), or by non-classical antigen presentation (Rossjohn et al., 2014). While most research has focused on MHC-Ia restricted $CD8^+$ T cells that have integral roles in classical adaptive immunity, the role of non-classically restricted T cells seems to bridge innate and adaptive immunity (Rodgers and Cook, 2005). In general, non-classical antigen presentation systems utilize non-polymorphic molecules such as MR1 and are shared by all individuals in a species (Adams and Luoma, 2013; Edholm et al., 2013; Huang et al., 2009). Non-classically restricted T cells can also display limited T cell receptor (TCR) repertoire diversity (Godfrey et al., 2015). Finally, in contrast to MHC-Ia restricted T cells that require priming and clonal expansion to respond to an infection, MR1-restricted T cells can emerge from the thymus as effector T cells (Gold et al., 2013). Therefore, they have the opportunity to play an initial role in rapidly recognizing a primary infection alongside the innate immune system.

Like MHC-Ia restricted T cells, MAIT cells develop in the thymus. There, MAIT cells first perform TCR gene rearrangement by somatic recombination and undergo positive selection. However, while conventional $CD8^+$ T cells are positively selected on cortical thymic epithelial cells expressing MHC-Ia, non-classically-restricted T cells are selected on $CD4^+CD8^+$ "double positive (DP)" hematopoietic cells (Bendelac, 1995; Cho et al., 2011; Treiner et al., 2003; Urdahl et al., 2002). As it specifically relates to MAIT cells, the near absence of detectable MR1 surface expression in general has slowed efforts to identify the cell type involved in the intrathymic selection of MAIT cells. Nevertheless, several lines of evidence show that their selection depends upon MR1 on DP hematopoietic cells (Seach et al., 2013; Treiner et al., 2003). However, only uniform low MR1 expression was detected in these murine DP thymocytes; leading the authors to hypothesize that this cell type is important for MAIT cell selection for reasons other than high MR1 expression. DP $MR1^+$ thymocytes could possibly uniquely present an

endogenous MR1 ligand in the thymus to developing MAIT cells. Alternatively, DP thymocytes could express a unique co-stimulatory protein necessary for MAIT cell development.

The human thymus also contains developing non-classically restricted T cells with innate effector capabilities (Gold et al., 2008). A proportion of these cells are MAIT cells, with a naïve phenotype (Martin et al., 2009a). In contrast to the mouse thymus, the human thymus contains a population of hematopoietic DP T cell thymocytes that express high levels of surface MR1 (Gold et al., 2013). However, the characteristics of these cells, whether they can act as antigen presenting cells, and whether they are the cell type responsible for selecting MAIT cells remains to be determined. Taken together, these data suggests human MAIT cells are selected in a similar manner to murine MAIT cells, but this remains to be definitively demonstrated.

The TCR repertoire of MR1-restricted T cells during development in the human thymus is unclear because most studies have focused on defining the repertoire in peripheral blood. Human MAIT TCRs typically comprise a *TRAV1-2/TRAJ33* gene-encoded α-chain paired with a limited array of TCR β-chains (Gold et al., 2014; Lepore et al., 2014a; Porcelli et al., 1993; Tilloy et al., 1999; Treiner et al., 2003). However, as I describe in more detail in Chapter 3, other TCRs outside of this archetype can recognize MR1 presented antigens (Gherardin et al., 2016; Meermeier et al., 2016). Gherardin and colleagues also described TRAV1-2 negative TCRs that bound selectively to MR1 tetramers loaded with previously described MR1 ligands: riboflavin metabolites and folate derivatives (Gherardin et al., 2016). Collectively, these "TRAV1-2 negative" TCRs represent unprecedented diverse TRAV and TRBV usage by MR1-restricted T cells. These findings display that MR1-restricted T cells can use diverse TCRs to recognize microbial infection by MR1 antigen presentation. MAIT cells are rare in the thymus when identified by their expression of TRAV1-2 and production of IFN-γ to bacterially infected cells *ex vivo*.

It is clear that usage of TRAV1-2 TCR by MR1-restricted T cells far dominates other TCRs in the blood; <2% of MR1-restricted T cells in the blood use alternative TCRs (Thesis Chapter 3). A mechanism

must exist that favors TRAV1-2 among MR1-restricted TCRs. So in this chapter, I will try to address whether the MR1-restricted semi-invariant TCR repertoire, as observed in the peripheral blood, is generated by development in the thymus or if it is focused in the periphery presumably due to exposure to exogenous antigen.

Chapter 2 Results

The majority of MR1 Tetramer$^+$ cells in the human infant thymus use TCRα chains not associated with MAIT cells in peripheral blood

To determine the prevalence of MAIT cells in the thymus and address the hypothesis that the semi-invariant TRAV1-2 TCR is favored during MAIT cell development, human thymocytes were co-stained with an MR1 tetramer and antibody to TRAV1-2. The MR1 tetramer was loaded with the universal MAIT antigen 5-OP-RU, which is a synthetic intermediate of riboflavin, and has been shown to label all MAIT cell in the peripheral blood (Reantragoon et al., 2013). MR1-tetramer+ thymocytes were analyzed by flow cytometry (Figure 2.1A). All thymuses tested contained MR1 tetramer$^+$ thymocytes, as shown in Figure 2.1B and summarized on the right, where the median frequency of total thymocytes was 0.023%, n = 6. The gated MR1/Ag tetramer+ cells did not have uniform expression of CD4 or CD8 or CD45RO, indicating that they were actively undergoing different stages of maturation (Fig. 2.1C). Importantly, we also observed that a minority of the MR1/Ag tetramer+ cells expressed TRAV1-2 (Fig. 2.1C). The median frequency of tetramer$^+$ thymocytes that expressed TRAV1-2 TCR was 3.48% (range 0.99% - 19.2%), summarized to the right. This data suggested that other TCR alpha chains could recognize MR1/Ag in the thymus.

To establish which TCRα chains were used by MR1/Ag tetramer+ cells in the thymus, this population was sorted from three thymuses and the TCRα genes were sequenced. Shown in Figure 2.2 are the productive TCRα variable (TRAV) genes used by MR1/Ag tetramer$^+$ CD4$^+$CD8$^+$ thymocytes in each thymus. The TCRα joining genes displayed similar diversity (data not shown). This sequencing suggests that diverse TCRα gene rearrangements can be used by thymocytes to interact with MR1/Ag.

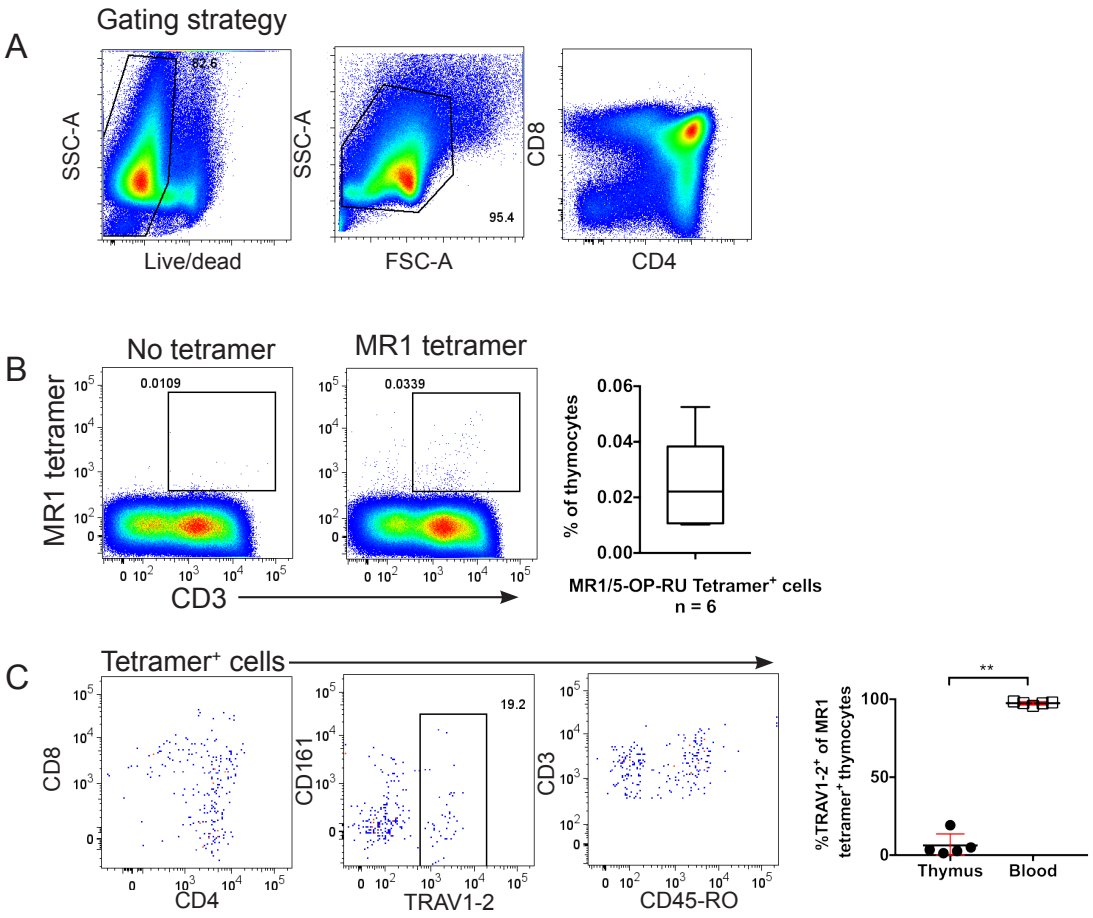

A
Gating strategy
SSC-A
Live/dead
82.6
SSC-A
FSC-A
95.4
CD8
CD4
B
No tetramer
MR1 tetramer
MR1 tetramer
CD3
0.0109
0.0339
% of thymocytes
MR1/5-OP-RU Tetramer+ cells
n = 6
C
Tetramer+ cells
CD8
CD4
CD161
TRAV1-2
19.2
CD3
CD45-RO
%TRAV1-2+ of MR1 tetramer+ thymocytes
Thymus
Blood
**

Figure 2.1. MR1-restricted T cells are rare in the human thymus and express diverse T cell receptors

(A) Representative flow cytometry dot plot showing the gating used for analysis of thymocytes. Dead cells were excluded based on Live-dead viability dye, then thymocytes were gated on lymphocyte shape by FSC-area and SSC-area.

(B) Flow cytometry dot plots showing all MR1-restricted T cell by staining with the MR1-5-OP-RU tetramer (y-axis) by CD3 (x-axis), representative of six donors, examined across 3 independent experiments. The summary on the right is a box plot of six data points showing the maximum and minimum values with the box around the 25^{th} and 75^{th} percentiles. The staining with no tetramer has been performed once on a paired sample with tetramer staining.

(C) Representative flow cytometry dot plot showing CD4 and CD8 expression of MR1-tetramer+ thymocytes (left), and TRAV1-2 T cell receptor and CD161 expression (middle), and CD45-RO isoform and CD3 (right). The graphical summary on the right shows the expression of TRAV1-2 on MR1-tetramer+ thymocytes across five donors in two independent experiments. ** indicates a p value less than 0.01 by an unpaired nonparametric Mann Whitney T test.

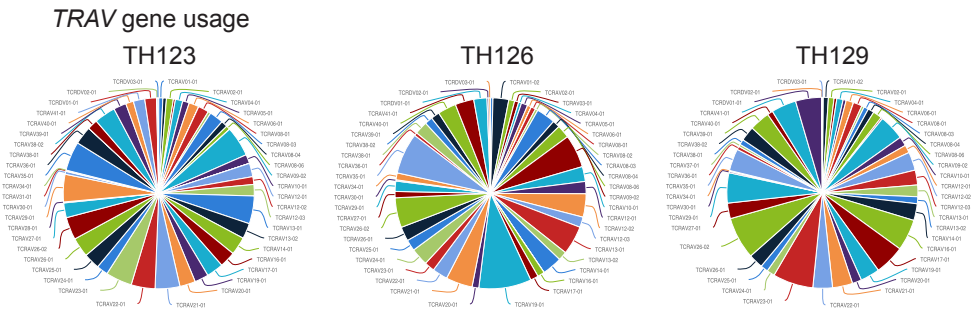


Figure 2.2 Diverse TRAV Gene Usage of MR1-restricted Thymocytes

Pie-charts showing distribution of TRAV genes used by T cells sorted from three infant thymuses. The cells were sorted based on live, $CD8^+$, MR1-5-OP-RU $tetramer^+$ thymocytes.

Thymic MAIT T cell clones express TRAV1-2$^+$ TCRs

Thymocytes need to pass developmental thresholds in the thymus to survive/mature or they face apoptosis. The default is that thymocytes die without a threshold of signaling, via Notch, then via the TCR. The requirements for MAIT cell development and their unique early effector function are still unclear. In order to determine the characteristics of MR1-restricted T cells in the thymus, MR1-tetramer$^+$ T cell clones were generated by a limiting dilution approach (Figure 2.3). The rationale for this approach was that we could focus on thymocytes that had the ability to proliferate, perhaps by a survival signal, and would grow in the limiting dilution assay culture conditions. Yet these clones would exhibit a TCR repertoire unchanged by peripheral antigen exposure. The second benefit to studying T cells by limiting dilution cloning is the ability to analyze features of individual T cells in isolation, such as paired T cell receptor use and effector functions. MR1/Ag tetramer+ cells from thymus donor 126 were sorted (Fig. 2.3A) and we found these cells to express different levels of co-receptor (Fig. 2.3B). Next, cells were sorted and cultured at one cell per well with limiting dilution assay conditions and polyclonal stimulation to non-specifically expand each thymocyte. Through this process ten T cell clones were obtained (Table 2.1).

Each T cell clone was given a name based on its co-receptor expression at the time of cell sorting from the thymus (column 1 of Table 2.1). The majority of the thymus-MAIT clones were derived from double positive thymocytes. In order to determine the unique TCRα and β chains used by each clone, we performed TCR sequencing at each locus. The TCR gene usage and CDR3α and β hypervariable amino acid sequences are shown in Table 2.1. In regards to the TCR α chain, all ten thymus-MAIT clones used *TRAV1-2 and TRAJ33 or TRAJ20*, as has been described as the predominant TCRα rearrangement for MAIT cells in the periphery. All ten CDR3α sequences had MAIT match scores greater than 0.9,

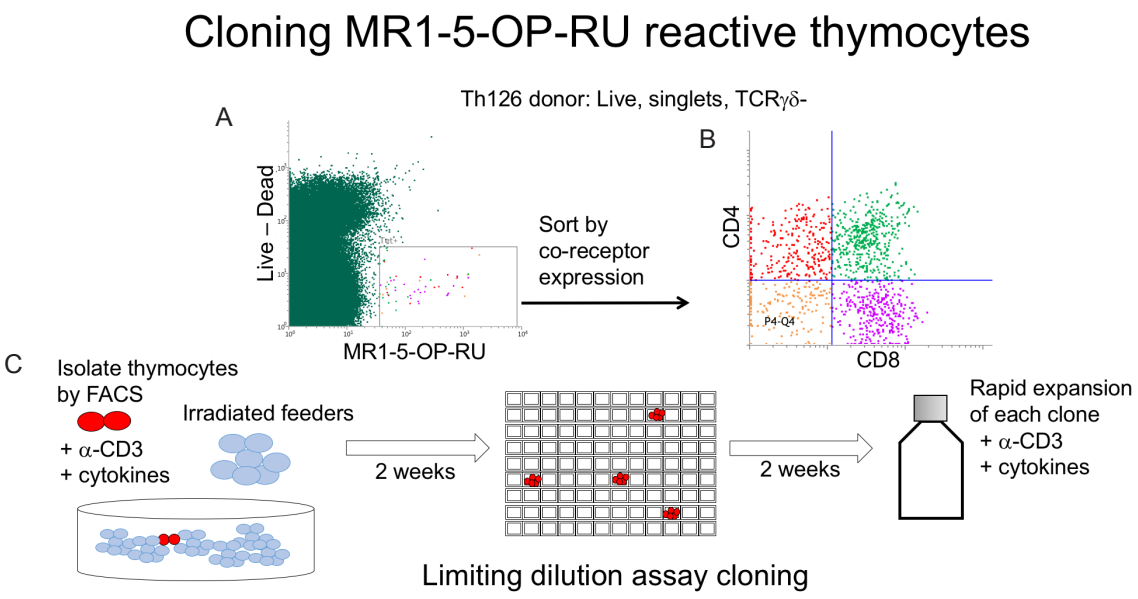


Figure 2.3 Isolation of MR1-restricted thymocyte T cell clones

(A) FACS dot plot of thymocytes from donor 126 gated on single events that were γδTCR-, with MR1-tetramer staining on the x-axis and viability staining on the y-axis. This dot plot shows 1e6 cells from this sample. The gate shows the cells that were sorted and put into culture.

(B) The FACS sorted MR1-tetramer^{+} cells were divided into four populations based on their expression of CD4 or CD8 based on this gating plot.

(C) Schematic of T cell cloning procedure

Chapter 2 Table 2.1

Clone name	TRAV	CDR3	TRAJ	MAIT Match Score	TRBV	CDR3	TRBJ
DP EM-B11	1-2	CAATDSNYQLI	33	0.9412	20-1	CSAGGGGGLNEQF	2-1
DP EM-C5	1-2 *2-1*	CAVRDGDYKLS CAVEENQAGTALI	20 15	0.9628	15-1	CATSRVTGSYYNEQF	2-1
DP EM-D5	1-2	CAAMDSNYQLI	33	0.9576	6-4	CASSDRGAGGEVTEAF	1-1
DP EM-D6	1-2	CAVKDSNYQLI	33	0.9577	4-1	CASTPGGEAGELF	2-2
DP MG-A4	1-2	CAVFDSNYQLI	33	0.9336	6-2/6-3	CASSHLAGFPTDTQY	2-3
DP MG-A11	1-2 9-2	CAPMDSNYQLI CALSAPPRSSASKII	3 33	0.9453	20-1	CSARGGGPSEKLF	1-4
DP MG-B4	1-2	CAVLDSNYQLI	33	0.9581	20-1	CSARGDPVEQY	2-7
SP IK-B12	1-2 13-1	CAVRDSNYQLI IYNFNKFY	33 21	0.9567	6-4 19-1	CASSDSNAREQF CASLRDPGYT	2-1 1-2
SP IK-C1	1-2	CAVKDSNYQLI	33	0.9577	6-1	CASSEPTGSTEAF	1-1
DP JK-A8	1-2	CAVWDSNYQLI	33	.9094	6-4	CASSDPRAYNEQF	1-2

Table 2.1 Table of T Cell Clones Isolated from Thymus 126

The clone name begins with an abbreviation to describe which FACS sorted population they came from, double positive for CD4 and CD8 (DP) or single positive (SP). The MAIT match score is a similarity score to published TCR sequences of MAIT cells (with 1 being a perfect match to published sequences; http://www.cbs.dtu.dk/services/MAIT_Match/). This method was developed by Morton Nielsen at the Technical University of Denmark.

indicating that they were similar to published CDR3α sequences of mature MAIT cells found in the blood (http://www.cbs.dtu.dk/services/MAIT_Match/) (Table 2.1). The uniform expression of TRAV1-2 and CD3 by all ten clones indicated that they were clonal. However, three of the ten thymic clonal MAIT cells expressed two TCR alpha chains simultaneously and reasons for this phenomenon will be addressed in the discussion. Finally, the thymus MAIT T cell clones used TCR β chains that have been associated with mature MAIT cells (Gold et al., 2014; Lepore et al., 2014a; Reantragoon et al., 2013).

Thymus MAIT T cell clones express similar receptors as blood MAIT cells

To phenotypically characterize each thymus MAIT clone, we stained them with antibodies to CD3, CD4, and CD8. We observed uniform expression of CD3 but variable expression of CD4 and CD8 among the clones (Figure 2.4). Clones MG-A4, MG-A11, MG-B4, IK-C1, EM-B11, and EM-D6 were double positive for CD4 and CD8. We observed that clones EM-D5, EM-C5, and IK-B12 were mainly double positive but also had some cells in the transition to becoming CD8 single positive. Surprisingly, the clone JK-A8 only expressed CD4 – peripheral MAIT cells have never been described within the CD4 helper T cell lineage. As shown in the second column, all of the T cell clones exhibited uniform expression of TRAV1-2. MAIT cells can also be characterized by high expression of the C-type lectin receptor, CD161 (or KLRB1) (Martin et al., 2009a). Although the physiological function of CD161 is still not known, its co-expression with TRAV1-2 has been widely used to identify MAIT cells and it has been shown to be necessary for MAIT cell activation (Le Bourhis et al., 2013a). We observed that all thymus MAIT clones expressed variable amounts of CD161 (Fig. 2.4). Other cell surface markers have been associated with MAIT cells, including the dipeptidyl peptidase-4 or CD26 (Sharma et al., 2015a). Similarly, all of the thymus MAIT clones also expressed CD26 (Fig. 2.4). Collectively, these data show that immature MAIT cells located in the thymus, already express certain surface proteins used to characterize mature MAIT cells in the blood.

Figure 2.4 Phenotype of MR1-restricted thymocyte T cell clones

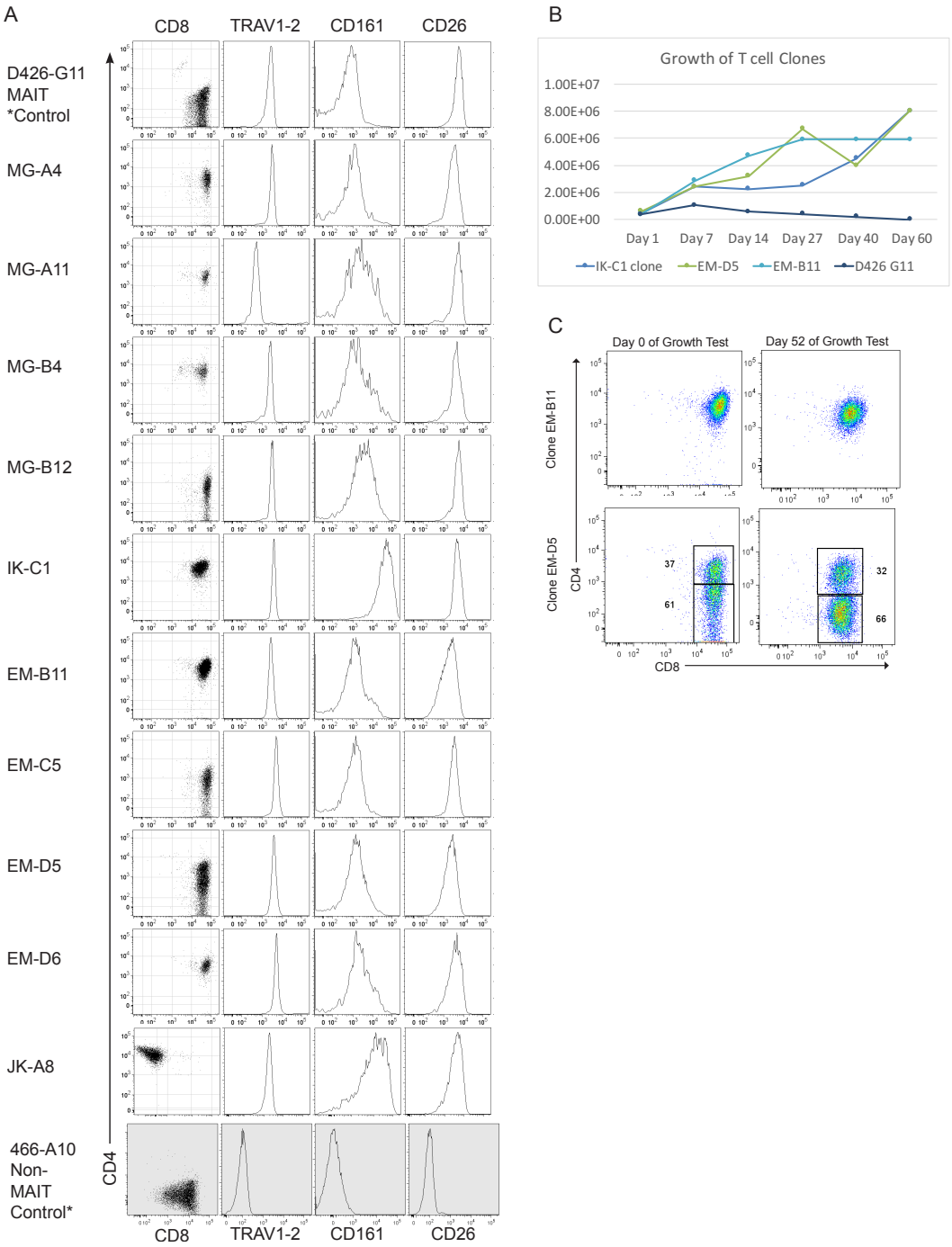


Figure 2.4 Phenotype of MR1-restricted thymocyte T cell clones

(A) The left column displays a flow cytometry dot plot of CD8 (x-axis) and CD4 staining (y-axis). The next column displays a histogram of TRAV1-2; then CD161; and finally CD26 staining for each clone. D426-G11 T cell clone was used as a positive control MAIT T cell clone, while D466-A10 T cell clone (gray plots) was used as a negative control for TRAV1-2, CD161, and CD26 staining as is an HLA-B45 restricted CD8+ T cell clone. Experiment is representative of two independent experiments (one in the T cell clone buttons, one on the rapid expansion of the T cell clone).

(B) Line graph of the growth or decline of four T cell clones cultured in RPMI 10% fetal bovine serum over time. T cells were grown in 10 mL and received one dose of human IL-2 [0.3 ng/ml] on Day 1. IK-C1, EM-D5, EM-B11 are thymus-derived MAIT cells, whereas D426-G11 is a peripheral blood derived MAIT T cell clone. This data is representative of two independent growth curves for IK-C1 and D426-G11.

(C) Flow cytometry dot plots showing the expression of CD4 and CD8 co-receptor proteins on the surface of MAIT thymocyte clone EM-B11 (top) and EM-D5 (bottom) at the beginning and near the end of the growth experiment in (B). All events were first gates on single, live, CD3+ events. This experiment has only been done once.

By happenstance, we observed that the MAIT thymocytes were continually proliferating. Growth patterns over sixty days are shown in Figure 2.4B for thymic MAIT clones compared to a traditional MAIT clone D426-G11. In these experiments, we thawed from MAIT clones and placed in culture with RPMI media supplemented with 10% heat inactivated fetal bovine serum and antibiotics. The clones were not given any stimulation but were given one dose of 2.5 ng/ml human IL-2 on day 0, however we have not tested whether the T cell clones create their own IL-2. The three thymocyte MAIT clones proliferated over the 60 day period while a traditional MAIT T cell clone did not. This signified that the thymic MAIT clones lack cell cycle regulation or have a proliferation mechanism that traditional MAIT cells do not. Interestingly, the surface markers observed on each T cell clone shown in Fig. 2.4A, including their co-receptor expression, did not change over this time period.

Thymus MAIT T cell clones have selective affinities for MR1/antigen tetramers

The clonality and MR1 reactivity of the thymus MAIT clones was confirmed by staining with the MR1-Ag tetramer loaded with the MAIT activating ligand, 5-OP-RU (Fig. 2.5 A, B). Notably, the thymus MAIT clones bound the MR1-Ag tetramer with the same level of intensity as the TRAV1-2^{+} MAIT cell clone D426-G11 (Gold et al., 2010a), while the MHC-1a-restricted clone D480-F6 (Lewinsohn et al., 2007) did not bind the tetramer. In contrast none of the T cell clones bound the MR1 tetramer loaded with the MAIT cell antagonist, 6-formyl pterin. We also tested the ability of each clone to bind MR1 tetramer loaded with MR1 ligands from *E.coli.* We observed highly variable TCR affinities for MR1/*E.coli* tetramer between the different clones (Fig. 2.5C). Clones EM-C5, EM-D5, and EM-B11 did not stain above background. Clones MG-A4 and MG-A11, stained dimly with this tetramer (MFI < 1000). The positive control MAIT clone D426-G11 and clone IK-B12 stained with the tetramer at similar levels (MFI ~ 1000). However, clones MG-B4, IK-C1, and EM-D6 exhibited an MFI > 6000 for tetramer staining, indicating a selective affinity for MR1/*E.coli* tetramer. Collectively, the pattern of recognition of MR1 tetramers by

Figure 2.5 MR1 Tetramer Binding by MR1-restricted thymocyte T cell clones

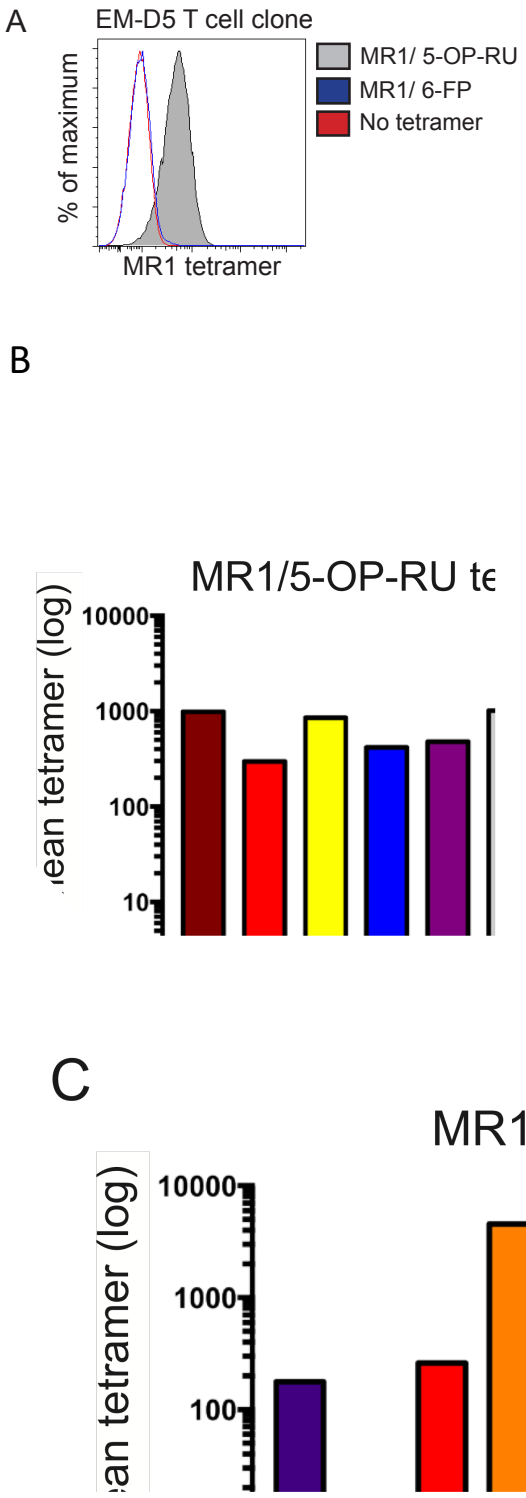


Figure 2.5 MR1 Tetramer Binding by MR1-restricted thymocyte T cell clones

(A) Representative histogram overlay of T cell clone EM-D5 stained with MR1 tetramer-5-OP-RU (shaded), MR1-6-formyl pterin (blue), or FMO control (red). Similar staining was observed with all clones tested, as shown in (B) (n = 6).

(B) Bar graph depicting the staining of eight T cell clones with the tetramer from (A) bound to 5-OP-RU. The geometric mean of the fluorescence intensity is plotted on the y-axis

(C) Bar graph depicting the level of staining of MR1/E.coli tetramer with each T cell clone by the geometric mean of the fluorescence intensity (y-axis). T cell clone JK-A8 was not tested. Other T cell clones listed with no bar did not stain the tetramer. MAIT T cell clone D426-G11 was used as a positive control, while D480-F6, a classically restricted T cell clone was used as a negative control.

thymus-derived MAIT clones confirms the presence of different antigen-selective MAIT TCRs during maturation.

Thymus MAIT T cell clones are MR1 restricted and recognize microbial infection

To confirm our previous finding that MAIT cells in the thymus are microbe reactive effectors (Gold et al., 2013), we tested the ability of each thymus MAIT clone to respond to *M. smegmatis*-infected epithelial cells. As shown in the blue bars of Figure 2.6, all of the clones responded by producing variable amounts of IFN-γ cytokine when incubated with infected A549 cells. These responses were all dependent upon MR1 as observed by the abrogation of response when an $MR1^{-/-}$ cell line was used as APC (Fig. 2.6). To determine if these T cell clones share the broad pathogen reactivity of MAIT cells, we would also need to test other microbial infections. These results suggest that, as with mature peripheral MAIT cells, thymus-derived MAIT cells are microbe-reactive and dependent upon MR1 antigen presentation.

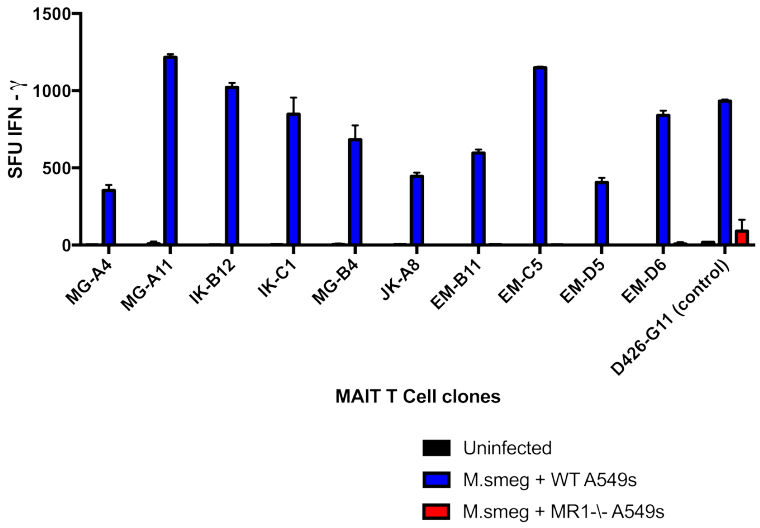


Figure 2.6 MR1-restricted thymocyte T cell clones recognize *M. smegmatis* infection

Bar graph depicting the cytokine response (spot forming units of IFN-γ) of each thymocyte MAIT clone (1e5/well) in response to *M. smegmatis* (MOI = 1) infected A549 cell line (1e5/well) by ELISPOT in blue. Red bars indicate wells where an $MR1^{-\backslash-}$ A549 cell line was used. Black bars represent the response to uninfected A549 cells. Error bars refer to the SEM of two replicate wells. These data are representative of two independent replicates.

Table 2.2 Summary of MR1-restricted thymocyte T cell clones

Clone name	Co-receptor expression	5-OP-RU	6-FP	TRAV1-2^{+} T cell receptor	Recognition of infection	MR1-restricted
DP EM-B11	Double positive (DP)	Yes	No	Yes	Yes	Yes
DP EM-C5	DP & CD8	Yes	No	Yes	Yes	Yes
DP EM-D5	DP & CD8	Yes	No	Yes	Yes	Yes
DP EM-D6	DP	Yes	No	Yes	Yes	Yes
DP MG-A4	DP	Yes	NT*	Yes	Yes	Yes
DP MG-A11	DP	Yes	No	Yes	Yes	Yes
DP MG-B4	DP	Yes	NT	Yes	Yes	Yes
SP IK-B12	CD8	Yes	NT	Yes	Yes	Yes
SP IK-C1	DP	Yes	NT	Yes	Yes	Yes
DP JK-A8	CD4	Yes	No	Yes	Yes	Yes

*NT = not tested

Chapter 2 Summary and Discussion

In these experiments, we analyzed the MR1-restricted TCR repertoire in development, prior to foreign antigen exposure. Using a different approach than past studies of MAIT cell thymic development, we enumerated MR1-restricted T cells in the thymus with a MR1-tetramer. We found they are rare in frequency compared to the peripheral blood. We also observed that the MR1-Ag tetramer stains a variety of TCRs expressed on thymocytes; of which the minority used the semi-invariant TRAV1-2 alpha chain associated with MAIT cells in the periphery. We generated ten MR1-restricted T cell clones from a human thymus, as identified by the same MR1-Ag tetramer, by limiting dilution based cloning. TCR sequencing and flow cytometry performed on thymus MAIT clones demonstrated similar TCR usage to MAIT cells in peripheral blood, including the uniform use of the TRAV1-2 alpha chain. We quantified expression of CD26 and CD161, markers used to identify MAIT cells in the peripheral blood. We found consistent expression of CD26 across the T cell clones, while CD161 expression was variable. Most of the MAIT thymocytes expressed both CD4 and CD8, while some were CD8 single positive and one was CD4 single positive. These patterns of co-receptor expression remained stable after T cell clone rapid expansions. Unexpectedly, we also observed that the MAIT thymocyte clones displayed continuous proliferation and did not depend upon TCR stimulation for their expansion *in vitro*. All of the T cell clones uniformly bound the MR1-Ag tetramer that they were originally isolated with by FACS, but displayed differential recognition to a MR1-tetramer bound to undefined *E. coli* antigens . Lastly, all of the clones were able to recognize *M. smegmatis*-infected epithelial cells in a manner dependent upon MR1, and responded by secretion of IFN-γ. This functional response by MR1-restricted thymocytes clones is consistent with previous publications that MAIT cells acquire effector function in the thymus.

What is the prevalence of MR1-restricted T cells in the human thymus?

We observed that MR1-tetramer$^+$ CD3$^+$ thymocytes comprised ~0.02% of total live thymocytes (Fig. 2.1), whereas MR1-tetramer$^+$ cells can be as abundant as 1-3% of live lymphocytes in the peripheral blood (Meermeier et al., 2016; Reantragoon et al., 2013). Our lab has previously published that when measured as a proportion of the non-classically restricted CD8$^+$ T cell response to *M. tuberculosis*, MAIT cells comprise ~6%, 16%, and 27% of the thymus, cord blood, and peripheral blood lymphocytes respectively (Gold et al., 2013). Taken together with the data that MAIT cell peripheral expansion depends upon microbiota (Treiner et al., 2003), the results presented here fit with current literature that MAIT cells are rare in development but expand upon thymic egress.

However, there are limits to the current methods used to enumerate MAIT cells that could bring bias into the data to answer this question. The methods that have been used to address MAIT cell frequencies rely upon (1) the use of an antibody or primers to TRAV1-2 which assumes all MR1-restricted T cells use this TCRα, or (2) the use of a specific antigen (5-OP-RU) or family of antigens from a microbe (*M. tuberculosis*) bound to MR1 (Gold et al., 2013; Martin et al., 2009a; Treiner et al., 2003). Therefore, MR1-restricted T cells that utilize alternative TCRs or recognize alternative ligands bound to MR1 would be missed in these analyses. The evidence for the existence of TRAV1-2 negative MR1 restricted TCRs and alternative MR1 ligands was published recently (Gherardin et al., 2016; Meermeier et al., 2016). In support of this notion, our laboratory published that a majority (~85%) of the non-classically restricted T cell response to *M. tuberculosis ex vivo*, used a TCR other than TRAV1-2 (Gold et al., 2010b). Furthermore, these T cells required CD8 for their functional response suggesting that they require intact antigen presentation and are not merely activated by soluble mediators of inflammation. The identity and regulation of this T cell population, and whether they are alternative MR1-restricted T cells, remains to be determined.

The finding that MR1-tetramer$^+$ T cells are rare in the thymus presents the opportunity to track them over time. To address whether MR1-restricted T cells have memory, it would be informative to

follow them over immunological experience: from the thymus or cord blood to infant to young adult. We know they expand by frequency (Gold et al., 2010a) but do MAIT cells stably change the repertoire of antigens recognized? Because MAIT cells recognize such a broad array of microbes, it could be assumed that MR1-restricted T cells had been exposed to antigen constantly over that time period – did they expand and change the TCR repertoire, or does the repertoire contract to what is observed early on?

How does the TCR repertoire of MR1-restricted T cells compare between the thymus and peripheral blood?

The recent discovery that TRAV1-2 negative MR1-restricted T cells exist in circulation at a much rarer frequency than TRAV1-2$^+$ MR1-restricted T cells, begged the question of: Why does the use of TRAV1-2 TCR dominate on MR1-restricted T cells? One approach to answer this question is to analyze the TCR repertoire of MR1-restricted T cells in the thymus. While multiple studies have addressed MR1-restricted T cell development in the thymus, they have focused only on TRAV1-2$^+$ cells. Therefore, it is important to know whether TRAV1-2 dominates in the pool of MR1-restricted T cells generated through thymic development, and prior to exogenous antigen exposure.

We have measured the TCR repertoire of MR1-restricted thymocytes in two ways. First, we used an MR1-Ag tetramer to isolate MAIT cells directly *ex vivo* and sequenced their TCRα. We did not find specific TCR usage among the *TRAV or TRAJ* chains. While the data supplied in Figures 2.1 and 2.2 was not sufficient to measure the repertoire of MR1-restricted T cells allowed to survive selection from the thymus, it does suggest that MR1 can bind to diverse TCRs in the thymus. Second, we designed a LDA-based culture system to isolate single MR1-restricted T cells from a thymus and performed paired TCR$\alpha\beta$ sequencing and phenotypic and functional characterization. By using these two different methods we obtained two different answers to our overall question.

In using the directly *ex vivo* approach to quantify tetramer$^+$ cells (approach #1), we observed that TRAV1-2 is not the dominant TCR that can interact with MR1-Ag in the thymus. We observed that a median of 3.48% of MR1-tetramer$^+$ thymocytes expressed TRAV1-2 by antibody staining (Fig. 2.1C) and a similarly low frequency when measured by DNA TCR rearrangements (Fig. 2.2). As the vast majority of MR1-restricted T cells in the periphery express TRAV1-2, this result was unexpected. Our results could fit within the paradigm of peripheral MR1-restricted T cells using a semi-invariant TCR in at least three possible ways:

The first model that could explain this phenomenon is that the thymus contains a highly diverse repertoire of MR1-selected T cells, which becomes focused as the TRAV1-2$^+$ T cells expand upon peripheral antigen exposure. Positive selection of randomly generated clones on MR1 might be followed by ligand dependent clonal expansion in the periphery. The clonal expansion of TRAV1-2$^+$ T cells could be driven by a focused family of MR1-ligands that are ubiquitously present from the microbiota, and therefore would not be dependent upon pathogenic microbial exposure or disease. This model is supported by studies of MAIT cells in mice where MAIT cells might not come into contact with diverse peripheral antigen due to the uniform environment of lab mice. These studies have found that mice do not contain high frequencies of MR1-restricted T cells that use a semi-invariant TCR and these cells also express a naïve phenotype (Martin et al., 2009a; Treiner et al., 2003). Additionally, MR1-restricted T cells are absent in mice without microbiota (Treiner et al., 2003).

Given that the tetramer$^+$ TCRs were at different stages of T cell development as assessed by their co-receptor expression (Fig. 2.1C), it is possible that we were observing TRAV1-2 negative T cells that were going to be deleted by selection but had not yet. In this scenario, many TCRs could associate with MR1-endogenous ligand, but only those that get a certain threshold of signaling would survive. This threshold might only be reached by TRAV1-2 paired with the family of TCRβ chains observed on MAIT cells in the periphery; or might require unique co-stimulatory receptor signaling. In this case, we

analyzed the MR1-restricted repertoire before a critical selection event or death by neglect occurred in this population. To test this, we could compare levels of BCL-2 anti apoptotic factor between TRAV1-2^{+} and TRAV1-2^{-} MR1-tetramer^{+} thymocytes; higher levels of BCL-2 in thymocytes indicates that the cell has been selected and given a survival signal.

Lastly, there is a possibility that this experiment detected an artifact of tetramer staining. Perhaps we were detecting low affinity TCRs in this experiment and not accurately surveying MR1 'binding'. We do not currently have a control MR1 tetramer to use to detect background low affinity associations with T cells. Although we did observe ~ one log fold higher MFI in some cells that stained positively with the tetramer (Fig. 2.1B), so this is probably not the sole reason. We also note that the MR1-tetramer labeled a minority population CD3 negative thymocytes. As these cells would not have a TCR yet, this labeling is an indication of a certain level of nonspecific binding to thymocytes. In the future, we might improve the specificity of the MR1-tetramer binding if we could enrich for the T cell population of interest first, instead of staining for cells with such low frequency. This method might decrease any background or nonspecific binding by alternative cells.

A future experiment to address whether there are functional TRAV1-2 negative MR1-restricted T cells in the thymus would be to test their ability to recognize and respond to microbial infection *ex vivo*. Thymocytes would be stained for TRAV1-2, then co-cultured with or without MR1 blocking antibody and infected APCs. If there are functional TRAV1-2 negative cells whose function is blocked by anti-MR1, then this would display the presence of MR1-restricted TRAV1-2 negative T cells in the thymus. Secondly, T cells in infant cord blood represent T cells that have been fully developed in the thymus yet are still antigen naïve. It would be an important future experiment to compare our results from Figure 2.1 with a similar experiment using samples of cord blood. If the pattern is maintained where the MR1-tetramer binds diverse TRAV1-2 negative TCRs at a higher frequency than the blood, this would suggest that peripheral antigen exposure is important for the dominant use of TRAV1-2 by MAIT cells.

The second approach taken to determine whether TRAV1-2 dominates in the pool of MR1-restricted T cells generated through thymic development, involved the isolation of thymocyte clones. We designed a LDA-based culture system to isolate single MR1-restricted T cells from a thymus and performed paired TCR$\alpha\beta$ sequencing and phenotypic and functional characterization. We used FACS to sort thymocytes that were able to bind MR1-tetramer and then cultured single cells in an environment designed to support general T cell activation and expansion. One drawback to this approach is that the culture might not recapitulate what a thymocyte needs for survival because it was originally designed for blood lymphocytes. Upon cloning of MR1-tetramer$^+$ thymocytes, we cultured T cells that expressed TCRs matching those observed on MAIT cells in the peripheral blood (Table 2.1 compared to: (Gold et al., 2014; Held et al., 2015; Lepore et al., 2014a; Reantragoon et al., 2013). Furthermore, most of these T cells expressed both CD4 and CD8, indicating that they were undergoing the TCR selection process. All of the T cell clones expressed *TRAV1-2* recombined with *TRAJ33 or 20,* and beta chains within families that have been observed to be used by MAIT cells before, like TRBV6 family. All of the T cell clones also shared the expression of CD26 and CD161 which helps to identify MAIT cells in the periphery. All ten T cell clones responded to mycobacterial infection with the production of IFN-γ in an MR1-dependent fashion. Therefore, while this data fit more closely with what we would have expected as far as the MR1-restricted TCR repertoire, there are different reasons that could explain this phenomenon.

These results fit the hypothesis that thymic selection favors the use of TRAV1-2$^+$ TCRs by MR1-restricted T cells. It is plausible that the semi-invariant TRAV1-2$^+$ MAIT TCR is favored during selection because it has a correct affinity for the high levels of MR1 expressed by the small population of hematopoietic cells in the thymus (characterized in Kurtz I., et al *in preparation*). T cell development is a highly ordered and controlled process where thymocytes at each stage are restricted to certain amounts of cell division as they pass each step (Mingueneau et al., 2013). So, as it relates to our assay set-up,

perhaps only selected thymocytes have the capability to proliferate to anti-CD3 stimulation. To follow the rules of thymic selection, a T cell will survive if it paradoxically recognizes MHC-ligand during positive selection, but does not recognize MHC- ligand during negative selection. In this proposed model, all thymocytes have the chance to interact with MR1 in the thymus but only MAIT cells interact in the correct pattern. MAIT cells depend upon positive selection on MR1 for survival (Seach et al., 2013; Treiner et al., 2003). This model is also supported by data from the mouse model showing that the MAIT semi-invariant TCR can interact with MR1 without any added foreign ligand (Treiner et al., 2003); leading the authors to suggest that MAIT cells are mildly autoreactive. Additionally, there may be a mechanism in place for MR1 to present endogenous-ligands representative of peripheral self-metabolites during negative selection in order to protect from autoimmunity. This would be parallel to the finding that thymic epithelial cells use AIRE transcription factor to express a vast diversity of self-antigens. Because all of the known MR1 ligands to date are microbial derived small molecules, the most logical source of related self-ligands would be lysosomal breakdown of self-metabolites with a similar core scaffold or possibly even metabolites from mitochondria because they are ancestrally prokaryotic. These products could drive selection so that strongly autoreactive MAIT cells get deleted.

As mentioned above, the thymic MAIT T cell clones used TCR beta chains that have been associated with mature MAIT cells (Gold et al., 2014; Lepore et al., 2014; Reantragoon et al., 2013). Since beta chain rearrangement happens before the alpha chain in thymic development, what is it about MAIT cell development that results in this semi-invariant TCR? Firstly, *TRAV1-2* might pair more efficiently with the few beta chains used by MAIT cells. Moreover, TCR rearrangement plays a role in the relative abundance of the semi-invariant TRAV1-2 alpha chain. The MAIT alpha chain is easier and faster to rearrange due to the temporal availability of *TRAV1-2* and the MAIT family of *TRAJ* for immune machinery to bind and recombine (Dik et al., 2005; Greenaway et al., 2013). The efficiency of TCR rearrangement may allow T cells expressing TRAV1-2 to be more prevalent or enter the selection stage

sooner or enter into a different selection microenvironment where MR1 selection is favored. Additionally, MR1-restricted T cell positive selection might involve an endogenous-ligand that spans both pockets of MR1 binding groove (A' and F' pocket). In this case, the TCRβ would have a role in selection as it is oriented over the F' pocket in several crystal structures (Eckle et al., 2015), offering a potential explanation for why we observe such an invariant TCRαβ repertoire in the thymus.

Another way to explain why all of the MR1-restricted thymocytes clones expressed canonical MAIT TCRs, is due to an artifact in the experimental set-up. The tetramer reagent, which is MR1 bound to the stimulatory ligand derived from riboflavin, 5-OP-RU, might have activated the thymocyte TCRs to simulate a 'positive selection' signal and microenvironment that propagates T cells specific only to the 5-OP-RU antigen. Necessary costimulation would have been provided by the irradiated feeder PBMC of the LDA. In this explanation of the results, we artificially skewed the growth of MAIT cells expressing TCRs used by peripheral MAIT cells because we used a peripheral antigen to isolate them from the thymus. This potential artifact might have been averted had we been able to use an MR1 tetramer loaded with thymus derived-self MR1 ligand (or whatever might be presented by MR1 in the thymus).

The isolation of thymic MR1-restricted T cell clones was important because we could analyze the paired αβTCR usage and link this to specific functional antigen reactivity. In relation to the other future directions I have proposed for this research, I think it would be important to study the MR1-restricted functional capacity of T cells in healthy cord blood. If the functional MR1-restricted T cells, directly *ex vivo* from cord blood, express TRAV1-2, then this would support our hypothesis that TRAV1-2 is favored on MAIT cells during development.

These experiments yielded some unexpected findings. Three of the ten thymus MAIT clones expressed two TCRα chains simultaneously, including TRAV1-2. Using an antibody to TRAV1-2, we confirmed that each T cell clone expressed this TCR uniformly (Table 2.1). One explanation is that these

clones did not yet get signals from posttranslational regulation of allelic exclusion after positive selection or they have not undergone negative selection yet (Niederberger et al., 2003).

As displayed in Figure 5, we unexpectedly observed dramatic differences in the ability of each MR1-restricted thymocyte clone to bind the MR1/*E. coli* tetramer. EM-D5 and EM-C5 did not bind the tetramer at all, whereas, EM-C6, IK-C1, and MG-B4 exhibited an MFI of ~10^5. We do not know the exact antigens bound by this MR1-tetramer reagent, however, we know that it is bound to a multitude of ligands from *E.coli* (Harriff et al., manuscript in submission). It is unexpected that some MAIT cells expressing the semi-invariant TCR would not recognize MR1-ligands derived from *E. coli* as this is a typical MAIT activating bacterium that produces riboflavin. This result suggests that MAIT TCRs can inherently discriminate between microbial infections, due to their thymic development. This result also suggests the presence of more diverse MR1 ligands from the described 5-OP-RU riboflavin derived ligands; all of the T cell clones were validated to be able to bind the MR1-5-OP-RU tetramer they had been originally sorted with. The notion of microbial selectivity of MR1-restricted TCRs is the topic of Chapter 3 of this thesis.

Another unexpected finding of this project was that the MR1-restricted cloned thymocytes seem to be transformed in some way. The T cell clones could be slowly propagated in culture for up to 60 days (maximum time tested) without the need for additional IL-2 or TCR stimulation. This finding was unexpected as the *in vitro* expansion of T cells usually requires TCR stimulation, costimulation, and IL-2 cytokine. Using this rapid expansion procedure, T cell clones will divide and survive in culture for about one month. Two caveats to this observation are that we have not replicated this experiment with another thymus or with a non-MR1 restricted TCR to determine generalizability.

It is possible that this phenomenon is not unique to MAIT thymocytes but could result from T cell cloning of thymocytes in general. We might have sampled DP thymocytes that were undergoing proliferation after positive selection. Then, the T cell cloning culture conditions did not provide a

regulatory signal to stop proliferating which would have been provided by the thymus microenvironment. The proposition that the MAIT thymocyte clones are stalled at a certain phase of development is supported by the observation that they remained DP over time in culture as well.

We used a MR1-tetramer to isolate MR1-restricted thymocytes and perform LDA cloning. An alternative explanation for the immortalization of the T cell clones could be that the tetramer induced a TCR signal to the thymocytes it bound. In combination with the supportive conditions of the LDA, this caused the T cell to become activated and start proliferating uncontrollably.

It is possible that this phenomenon is a property of innate-like T cells undergoing development in the thymus. Innate like T cells, such as NKT cells, MAIT cells, and certain $\gamma\delta$ T cells express the transcription factor PLZF (pro-myelocytic leukemia zinc finger). PLZF can direct the effector differentiation of innate-like T cells independently of agonist TCR activation (Lu et al., 2015; Savage et al., 2011). Additionally, studies show that PLZF can repress negative regulators of the cell cycle, like *p53,* and in some cases, promote leukemia (Choi et al., 2014). In this way, expression of PLZF could favor a long lived MAIT cell, however, a mechanism for regulating this proliferation would be necessary for immune homeostasis.

Lastly, our method of isolating MR1-restricted T cells with a tetramer reagent and then placing them into a LDA culture well containing the non-specific activator, anti-CD3, induced robust TCR-proliferation. Intracellular signaling from the TCR activates the general AKT pathway that promotes survival and cell proliferation primarily through mammalian target of rapamycin (mTOR). The role of mTOR is to act as an energy sensor and activate translation of proteins. One study has shown that prolonged TCR ligation with costimulation can sustain IL-2-independent lymphocyte proliferation through signaling by mTOR (Colombetti et al., 2006). Therefore, our culture system described in Fig. 2.3 may have provided persistent TCR activation to maintain clonal expansion, without the need for exogenous growth factors, even after removal from the stimulatory culture.

In our future research with these T cell clones, we could analyze the transcriptome of the thymus MAIT clones to determine if genes involved in the cell cycle or specific transcription factors are upregulated that could be causing this proliferation. To determine if this phenomenon is unique to innate like T cells, we could compare the thymus MAIT clone transcriptome or validated protein expression to other thymus T cell clones by RT-PCR or flow cytometry.

Chapter 2 Materials and Methods

Cell Lines and cell culture

A549 (ATCC CCL-185) and MR1$^{-/-}$ A549s (Laugel et al., 2016) were cultured in F12K medium with 10% heat inactivated fetal bovine serum and gentimicin antibiotics. All thymocytes and human T cell clones were cultured in RPMI medium with 10% heat inactivated, pooled human serum and gentimicin.

Thymus Organ Processing

Thymus tissue was obtained from infants undergoing cardiac surgery at Doernbecher Children Hospital under a protocol approved by the Institutional Review Board at Oregon Health and Science University. The thymus was made into a single cell suspension of thymocytes using the following steps. The thymus was cut into 1cm^3 pieces and spun in a GentleMAX machine on the spleen setting twice. Next, DNase was added to the suspension and then filtered through a 100uM, and then 40uM mesh size filter. The flow through contained the thymocytes. This suspension was enriched for thymocytes further using a Ficoll gradient by centrifugation (Sigma). Lastly, the thymocytes were washed twice in DMEM media and stored in a liquid nitrogen freezer.

Isolation and limiting dilution cloning of MAIT cells in the thymus

Thymocytes from donor 126 were stained in tests of 4x10^6 with the MR1 tetramer (0.3nM), antibodies to CD4 (OKT4 clone, FITC, Biolegend, 1:50) and CD8 (SK8 clone, APC-Cy7, Biolegend, 1:500) and a viability stain (Live-Dead Aqua, Life Technologies). Each test was stained with the MR1 tetramer in a volume of 25ul for 45 minutes at room temperature. Then the rest of the stains were added in another 25ul for 15 minutes before washing twice. The staining buffer used was PBS with 2% heat inactivated fetal bovine serum. Live tetramer$^+$ cells were sorted on a BD Influx FACS machine (sort mode = yield) based on expression of each co-receptor and rested overnight in RPMI 1640 media with 0.5ng/ml rhIL-2.

Then the sorted thymocytes were plated under limiting dilution analysis conditions. Therefore, one cell per well was co-cultured with x-ray inactivated PBMC feeder ($150x10^5$/well) and LCL cells ($3x10^4$/well) in a 96-well round bottom plate. Cytokines were also added to the culture: IL-2 (5ng/ml), IL-12 (0.5ng/ml), IL-7 (0.5ng/ml), IL-15 (0.5ng/ml), and IL-21 (0.5ng/ml). Finally, anti-CD3 (0.03 μg/ml) was added to non-specifically expand each thymocyte. Plates were incubated at 37 C for 20 days before thymocytes were harvested for analysis.

Expansion of T Cell Clones

T cell clones were cultured in the presence of x-rayed (3000 cGray using X-RAD320, Precision X-Ray Inc.) allogeneic PBMCs, x-rayed allogeneic LCL (6000 cGray), and anti-CD3 mAb (20 ng/ml; Orthoclone OKT3, eBioscience) in RPMI 1640 media with 10% human serum in a T-25 upright flask in a total volume of 30 ml. The cultures were supplemented with IL-2 (5ng/ml) on days 1, 4, 7, and 10 of culture. The cell cultures were washed on day 5 to remove soluble anti-CD3 mAb.

IFN-γ ELISPOT assay

A MSHA S4510 96-well nitrocellulose-backed plates (Millipore, bought via Fisher Scientific) was coated overnight at 4°C with 10 μg/ml solution of anti-IFN-γ monoclonal antibody (Mabtech clone 1-D1K) in a buffer solution of 0.1M Na2CO3, 0.1M NaHCO3, pH = 9.6). Then the plate was washed three times with sterile PBS and blocked for 1 hour at room temperature with RPMI 1640 media containing 10% heat inactivated human serum pool. Then the antigen presenting cells and T cells were prepared as described below and co-incubated overnight. Briefly, thymocytes were used as APCs at $150x10^4$ per well and the A549 cell line was used as APCs at $1X10^4$ per well in ELISPOT assays. For all blocking ELISPOT assays, blocking antibodies were added for 2 h at 2.5μg/ml (α-HLA-I clone W6/32, α-CD1a, b, c, d (Branch Moody), and α-MR1 clone 26.5 (Ted Hansen) or appropriate isotype controls). T cell clones were added

at $1X10^4$ /well. The plate was incubated overnight at 37°C and then washed six times with PBS containing 0.05% tween. The plate was then incubated for two hours at room temperature with a 1 µg/ml solution of anti-IFN-γ-biotin secondary antibody (Mabtech clone 7-B6-1) in 0.5% BSA, 0.05% Tween in PBS. Finally, the plate was washed six times in PBS-tween, then PBS, and developed using an AEC Vectastain kit SK-4200 (Vector labs). The plate was read and analyzed using an ELISPOT-AID system.

Flow cytometry staining, sorting, and tetramer staining

Cells to be analyzed for cell surface marker expression were first incubated at 4°C in a blocking solution of PBS containing 2% normal rabbit serum (Sigma-Aldrich), 2% normal goat serum (Sigma-Aldrich), and 2% human serum to prevent nonspecific binding. Cells were washed in PBS and then incubated with Live/Dead discriminator and surface stains or isotype controls for 20 minutes in the dark at 4°C in a total volume of 50ul. Cells were then washed and fixed. Specifically for MR1 tetramer staining, $1x10^5$ T cell clones were stained with the MR1/5-OP-RU or MR1/6-formyl pterin tetramer at 0.3nM in 25ul volume for 45 minutes in PBS buffer containing 2% FBS at room temperature in the dark. Viability and surface stains were added on top of the tetramer stain for another 20 minutes. Samples were then washed twice in tetramer staining buffer. All flow cytometry was performed on a Fortessa 18-parameter flow cytometer (BD). All fluorescence activated cell sorting (FACS) was performed using an Influx 11-parameter flow cytometer (BD) by the OHSU flow cytometry core facility. Data were analyzed using FlowJo (v9.8.5). Fluorescence minus one (FMO) controls were used for optimal gating. Doublets were excluded based on FSC-H and FSC-A, SSC-H and SSC-A, lymphocytes were identified based on FSC-A and SSC-A and CD3 expression, and dead cells were excluded based on Aqua viability dye (Life Technologies).

***Ex vivo* Stimulation assay**

To observe the nonclassical T cell response, we used the A549 cell line as APCs because it does not express MHCII and is MHC-I-mismatched to the donor source of T cells. CD8+ T cells were positively selected from healthy donor PBMCs using magnetic bead separation according to the manufacturer's instructions (Miltenyi), added to uninfected WT or *M. smegmatis*-infected (MOI = 3, overnight infection) WT or MR1-/- A549 cells at a ratio of 3:1, and incubated overnight in the presence of Brefeldin A, and 0.5ng/ml rhIL-2 at 37°C. The following day, the cells were stained for surface phenotype markers and Live/Dead discriminator.

Microorganisms and infection of A549 cells

Adherent A549 cells were infected overnight with *M. smegmatis* (multiplicity of infection [MOI] 3) in the absence of antibiotics. *M. smegmatis* strain used was mc^2-155.

Analysis of TCR usage

Amplification and sequencing of TCRA CDR3 regions of bulk thymocytes in Figure 2.2 was performed using the immunoSEQ Platform (Adaptive Biotechnologies™, Seattle, WA). ImmunoSEQ combines multiplex PCR with high throughput sequencing and a bioinformatics pipeline for TCRA CDR3 region analysis (Carlson et al., 2013; Robins et al., 2009). Clonotypic analysis of thymus MAIT T cell clones were performed as described previously (Quigley et al., 2011). In brief, unbiased amplification of all expressed TRB or TRA gene products was conducted using a template switch–anchored RT-PCR with chain-specific constant region primers. Amplicons were subcloned, sampled, sequenced, and analyzed as described previously (Price et al., 2005). The IMTG nomenclature was used throughout the study(Lefranc, 2003).

Chapter 3 Human TRAV1-2 negative MR1-restricted T cells detect *S. pyogenes* and alternatives to MAIT riboflavin-based antigens

Abstract

MAIT cells detect microbial antigens presented by the HLA-Ib molecule MR1 through the exclusive use of a TRAV1-2-containing T cell receptor-α. We use MR1-tetramer staining and *ex vivo* analysis with mycobacteria-infected MR1-deficient cells to demonstrate the presence of functional human MR1-restricted T cells that lack TRAV1-2. We characterize an MR1-restricted clone that expresses the TRAV12-2 TCRα, which lacks residues previously shown to be critical for MR1-antigen recognition. In contrast to TRAV1-2$^+$ MAIT cells, this TRAV12-2-expressing clone displays a distinct pattern of microbial recognition by detecting infection with the riboflavin auxotroph *Streptococcus pyogenes*. As known MAIT antigens are derived from riboflavin metabolites, this suggests that TRAV12-2$^+$ clone recognizes unique antigens. Thus, MR1-restricted T cells can discriminate between microbes in a TCR-dependent fashion. We postulate that additional MR1-restricted T cell subsets may play a unique role in defence against infection by broadening the recognition of microbial metabolites.

Peer Reviewed Publications:

Meermeier, E.W., Laugel, B.F., Sewell, A.K., Corbett, A.J., Rossjohn, J., McCluskey, J., Harriff, M.J., Franks, T., Gold, M.C., and Lewinsohn, D.M. (2016). Human TRAV1-2-negative MR1-restricted T cells detect S. pyogenes and alternatives to MAIT riboflavin-based antigens. Nature Communications 7, 12506

Laugel, B., A. Lloyd, E.W. Meermeier, M.D. Crowther, T.R. Connor, G. Dolton, J.J. Miles, S.R. Burrows, M.C. Gold, D.M. Lewinsohn, and A.K. Sewell. 2016. Engineering of Isogenic Cells Deficient for MR1 with a CRISPR/Cas9 Lentiviral System: Tools To Study Microbial Antigen Processing and Presentation to Human MR1-Restricted T Cells. J Immunology

Effort Statement

All of the experiments contained in this chapter were performed by Erin Meermeier.

Chapter 3 Introduction

MAIT cells have been described as an abundant population of αβ-TCR T cells that display anti-microbial Th1-like cytotoxic capacity upon detection of a range of microbial infections (Gold et al., 2010a; Le Bourhis et al., 2013a; Le Bourhis et al., 2010). By definition, MAIT cells express a semi-invariant T-cell antigen receptor (TCR) that engages antigenic ligands presented by the HLA-Ib major histocompatibility complex (MHC)-related protein I (MR1). MR1 has been shown to present small compounds derived from folic acid and riboflavin biosynthesis, the latter of which can activate MAIT cells (Birkinshaw et al., 2014; Corbett et al., 2014; Kjer-Nielsen et al., 2012). In healthy humans, MAIT cells are abundant and account for 1 to 10% of $CD8^+$ T cells in peripheral blood. They are also abundant in liver and in a number of mucosal tissues (Dusseaux et al., 2011; Gold et al., 2010a; Jo et al., 2014; Leeansyah et al., 2014; Reantragoon et al., 2013; Treiner et al., 2003). Thymic selection and peripheral expansion of MAIT cells depends on MR1 (Seach et al., 2013; Treiner et al., 2003). Furthermore, MAIT cells with effector function have been found in the thymus, a finding that supports their definition as innate-like (Gold et al., 2013). While the role of MR1 and MAIT cells in human immunity is unclear, mouse studies have demonstrated their role in protection against bacterial infections including *Klebsiella pneumoniae*, *Mycobacterium bovis* BCG, and *Francisella tularensis* LVS (Chua et al., 2012; Georgel et al., 2011; Meierovics et al., 2013).

MR1 is an HLA-Ib MHC class I molecule thought to be highly conserved in mammalian evolution (Tsukamoto et al., 2013). MR1 can bind vitamin B-based precursors derived from folic acid (vitamin B9) and riboflavin (vitamin B2) biosynthesis that share a common pterin ring structure (Kjer-Nielsen et al., 2012). So far, only those from the riboflavin synthetic pathway have been shown to stimulate MAIT cells. These stimulating ligands can be derived from either pyrimidine-based early intermediates in riboflavin synthesis (5-A-RU) that form adducts with other small metabolites (eg. 5-OP-RU) or the direct lumazine precursors of riboflavin (eg. ribityllumazine-6,7-diMe) (Corbett et al., 2014; Kjer-Nielsen et al.,

2012). Because riboflavin synthesis does not occur in humans, riboflavin metabolites presented in the context of MR1 have been suggested to be pathogen associated molecular patterns (PAMPs). However, evidence supports the existence of additional MR1 ligands. For example, structural analysis suggests that plasticity in the MR1 binding groove could accommodate a range of different ligands (Corbett et al., 2014; Eckle et al., 2014; Lopez-Sagaseta et al., 2013b; Patel et al., 2013; Reantragoon et al., 2012; Rossjohn et al., 2014). As the pterin ring occurs commonly in the environment, it is feasible that other microbial or host molecules with common chemotypic properties could bind to MR1 and function as antigens for MR1-restricted T cells.

Although MAIT cells specifically recognize infection by pathogens with the capacity to synthesize riboflavin(Gold et al., 2010a; Le Bourhis et al., 2010), whether microbe specific MR1-ligands exist is unknown. We previously evaluated the *ex-vivo* human TCR repertoire of MAIT cells responsive to three riboflavin-synthesizing microbes, (Gold et al., 2014) finding that distinct MAIT TCR usage was associated with microbe selective responses within and across individuals. These data support the hypothesis that MR1 can present discrete microbial ligands, and that this presentation is in turn associated with selective clonal expansion of MAIT cells. However, it is not known whether each microbe synthesizes the same repertoire of riboflavin metabolites, but at varying proportions, or whether there are unique ligands.

The nature of the diversity in MR1-ligand repertoire suggests an accordingly diverse MAIT TCR repertoire to mediate ligand recognition. Human MAIT TCRα chains have been described as being invariant; comprised of *TRAV1-2/TRAJ12, 20, 33* genes paired with a limited array of TCR β-chains (Gold et al., 2010a; Gold et al., 2013; Porcelli et al., 1993; Tilloy et al., 1999; Treiner et al., 2003). However, other studies have identified greater TCR heterogeneity through more diverse TCRα and TCR chain usage(Fergusson et al., 2014; Gherardin et al., 2016; Gold et al., 2014; Lepore et al., 2014b; Reantragoon et al., 2013). Gherardin *et al*. described TRAV1-2 negative TCRs that bind selectively to MR1 tetramers

loaded with 5-OP-RU (riboflavin metabolite), or 6FP/acetyl-6FP (folate derivative), or both (Gherardin et al., 2016). These TRAV1-2 negative TCRs represent unprecedented diverse TRAV and TRBV usage by MR1-restricted T cells. These findings suggest that MR1-restricted T cells could use diverse TCRs to recognize microbial infection; therefore, the full repertoire of TCRs that can be used by MR1-restricted T cells is unknown.

We describe microbe reactive MR1-restricted T cells that do not express TRAV1-2. Functional analysis reveals that these cells, although less prevalent than those that express TRAV1-2, can be found in PBMC from all individuals. Among MR1-Ag tetramer positive cells, 1-4% are TRAV1-2-negative. T-cell cloning confirms the usage of an alternative TCRα chain, TRAV12-2, by an MR1-restricted T-cell clone from one donor. In comparison to previously described TRAV1-2^{+} MAIT cells, this T-cell clone displays a unique pattern of ligand and microbial selectivity. Most notably, the TRAV12-2 T-cell clone could detect infection with *Streptococcus pyogenes* in a TCR-dependent fashion, a microbe that is not capable of synthesizing riboflavin. These data, provide direct evidence of the ability of MR1 to present a diverse array of ligands, which in turn is associated with selective TCR usage. Finally, our findings challenge the current paradigm of sole usage of TRAV1-2 in conjunction with the recognition of riboflavin metabolites being the defining feature of MR1-restricted T cells.

Chapter 3 Results

Enumeration of functional TRAV1-2-negative MR1-restricted T cells

MAIT cells can detect a wide range of bacteria and fungi through recognition of riboflavin metabolites presented by the HLA-Ib molecule MR1. In this context, we sought to explore the relative contribution of MR1 to the entire HLA-Ib restricted $CD8^+$ T cell response to microbial infection. In order to quantify and characterize these responses directly *ex-vivo*, we have developed a functional *ex-vivo* assay that relies upon cytokine production by $CD8^+$ T cells in response to microbial infection of HLA-mismatched A549 cells (Gold et al., 2010a). The flow cytometry gating scheme used to analyze this response is shown in Appendix Figure 1. Using this approach, we have consistently been able to enumerate MAIT cells ($TRAV1\text{-}2^+$) responsive to a number of microbes such as Mtb (Gold et al., 2010a; Gold et al., 2013; Gold et al., 2014), *Candida albicans* and *Salmonella typhimurium* infections(Gold et al., 2014). However, we also consistently observed TRAV1-2 negative cells that are reactive to these same microbes. For example, nearly 50% of the $CD8^+$ HLA-Ib response to *M. smegmatis* was TRAV1-2 negative in a representative donor, D462 (Figure 3.1).

To address the hypothesis that TRAV1-2 negative cells were MR1-restricted, we generated an MR1-knockout A549 cell line (Laugel et al., 2016). Briefly, CRISPR/ Cas9 technology was used to generate a single base pair-deletion and a 125-bp deletion separately on each allele to ablate expression of MR1. To confirm the absence of MR1 in a functional assay, the WT and $MR1^{-/-}$ cell lines were infected with mycobacteria and T cell responses evaluated by IFN-γ ELISPOT. As shown in Figure 3.2A, activation of the $TRAV1\text{-}2^+$ MR1-restricted clone (D426-B1(Gold et al., 2014)) was ablated, while activation of the HLA-E restricted clone (D160-1-23) was unaffected, indicating that lack of MR1 did not affect infectivity or a separate antigen presentation pathway.

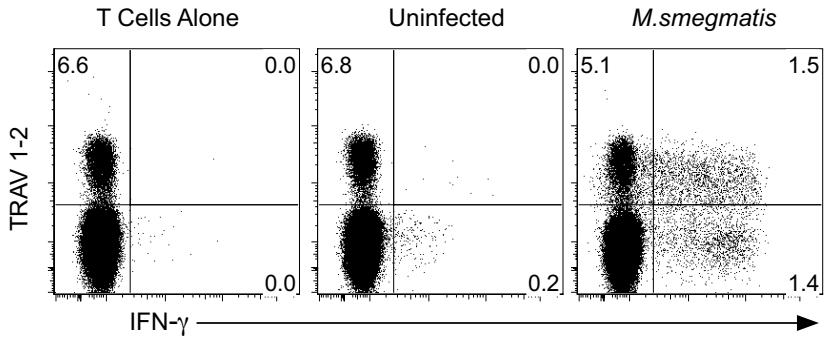


Figure 3.1

***M. smegmatis* infection elicits a response from MAIT cells and TRAV1-2$^-$ HLA-Ib restricted CD8$^+$ T cells**

PBMC-derived CD8$^+$ T cells from adult donor (D462) were stimulated *ex vivo* by A549 cells infected with *M. smegmatis* to identify mycobacteria-reactive HLA-Ib T cells. Functional MAIT cells were identified by production of IFN-γ and expression of TRAV1-2 TCR. Numbers in quadrants indicate the % of live CD3$^+$ cells. Experiments were performed three independent times with similar results. Representative results are shown.

To establish the prevalence of MR1-restricted T cell responses *ex-vivo*, WT and MR1$^{-/-}$ A549 cells were infected with *M. smegmatis* and used as stimulators for CD8^{+} T cells isolated from PBMC of five healthy individuals (Figure 3.2B). Intracellular IFN-γ production was assessed by flow cytometry. As expected TRAV1-2^{+} cells produced IFN-γ in response to *M. smegmatis*-infected WT A549 cells. Furthermore, the majority of the response was MR1-dependent (mean 85.21% MR1-restricted, range 45.3 to 97.22, n = 5, tested in duplicate). Each donor also had a proportion of TRAV1-2 negative cells whose production of IFN-γ was MR1-dependent (mean 25.83% MR1-restricted, range 10 to 41.03, n = 5, tested in duplicate) (Fig. 3.2B). The average of the cytokine responses by the TRAV1-2 negative population for each donor across experiments was plotted in Fig. 3.2B (right panel). In order to confirm the presence of TRAV1-2 negative *M. smegmatis*-reactive T cells, we repeated this assay using PBMC that were FACS selected on CD8 but depleted of TRAV1-2^{+} cells. Then we stimulated the T cells with WT infected A549 cells. From all donors we observed a detectable IFN-γ producing population. Therefore we conclude that not all microbe-reactive MR1-restricted T cells are TRAV1-2^{+}.

Cloning of TRAV1-2 negative MR1-restricted T cells

To further characterize TRAV1-2-negative MR1-restricted CD8^{+} T cells in each of the five donors, we used *M. smegmatis*-infected APC to generate CD8^{+} TRAV1-2 negative T cell lines that were both reactive to *M. smegmatis* and whose activation was blocked by the addition of MR1-antibody. Subsequent cloning of the T cell line from one donor, D462, using α-CD3 stimulation was used to establish the T cell clone, D462-E4. As shown in Figure 3.3A, D462-E4 was characterized by the uniform expression of CD8α and the absence of TRAV1-2. In comparison to TRAV1-2^{+} MAIT cell clones, D462-E4 expressed equivalent levels of TCR and co-stimulatory receptors (Fig. 3.3B). This T cell clone also expressed CD26 (Sharma et al., 2015b) but did not express CD161 (Dusseaux et al., 2011), although CD161 may have been down-

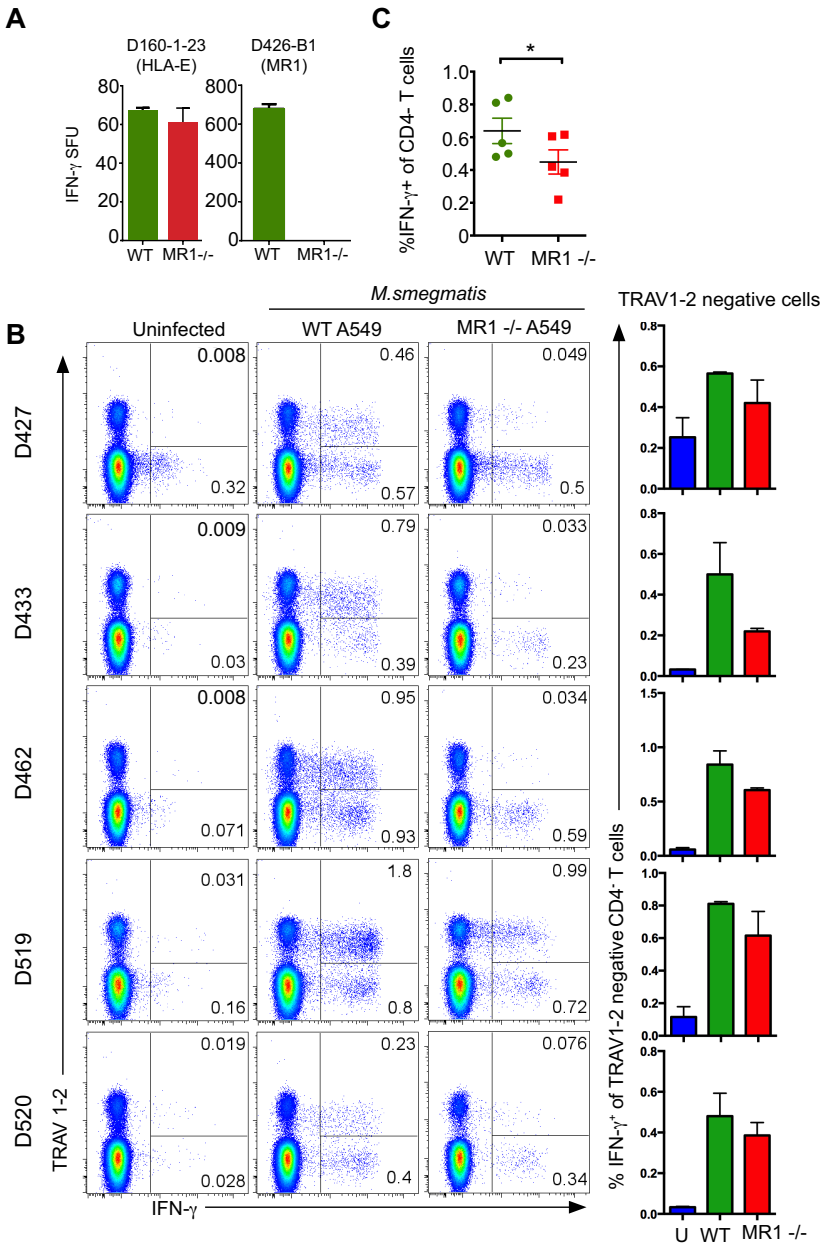


Figure 3.2: MR1-restricted microbial-reactive CD8$^+$ T cells from blood do not exclusively express TRAV1-2. Legend on following page

Figure 3.2 MR1-restricted microbial-reactive CD8$^+$ T cells from blood do not exclusively express TRAV1-2. (A) IFN-γ production by T cell clones D160-1-23 (restricted by HLA-E) and D426-B1 (restricted by MR1) in response to mycobacteria-infected WT and MR1-/- A549 cell line. (B) Positively selected CD8$^+$ T cells from PBMC were tested for *ex vivo* IFN-g responses to *M. smegmatis* infected WT or MR1-/- A549 cell line. Events are gated on live CD3$^+$CD4- cells. IFN-g and TRAV1-2 expression are shown on the x and y-axis, respectively. To the right, is a summary of the TRAV1-2 negative response from each donor across experiments (C) Frequency of IFN-g+ CD4- cells from each donor (represented by one dot) when stimulated by *M. smegmatis* infected WT or MR1 -\- A549s, n = 5 biological replicates, with n = 3 technical replicates. Statistical significance of difference between groups was determined using the nonparametric Mann-Whitney U test. Error bars are the SEM of triplicates in A and B. Experiments in this figure were performed at least twice with similar results. Representative results are shown. *p-value of less than 0.05 was considered significant.

regulated as the result of expansion with anti-CD3 (Leeansyah et al., 2013; Sharma et al., 2015b) (Fig. 3.3B). TCR sequence analysis of D462-E4 demonstrated the unique expression of *TRAV12-2/TRAJ39* TCRα and *TRBV29-1/TRBJ1-5* TCRβ (Fig. 3.3C and Appendix Table 1). The expression of the TCRβ chain was confirmed by antibody staining, which showed staining on D462-E4 but not by MAIT T cell clone, D481-A9 that expresses TRBV20-1 (Gold et al., 2010a). We confirmed that the clone was *M. smegmatis*-reactive using infected epithelial cells, and restricted by MR1 using antibody blockade (Fig. 3.3D). The D462-E4 clone retained the ability to recognize *M. smegmatis* infected cells in a manner that was blocked by α-MR1, but not by the pan-HLA I antibody W6/32, nor by any of the CD1 blocking antibodies. These results are similar to the pattern seen by the known MR1-restricted T cell clones (D481-F12, Fig. 3.3D). The clonality and MR1 reactivity of D462-E4 was confirmed by staining with the MR1-Ag tetramer loaded with the MAIT activating ligand, 5-OP-RU (Fig. 3.3E). Notably, clone D462-E4 bound the MR1-Ag tetramer with the same level of intensity as the TRAV1-2$^+$ MAIT cell clone D426-B1 (Gold et al., 2010a), while the HLA-B45-restricted clone D466-D6 (Lewinsohn et al., 2007) did not bind the tetramer. In contrast neither MR1-restricted clone bound the MR1 tetramer loaded with the MAIT cell inhibitor, 6-FP. Collectively, isolation of clone D462-E4, confirms the presence of microbe-reactive MR1-restricted CD8$^+$ T cells that detect infection using an atypical MAIT TCR TRAV12-2.

Differential antigen recognition by MR1-restricted T cells

Because we had isolated D462-E4 from an *M. smegmatis*-reactive T cell line, we wanted to compare the *M. smegmatis*-reactivity of TRAV1-2$^+$ and TRAV12-2$^+$ T cell clones over a range of MOI. In Figure 3.4A, we tested clone D426-G11 and D462-E4 in an IFNγ ELISPOT for their reactivity to infected dendritic cells. Although both clones recognized the infection, our data shows a higher potency of antigen dose in regards to the TRAV1-2$^+$ clone (D426-G11), while both clones displayed similar maximal efficacy (cytokine release). This result suggested that the TRAV12-2 TCR either had lower TCR avidity or

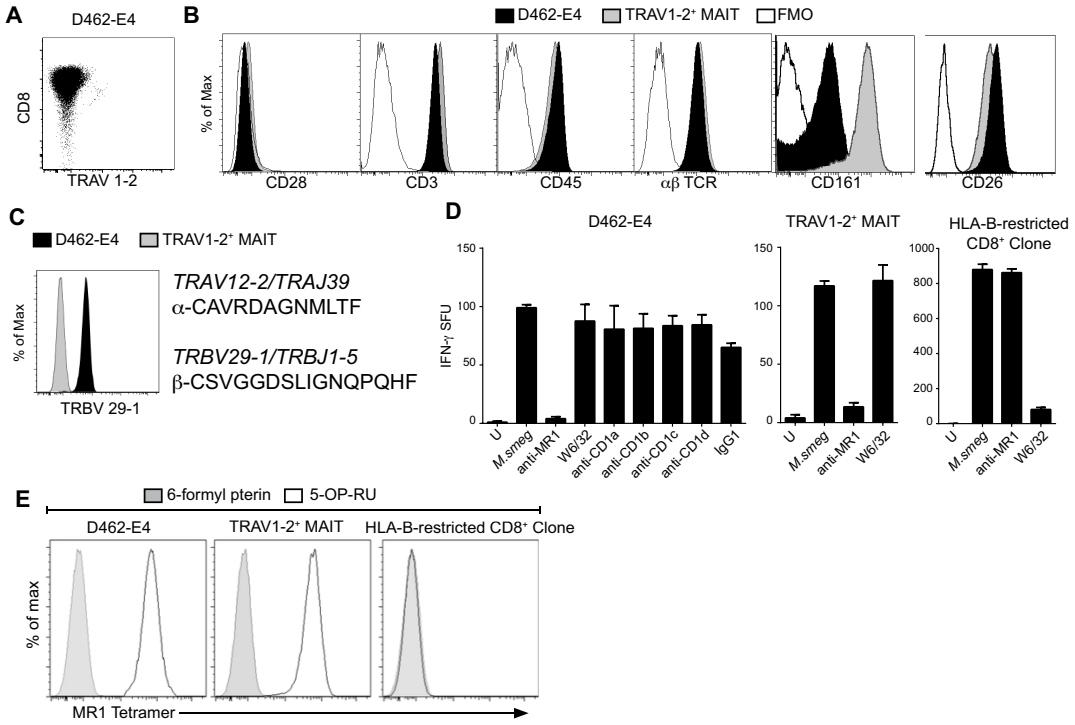

A
D462-E4
CD8
TRAV 1-2
B
D462-E4
TRAV1-2+ MAIT
FMO
% of Max
CD28
CD3
CD45
αβ TCR
CD161
CD26
C
D462-E4
TRAV1-2+ MAIT
% of Max
TRBV 29-1
TRAV12-2/TRAJ39
α-CAVRDAGNMLTF
TRBV29-1/TRBJ1-5
β-CSVGGDSLIGNQPQHF
D
D462-E4
TRAV1-2+ MAIT
HLA-B-restricted CD8+ Clone
IFN-γ SFU
U
M.smeg
anti-MR1
W6/32
anti-CD1a
anti-CD1b
anti-CD1c
anti-CD1d
IgG1
E
6-formyl pterin
5-OP-RU
D462-E4
TRAV1-2+ MAIT
HLA-B-restricted CD8+ Clone
% of max
MR1 Tetramer

Figure 3.3

Isolation of an MR1-restricted T cell clone that reacts to bacteria independently of the TRAV1-2 TCR. Generation of a $CD8^+$ T cell line and then T cell clone, D462-E4, through FACS sorting on $CD8^+$, $\gamma\delta TCR^-$, TRAV1-2 negative T cells from donor in (Fig.1) and co-culturing for 7 days with *M. smegmatis (M. smegmatis)* infected autologous dendritic cells. The line was stained with CFSE and co-cultured for 7 days with *M. smegmatis* infected autologous macrophages. (A) Staining of TRAV1-2 and co-receptor CD8 on T cell clone D462-E4. (B) Comparison of T cell surface marker expression on TRAV1-2 negative clone D462-E4 (black) and TRAV1-2^+ MAIT clone D426-G11 (gray) and (C) of TRBV 29-1 on D462-E4 and D481-A9 TRAV1-2^+ MAIT clone. TCR CDR3 sequences and gene names of D462-E4 are shown, right. (D) BEAS2B cell line infected with mycobacteria and tested for their ability to stimulate T cell clones in the presence of blocking antibodies or isotype by IFN-γ ELISPOT assays, D462-E4, D481-F12 (TRAV1-2+ MAIT), and D466-A10 (HLA-B45). (E) Flow cytometry of clones with MR1-tetramer loaded with 6-formyl pterin (gray) or 5-OP-RU (clear). D462-E4, D481-A9 MAIT clone, D466-A10 HLA-B45 restricted T cell clone. Error bars represent the SEM of triplicates. Experiments were performed at least two times with similar results. Representative results are shown.

could recognize fewer MR1-ligands from the infected cell. Given prior evidence of MR1 ligand discrimination (Corbett et al., 2014; Gherardin et al., 2016; Gold et al., 2010a; Gold et al., 2014; Patel et al., 2013), we tested whether the TRAV12-2 TCR had different ligand selectivity in comparison to the TRAV1-2 MAIT TCR. To define the MR1 ligands recognized by the TRAV12-2^{+} TCR, we first tested D462-E4 for its ability to recognize the A549 cell line loaded with MAIT ribityllumazine antigens RL-6,7-diMe and RL-6-Me-7-OH (Kjer-Nielsen et al., 2012) in the presence or absence of MR1 blockade. As shown in Figure 3.4B, D462-E4 detected both antigens in an MR1 dependent fashion. However, D462-E4 was preferentially stimulated by RL-6-Me-7-OH. In contrast, two previously characterized MR1-restricted clones D481-F12 and D426-G11 (Gold et al., 2010a; Gold et al., 2014) were preferentially stimulated by the RL-6,7-diMe antigen. To better understand whether this differential response was due to TCR avidity, T cell activation was tested over a range of antigen concentrations. We found that the differential responses by D462-E4 compared to the other MR1-restricted T cell clones were maintained over a wide range of concentrations (Figure 3.4C). In these experiments, we observed similar antigen potency (antigen concentration of half maximum response) yet different maximal efficacy (cytokine release) in response to the two-ribityllumazine antigens between the three MR1-restricted T cell clones. These responses may be indicative of different levels of antigen cross reactivity between these TCRs. Therefore, we concluded that D462-E4 displayed ligand discrimination between MAIT ribityllumazine antigens.

Differential recognition of microbes by MR1-restricted T cells

To establish the repertoire of microbes recognized by D462-E4, a diverse array of microbes were tested. Dendritic cells (DC) and epithelial cells were infected with microbes at optimized MOI and used to compare the ability of T cell clones D462-E4 (TRAV12-2) and D426-G11 (TRAV1-2) to recognize these targets (Figure 3.4D). We observed four patterns of responses from the MAIT T cell clones: 1) microbes

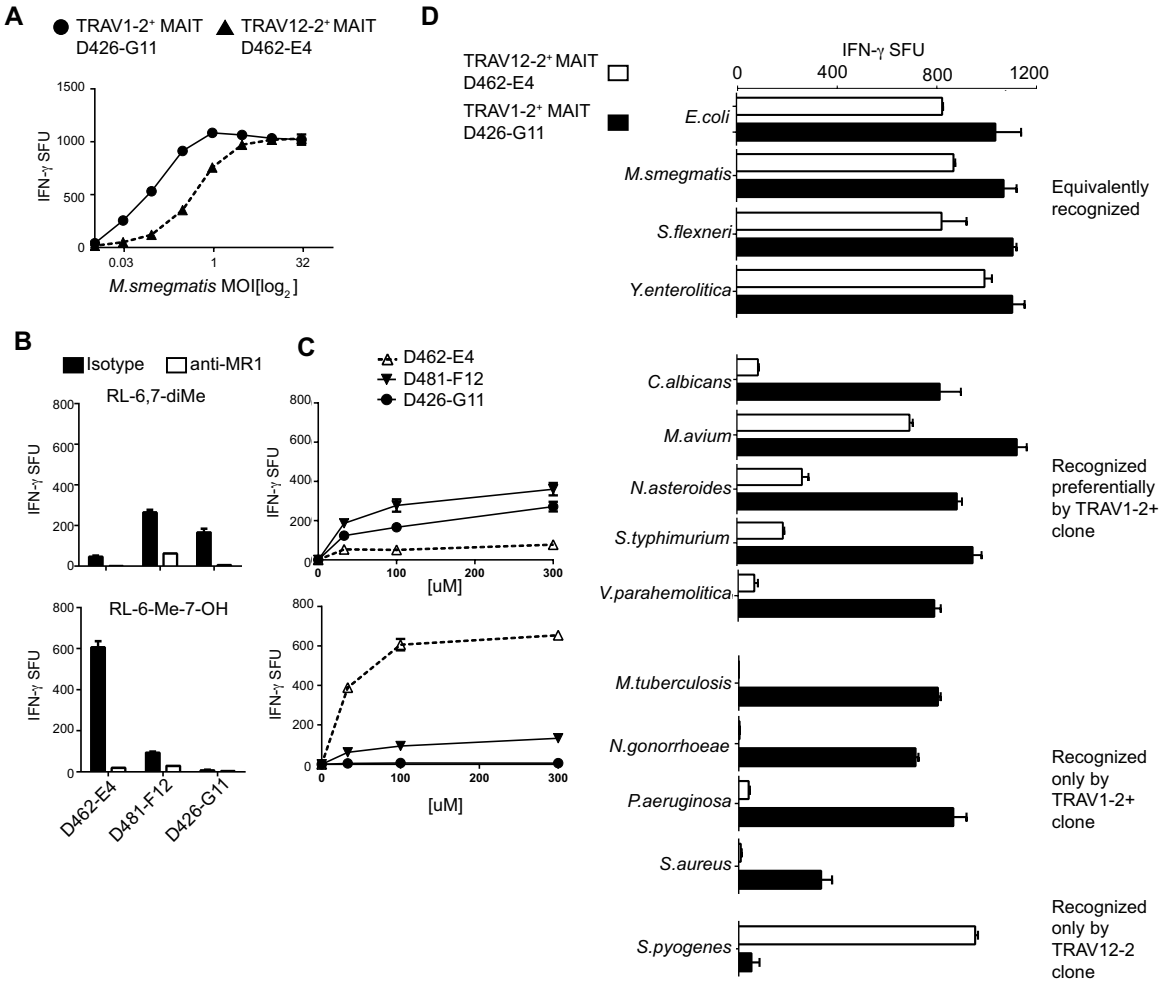


Figure 3.4 T cell clone D462-E4 displays ligand and microbial infection selectivity. Legend on following page

Figure 3.4 T cell clone D462-E4 displays ligand and microbial infection selectivity

(A) Dendritic cells were infected with *M. smegmatis* at a range of MOI (x-axis) and then co-incubated with indicated MR1-restricted T cell clones. (B) A549 cells were pulsed for 2 hr with 100 uM ribityllumazine (RL)-6,7-diMe (top) or RL-6-Me-OH (bottom) and tested for their ability to stimulate MR1-restricted T cell clones in the presence of -MR1 or isotype antibody by IFN-γ ELISPOT. (C) A549 cells were pulsed for 2 hr with a range of concentrations of RL-6,7-diMe (top) or RL-6-Me-OH (bottom) and tested for their ability to stimulate MR1-restricted T cell clones by IFN-γ ELISPOT. (D) Dendritic cells were infected at optimized MOIs with pathogens listed on the y-axis, except for *Yersinia* and *Shigella* where the A549 cell line was used, then co-incubated overnight with the indicated MR1-restricted T cell clones. IFN-γ production was quantified by ELISPOT. Results are grouped by comparative response by MR1-restricted T cell clones. Pattern of recognition was maintained over a range of MOI. Error bars are the SEM of triplicates. Assays were performed twice, with similar results. Representative results are shown.

recognized equivalently by both T cell clones (*M. smegmatis, E. coli, Yersinia enterolitica, Shigella flexneri*), (2) microbes preferentially recognized by the TRAV1-2^{+} T cell clone (*M. avium, Salmonella Typhimurium, Candida albicans*, *Nocardia asteroides, Vibrio parahemolytica*), 3) microbes only detected by the TRAV1-2^{+} T cell clone (*M.tuberculosis, Staphylococcus aureus, Pseudomonas aeruginosa, Neisseria gonorrhoeae*) and 4) microbes only recognized by TRAV12-2^{+} T cell clone (*Streptococcus pyogenes)* (Fig. 3.4D). Neither clone responded to the riboflavin auxotroph *Enterococcus faecalis.* Thus, the TRAV1-2^{+} MAIT clone, D426-G11, only responded to microbes with the capacity to produce riboflavin. While the D462-E4 clone was also able to recognize many of these microbes (Fig. 3.4D), it was distinguishable in its ability to respond to infection with *Streptococcus pyogenes.*

S.pyogenes is a bacterium that does not encode the genes necessary for riboflavin synthesis, such as *ribA, ribB, ribD, ribH,* and *ribE* (Ferretti et al., 2001; Minogue et al., 2014; Vitreschak et al., 2002). Initially, we confirmed that *Streptococcus pyogenes* was auxotrophic for this vitamin (Fig 3.5A). To demonstrate the selectivity of the TRAV12-2 T cell clone for *S. pyogenes*, clones were tested over a broad range of MOIs. The TRAV12-2^{+} T cell clone responded over a range of *S. pyogenes* MOI in dendritic cells (Fig. 3.5B) while the TRAV1-2^{+} clone did not respond to this infection. To exclude a non-specific effect on the TRAV12-2 clone, cells were incubated with *S. pyogenes* or filtered culture supernatant without an APC. No activation was observed (Fig. 3.5C). In order to establish whether the T cell clone was activated by soluble factors from the APC, we used two approaches. First, we tested whether conditioned media from DCs pulsed with *S.pyogenes* culture supernatant could activate D462-E4. No activation was observed (Fig. 3.5C). Second, we tested whether pulsed fixed DCs would maintain the same pattern of eliciting T cell clone activation as unfixed pulsed DCs (Fig. 3.5C). In this experiment, DCs that had been pulsed with *S.pyogenes* supernatant overnight and then fixed were still able to activate T cell clone D462-E4, suggesting the response depends upon antigen presentation and not soluble factors. We next sought to determine whether the response to *S.pyogenes* was dependent upon the TCR.

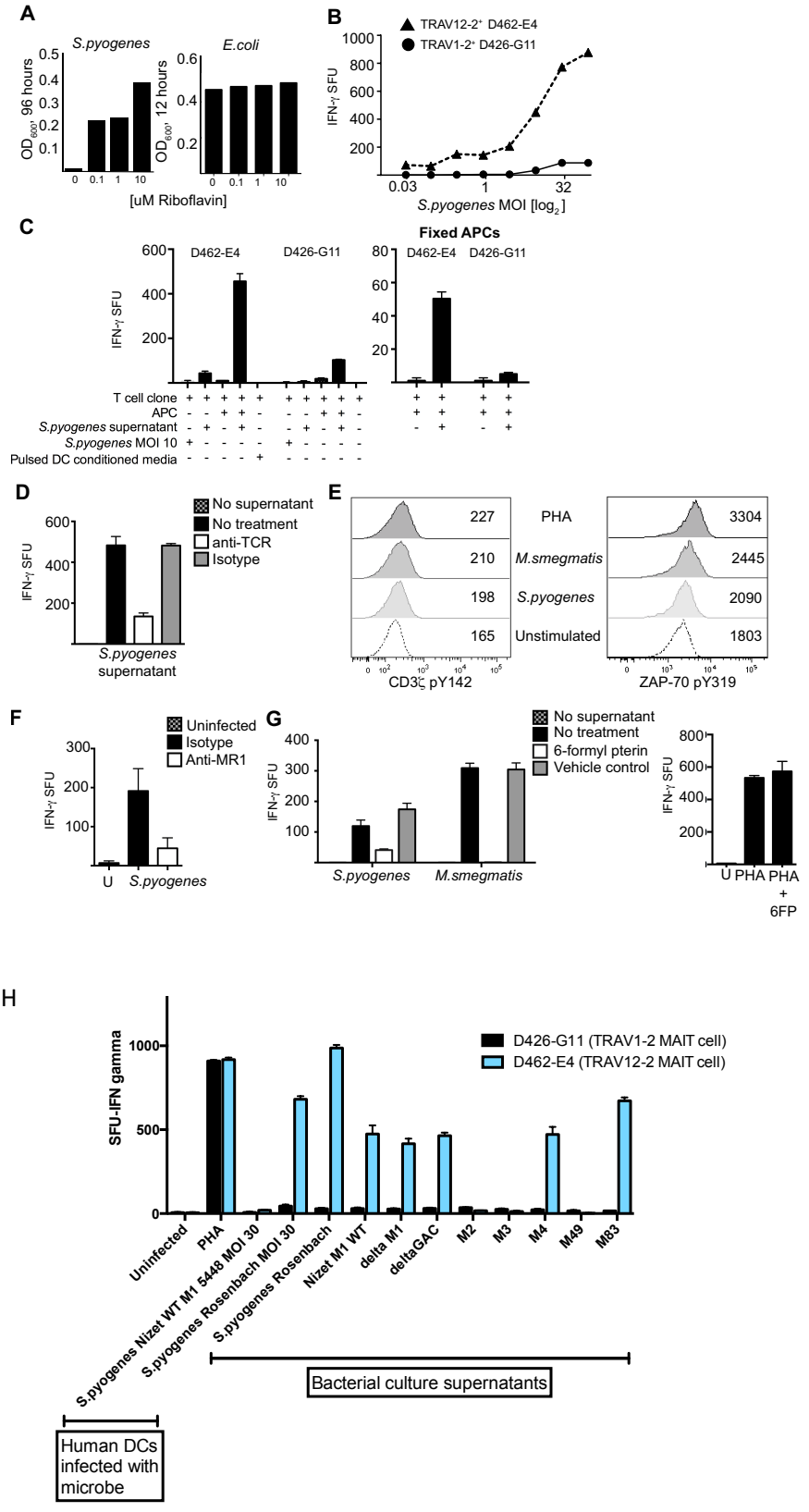

A
S.pyogenes
E.coli
OD600, 96 hours
OD600, 12 hours
[uM Riboflavin]
B
TRAV12-2+ D462-E4
TRAV1-2+ D426-G11
IFN-γ SFU
S.pyogenes MOI [log2]
C
Fixed APCs
D462-E4
D426-G11
IFN-γ SFU
T cell clone
APC
S.pyogenes supernatant
S.pyogenes MOI 10
Pulsed DC conditioned media
D
No supernatant
No treatment
anti-TCR
Isotype
IFN-γ SFU
S.pyogenes supernatant
E
PHA
M.smegmatis
S.pyogenes
Unstimulated
227
210
198
165
3304
2445
2090
1803
CD3ζ pY142
ZAP-70 pY319
F
Uninfected
Isotype
Anti-MR1
IFN-γ SFU
U
S.pyogenes
G
No supernatant
No treatment
6-formyl pterin
Vehicle control
IFN-γ SFU
S.pyogenes
M.smegmatis
U
PHA
PHA + 6FP
H
SFU-IFN gamma
D426-G11 (TRAV1-2 MAIT cell)
D462-E4 (TRAV12-2 MAIT cell)
Uninfected
PHA
S.pyogenes Nizet WT M1 5448 MOI 30
S.pyogenes Rosenbach MOI 30
S.pyogenes Rosenbach
Nizet M1 WT
delta M1
deltaGAC
M2
M3
M4
M49
M83
Bacterial culture supernatants
Human DCs infected with microbe

Figure 3.5 Selective recognition of *Streptococcus pyogenes* by a TRAV1-2 negative T cell clone D462-E4

(A) *S.pyogenes* or *E.coli* was cultured in minimal broth without riboflavin or with a titration of riboflavin over the course of 96 hours (*S.pyogenes*) or 12 hours (*E.coli*) and growth was monitored by optical density readings. (B) Dendritic cells were infected with *S.pyogenes* at a range of MOI (x-axis) and then co-incubated with indicated MR1-restricted T cell clones. (C) T cell clones D462-E4 and D426-G11 were incubated with (left to right) *S. pyogenes* (MOI = 10), *S.pyogenes* culture supernatant (SN) 30ul, unloaded DCs, DCs with *S.pyogenes* SN, or pulsed DC conditioned media overnight. On the right, D462-E4 and D426-G11 were incubated with paraformaldehyde fixed DCs that had been pulsed with *S. pyogenes* SN. IFN- production was quantified by ELISPOT. (D) T cell clone D462-E4 was blocked with anti-pan TCR$\alpha\beta$, isotype control or not treated, and then co-incubated with DCs loaded with *S. pyogenes* supernatant (30ul). IFN- production was quantified by ELISPOT. (E) Phosphorylation of the CD3-ζ chain of the TCR/CD3 complex at tyrosine 142 or ZAP-70 at tyrosine 319 was quantified by flow cytometry after T cell clone D462-E4 was co-incubated with DCs infected with *S. pyogenes or M. smegmatis* MOI = 10, PHA (20 μg/ml) or left untreated (unstimulated condition). Numbers on the overlay indicate the geometric mean fluorescence intensity of at least 30,000 clones. (F) DCs were infected with *S. pyogenes* at MOI = 3, blocked with anti-MR1 or isotype control (10 μg/ml), and then co-incubated with D462-E4 T cell clone. IFN-γ production was quantified by ELISPOT. (G) DCs were either blocked with 6-formyl pterin (50 μg/ml) or 0.01M NaOH vehicle control or nothing, and then loaded with *S. pyogenes or M. smegmatis* supernatant (15 ul) or PHA at 10 μg/ml. The DCs were then used to stimulate T cell clone D462-E4 and IFN-γ production was quantified by ELISPOT. (H) T cell clones D462-E4 (blue) and D426-G11 were incubated with (left to right) uninfected DCs, DCs and PHA, DCs infected with highly invasive *S. pyogenes* strain M1-5448 (gift of Nizet Lab) or ATCC Rosenbach strain, DCs loaded with cell-free bacterial culture supernatant generated from *S. pyogenes* strains and different M protein-serotypes (gifts from Nizet Lab). deltaM1 = *S. pyogenes* without any M-protein, deltaGAC = *S. pyogenes*

that cannot create group A carbohydrates. Error bars represent the SEM of at least duplicates. The experiment in 5H has been performed once while all other assays were performed three times, with similar results. Representative results are shown.

As shown in Figure 3.5D the response to *S. pyogenes* could be blocked using the pan αβ-TCR non-activating antibody but not an isotype control. Furthermore, increased phosphorylation of CD3ζ the TCR-CD3 complex, (the primary intracellular signal transducing subunit (Alberola-Ila et al., 1997)) was observed in D462-E4 following incubation with DCs infected with *M. smegmatis* or *S. pyogenes* (Fig 3.5E). ZAP-70 is a tyrosine kinase that, upon TCR stimulation, is recruited to the TCR-CD3 complex by phosphorylation of the ITAMs of CD3 (Wang et al., 2010). After TCR engagement, the tyrosine residues Y315 and Y319 of ZAP-70 are phosphorylated. In order to provide further evidence of TCR stimulation upon recognition of bacterially infected DCs, we also compared the level of ZAP-70 pY319 between treatments. We also observed increased phosphorylation of this key residue of ZAP-70 in D462-E4 following incubation with DCs (Fig 3.5E). Cumulative results from multiple experiments measuring the intracellular phosphorylation status are summarized in the Appendix Figure 2. To determine whether the recognition of *S. pyogenes* was dependent on MR1, two approaches were employed. First, we tested whether the clone's response to *S. pyogenes* could be blocked with antibody to MR1 (Fig. 3.5F). Recognition was efficiently inhibited by addition of anti-MR1 but not isotype control. Second, we employed the MR1 antagonist, 6-formyl pterin (Fig. 3.5G). The response to *S. pyogenes*, but not mitogen PHA, was also blocked by addition of 6-formyl pterin over the vehicle control. Finally, to test whether D462-E4 could broadly react to *S. pyogenes*, we tested its and a control MAIT cell clone's recognition of different strains and serotypes, that were gifts from Dr. Victor Nizet's Lab (Figure 3.5H). The 5448 "M" serotypes are highly invasive compared to the ATCC Rosenbach strain used in all other experiments. Neither T cell clone could recognize DCs infected with any of these strains and we hypothesize this could have been due to toxicity of the cells. Therefore, we prepared bacteria free-culture supernatant from each strain, co-incubated it with DCs, and tested whether this provided stimulation to D462-E4 (blue) or D426-G11 (black). While the MAIT cell clone did not react to any of the stimuli, D462-E4 responded to some and not others. This suggested to us that either M2, M3, M49 did not contain the necessary

antigen or contained toxic molecules that killed the cells. Further experiments are needed to differentiate between these explanations.

Thus, clone D462-E4 detects both ribityllumazines MAIT antigens and an unidentified streptococcal-derived antigen in an MR1 and TCR-dependent fashion. This unique detection pattern of infection and ligand recognition by TRAV12-2^{+} D462-E4 compared to TRAV1-2^{+} MAIT cells indicates a greater diversity in microbial MR1 ligands.

Tetramer staining of TRAV1-2-negative MR1-restricted T cells

To establish the prevalence of TRAV1-2 negative MR1 restricted T cells across healthy individuals we first stained PBMC with the MR1-Ag (5-OP-RU) tetramer, followed by sequential staining with antibodies to MAIT-associated surface markers (Appendix Fig. 3). As human MAIT cells have been defined as either CD8^{+} or CD8^{-}CD4^{-} T cells (Gold et al., 2010a; Porcelli et al., 1993), we quantified tetramer staining within the CD4-negative population. Frequencies of MR1-Ag tetramer^{+} cells ranged from 0.98% to 4.30% (mean 2.30%, n = 5) of the CD4-negative lymphocytes (Figure 3.6, top row). In line with previously published data (Reantragoon et al., 2013), the majority of MR1-Ag tetramer^{+} cells expressed the TRAV1-2 TCR (Fig. 3.6, second row). However, on average 2.57% of MR1-Ag tetramer^{+} cells did not express TRAV1-2 (red events in Fig. 3.6). Furthermore, these were present in all donors and ranged in frequency from 1.40% to 4.22% of tetramer^{+} cells. TRAV1-2^{+} MAIT cells have been phenotypically characterized as cells with high expression of CD161 (Fergusson et al., 2014; Martin et al., 2009b) and CD26 (Dusseaux et al., 2011; Sharma et al., 2015b). In line with this, the majority of the TRAV1-2^{+} MR1-Ag tetramer^{+} cells co-expressed CD26 and CD161 (mean 96.2%, S.D. 2.5%) (Fig. 6, bottom row). In contrast, a smaller proportion of tetramer^{+}, TRAV1-2 negative cells expressed CD161 and CD26, although there was considerable heterogeneity between donors (mean 60.1%, S.D. 21.6%, range 37.8%-86.2%). To exclude the possibility that tetramer binding masked the staining of TRAV1-2, we depleted

the TRAV1-2^{+} cells by FACS in three of the above donors, and then stained the remaining TRAV1-2 negative cells with the MR1 tetramer. In this case, the estimated TRAV1-2 negative tetramer frequencies were nearly identical to those seen prior to TRAV1-2 depletion (Fig. 3.6 bottom). This experiment verified the frequencies of TRAV1-2- MR1 restricted T cells from each donor. In sum, these data confirm the presence of T cells capable of interacting with MR1-Ag that do not express the TRAV1-2 TCRα in healthy human blood.

Finally, in order to test the generalizability of the antigenic reactivity observed by clone D462-E4, we generated CD8^{+} TRAV1-2 negative MR1 tetramer^{+} T cell lines from the four additional donors used in Figure 3.6. Importantly, these four T cell lines were enriched to a range of 84-97% TRAV1-2 negative of MR1 tetramer^{+} cells by flow cytometry analysis. We observed MR1-restricted IFN-γ reactivity to *M. smegmatis* and *S.pyogenes* infection by each T cell line. Taken together, the confirmation of our finding from four additional PBMC donors supports generalized antigenic reactivity of TRAV1-2 negative MR1-restricted T cells across individuals.

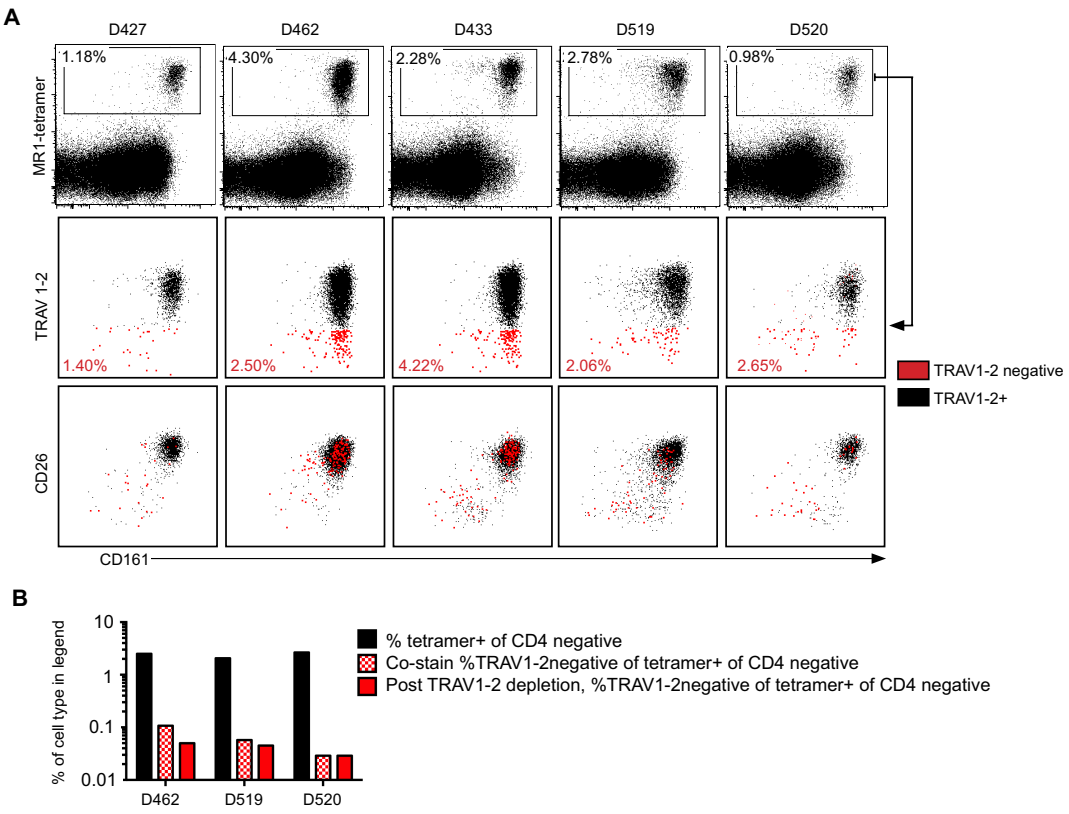


Figure 3.6 MR1-Ag Tetramer$^+$ CD8$^+$ T cells from peripheral blood do not exclusively express TRAV1-2

PBMCs were stained with antibodies to CD3, CD4, CD8, TRAV1-2, CD26, CD161, viability stain, and human MR1-Ag (5-OP-RU) tetramer. All plots are gated on live, CD3+, CD4 negative cells. The x-axis represents CD161 expression, and the y-axis represents MR1-tetramer (top row), TRAV1-2 (middle row), CD26 (bottom row). MR1 tetramer$^+$ cells were gated as indicated by the gate in the top row. MR1 tetramer$^+$ cells were sub-gated based on TRAV1-2 expression where TRAV1-2 negative events and their frequencies are shown in red. The second and third row of plots are overlays of TRAV1-2$^+$ in black and TRAV1-2negative in red. (B) PBMC were depleted of TRAV1-2+ cells by FACS and then stained with MR1-Ag tetramer and T cell lineage markers (red bars). The black bars and red patterned bars indicate frequencies presented in (A) for comparison. The experiment (A) was performed twice with similar results and (B) was performed once. Representative results are shown.

Chapter 3 Discussion

While MAIT cells have been defined by their usage of the TRAV1-2 TCR, in this report we demonstrate unambiguously the presence of MR1-restricted T cells that are TRAV1-2 negative, demonstrate the specific usage of the TRAV12-2 TCR by a clone, and find that these cells are capable of recognizing both the previously demonstrated ribityllumazine riboflavin intermediates, as well as unique ligands derived from *Streptococcus pyogenes*, a bacterium incapable of riboflavin biosynthesis. As a result, our study demonstrates considerable promiscuity in MR1-restricted T cells, at the level of the ability of their TCR to recognize antigens, and in the ability of MR1 to present these ligands.

We find that TRAV12-2 MR1-restricted T cells can be stained with the MR1-Ag tetramer, and have the ability to recognize both known ribityllumazine antigens as well as an antigen derived from *Streptococcus pyogenes*. The observation that the TRAV1-2 MR1-restricted T cell cannot recognize this bacterium provides definitive evidence that the antigen being recognized is distinct. These data, then, would suggest a model in which the MAIT cell TCR confers selectivity, but not stringent specificity. Similarly, our data support the hypothesis that MR1 is capable of presenting an array of ligands. The observation that MR1-restricted T cells of varying TCR usage and antigenic selectivity can be broadly defined by staining with the MR1-Ag (5-OP-RU) tetramer is reminiscent of CD1d-restricted T cells defined by staining with the aGalCer tetramer (Gumperz et al., 2002; Kawano et al., 1997; Lee et al., 2002). In these initial studies, human NKT cells were enumerated using CD1d-αGalCer tetramers and found to stain with anti-Vα24 TCR antibody. More recently, populations of NKT cells expressing alternative semi-invariant TCRs that bind to αGalCer-loaded CD1d tetramers have been identified (Brigl et al., 2006; Gadola et al., 2002). Elucidation of the crystal structure of one of these alternative TCRs to αGalCer-loaded tetramer showed a similar binding mode to that of the Vα24/Jα18 TCR (Lopez-Sagaseta et al., 2012). Taken together, this shared phenomenon between MR1 and CD1d antigen presentation

must be what allows selective activation within the confines of microbial pattern recognition by unconventional T cells.

The use of TRAV12-2 TCR by MR1-restricted T cells necessarily challenges the existing paradigm of how the MAIT cell TCR interacts with MR1. Prior studies have defined the structural and functional requirements of the semi-invariant TRAV1-2 TCR for MAIT cell activation in the context of MR1 and bound ligand (Corbett et al., 2014; Eckle et al., 2014; Lopez-Sagaseta et al., 2013b; Patel et al., 2013; Reantragoon et al., 2012). These studies define a clear role for the CDR3α and possibly the CDR3β loop in ligand recognition. Specifically these studies suggested a conservation of key patterns of TCR residues in the TCR α-chain including the conserved amino acid, Tyrα95(Adams and Luoma, 2013; Eckle et al., 2014; Lopez-Sagaseta et al., 2013b; Patel et al., 2013; Reantragoon et al., 2012; Rossjohn et al., 2014).The critical tyrosine at position 95 in the CDR3 of the TCRα chain allows for the formation of a hydrogen bond with MAIT activating but not non-activating ligands. For example, MR1 binding of the RL-6-Me-7-OH ligand, that is selectively recognized by D462-E4, allows for a single TRAV1-2^{+} TCR contact with TRAV1-2: Tyr95 of the CDR3α loop (Patel et al., 2013). While this residue is highly conserved between MAIT TCRs (Greenaway et al., 2013; Tilloy et al., 1999), sequence analysis of TRAV12-2^{+} D462-E4 demonstrates this clone lacks a tyrosine residue in its CDR3α region. We note that we have previously reported that a proportion of microbial-reactive, TRAV1-2 expressing MR1-restricted T cells do not contain the Tyrα95 (Gold et al., 2014). A recent study by Gherardin et al., observed that a TRAV1-2 negative MR1-restricted TCR (TRAV36/TRAJ34) could instead use an asparagine residue of its CDR1a to contact 5-OP-RU activating ligand(Gherardin et al., 2016). This elegant study highlights alternative molecular interactions can mediate atypical TCR recognition of MR1-Ag. In the future we would like to elucidate the crystal structure of the TRAV12-2 MR1-restricted TCR in a ternary structure with antigens derived from both *M. smegmatis* and *S. pyogenes* to determine the critical residues mediating the

binding. However, at present, we cannot comment on the critical residues that mediate the D462-E4 TCR interaction with MR1-ligand.

Our data clearly supports the hypothesis that MR1 can present a diverse array of ligands to MR1-restricted T cells. First, by comparing microbial recognition of the TRAV1-2 and TRAV12-2 TCR we have defined patterns of recognition that would imply the presence of more than one activating ligand. For instance, the ability of TRAV1-2 expressing T cells to uniquely recognize a microbe would suggest the presence of a ligand not recognized by TRAV12-2 TCR. Similarly, those microbes that are preferentially recognized by the TRAV1-2 T cells likely contain either a single ligand that is recognized preferentially analogous to our findings of ligand discrimination between ribityllumazines in Fig. 4B, or contain multiple ligands. Most striking, however, was the ability of the TRAV12-2^{+} MR1-restricted T cell to recognize *Streptococcus pyogenes*, an organism that cannot synthesize riboflavin. Because this pathogen is not recognized by the TRAV1-2+ T cells, these data would refute the hypothesis that differential MAIT cell recognition can be simply explained by differing proportions of riboflavin metabolites. At present, the MR1 ligand from *Streptococcus pyogenes* remains to be determined. Recent molecular analyses suggest that MR1 can accommodate a range of different ligands due to plasticity in ligand orientation of the binding cavity (Corbett et al., 2014; Eckle et al., 2014; Lopez-Sagaseta et al., 2013b; Patel et al., 2013; Reantragoon et al., 2012; Rossjohn et al., 2014). As the pterin ring occurs commonly in the environment, it is feasible that other microbial or host molecules with common chemotypic properties could bind to MR1, and function as antigens for MR1-restricted T cells. We hypothesize that diversity in MR1 ligands allows MR1-restricted T cells to recognize a wide range of microorganisms and their associated metabolomes.

In the future, we would first like to determine characteristics of the MR1 ligands in *S. pyogenes*. To do this, we would test whether antigenic activity remains upon filtering bacterial lysate through a series of molecular weight gradients. Secondly, we could treat the lysate with proteases, lipases, or

enzymes that cleave sugar to determine whether it is proteinaceous, lipid based, or sugar based, respectively. Both of these techniques assume that *S. pyogenes* MR1 ligands are uniform so therefore, it will be important to use a more general procedure as well. To determine the general chemical nature of MR1 ligands derived from *S. pyogenes*, we propose to load recombinant MR1 in the presence of *S. pyogenes* lysate. Then we will elute the MR1-ligands and use a combination of liquid chromatography and mass spectrometry (LCMS) techniques to further purify and characterize the different chemotypes. To control for endogenous or artificial ligands, we will run the same experiment in parallel with recombinant MR1 without bacterial lysate.

In contrast to conventional T cell populations, MAIT cells can be found in the thymus with effector pathogen-reactive capability (Gold et al., 2013) and their selection depends on hematopoietic rather than epithelial cells (Martin et al., 2009b; Seach et al., 2013; Treiner et al., 2005). It is unknown whether innate T cell function is a T cell intrinsic program or is a result of TCR signaling through selection in the thymus by MR1. Both functional data, and tetramer staining demonstrates the presence of MR1-restricted T cells in all donors. Because we do not have a TRAV12-2 antibody, the full TCR repertoire of these cells remains to be determined as well as whether they share the innate T cell attributes and selection pathway of TRAV1-2^{+} MR1-restricted T cells. In the future, to determine the repertoire of TRAV1-2 negative MR1-restricted T cells, we would utilize the MR1 tetramer (Fig. 3.6) to isolate and analyze this population of interest. At present, the MR1/5-OP-RU tetramer has been described by others to label all TRAV1-2^{+} MR1T cells, and at least a subset of the TRAV1-2 negative MR1-restricted cells. I would employ this reagent both in *ex vivo* TCR sequencing and in the generation of TRAV1-2 negative MR1 T cell clones. TRAV1-2 negative MR1/5-OP-RU tetramer+ cells would be sorted by FACS from PBMC donors. A fraction of these cells could be used for unbiased TCR alpha chain sequencing to see how diverse or restricted the repertoire is. The rest of the cells could be used to generate T cell clones which are vital for answering questions about TCR pairing and antigenic reactivity as it relates to a specific TCR.

We also note the preponderance of MR1-restricted T cells expressing the TRAV1-2 TCR, in line with prior observations (Reantragoon et al., 2013). This phenomenon could occur by a scenario where TRAV1-2$^+$ T cell selection in the thymus is favored over other TCRs. Alternatively, given evidence of ligand discrimination by MAIT TCRs, TRAV1-2$^+$ MAIT cells could dominate in the periphery due to selective microbial exposures. In line with this hypothesis, perhaps repeated exposures of environmental mycobacteria or gram-negative gut microbiota allow for preferential expansion of TRAV1-2$^+$ MR1 restricted T cells in the majority of individuals. MR1-restricted T cells might display a different TCR repertoire in different tissues based on selective trafficking, or clonal expansion, to detect the microbes their TCRs can specifically recognize.

Based on our findings, we propose that non-TRAV1-2 MR1-restricted TCRs contribute to immune defense against infection primarily by providing more diverse, and in some instances unique, microbial recognition. For instance, the TRAV12-2$^+$ MR1-restricted T cell clone can recognize infection with *Streptococcus pyogenes*. A variety of diseases are caused by infection by *Streptococcus pyogenes* or Group A streptococcus (Walker et al., 2014). These include the throat infection "strep throat", pneumonia, fasciitis, nosocomial wound infection, and glomerulonephritis. I hypothesize that MR1-restricted T cells expressing TRAV12-2$^+$ or other atypical TCRs selectively expand at tissue sites, such as the human mouth, tonsils, and skin, associated with streptococcal infection. In the future, I would propose to analyze the TCR repertoire used by MR1-restricted T cells at mucosal/lymphoid organs like the tonsils. Our prior finding of microbe selective clonal MR1-restricted T cell expansions within individuals (Gold et al., 2014), in conjunction with the data presented herein demonstrates the capacity of MR1-restricted T cells to discriminate between microbial infections, and supports the hypothesis that MAIT cells display antigen-driven clonal expansion.

In sum, we show that MR1-restricted T cells have the capacity to detect a greater diversity of microbes than previously shown. We have isolated a human T cell clone that expresses a TCR never

observed within MAIT cells before, TRAV12-2, demonstrating that MR1-restricted T cells do not use TRAV1-2 exclusively. This TRAV12-2 TCR displays MR1-Ag discrimination both with regard to the recognition of known ribityllumazine metabolites, and most notably in its capacity to uniquely detect *Streptococcus pyogenes*, a pathogen that lacks the capacity to synthesize riboflavin. Collectively, these data provide evidence that additional MAIT cell subsets may play a unique role in human defense against infection by broadening the recognition of microbes and their associated metabolites.

Chapter 3 Materials and Methods

Human subjects.

All samples were collected and all experiments were conducted under protocols approved by the institutional review board at Oregon Health and Science University. PBMCs were obtained by apheresis from healthy adult donors with informed consent.

Cell lines and reagents.

All cell lines used in this study have been confirmed to be mycoplasma-free. The A549 lung carcinoma cell line (ATCC CCL-185) was used as antigen-presenting cells for IFN-γ ELISPOT assays in Figure 1 and Figure 2, direct *ex vivo* ICS determination of microbe-reactive T cells, and in Figure 4C for infection with *Neisseria gonorrhoeae* and *Yersinia enterolitica*. The BEAS-2B bronchial epithelial cell line was used (ATCC CRL-9609) in Figure 3 for antibody blockade ELISPOT assays. Cell lines were maintained by continuous passage in F12K culture medium supplemented with 10% FBS. Both cell lines were confirmed to be mycoplasma free. Ribityllumazine (RL)-6,7-diMe and RL-6-Me-7-OH were purchased from WuXi Apptec and 6-formyl pterin (6FP) from Schick Laboratories. Live-dead aqua stain and CFSE were purchased from Life technologies. Unconjugated antibodies used in the study were the following: anti (α)-CD3 (clone OKT3), αβTCR (clone T10B9, BD), αMR1 (26.5, gift of Ted Hansen), αHLA-ABC (W6-32, AbD Serotec), αCD1a/CD1b/CD1c/CD1d (gift of Branch Moody), αIFN-γ for ELISPOT (Mabtech, see ELISPOT methods section below) and LEAF purified IgG2a, IgG1, and IgM isotype controls (Biolegend). Conjugated antibodies used in this study were the following: αCD3 (UCHT1, FITC or PerCP Cy5.5, 1:50 dilution, Biolegend, BL), αTRBV29-1 FITC (1:10 dilution, Beckman Coulter), αCD8α (APC-Cy7, 1:500 dilution, clone RPA-T8, BL), αCD26 (FITC, 1:50 dilution, clone BA5b, BL), αCD161 (PE-Cy7, 1:50 dilution, HP-3G10, BL), αCD4 (Brilliant violet 785, 1:50 dilution, OKT4, BL), αIFN-g FITC (1:25 dilution, Beckman Coulter), αTCR αβ (PE, 1:50 dilution, clone T10B9, BD), and IgG2a isotype conjugated to FITC (BD),

αTRAV1-2 (APC, 1:50 dilution, OF5A12)(Gold et al., 2013), αCD247 pY142 (PE, 1:10 dilution, BD Phosflow), αZAP-70 pY319 / Syk pY352 (PE, 1:10 dilution, BD Phosflow) and αTCR (FITC, 1:25 dilution, clone B1, BD). 5-OP-RU: 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil.

Monocyte-derived dendritic cells.

PBMC obtained by apheresis were resuspended in 2% human serum (HS) in RPMI and allowed to adhere to a T-75 flask at 37C for 1 h. On average, 30 million PBMC yielded 5 million monocyte derived DCs. After gentle washing twice with PBS, nonadherent cells were removed and 10% HS in RPMI containing 30 ng/ml of IL-4 (Immunex) and 30 ng/ml of GM-CSF (Immunex) was added to the adherent cells. The cells were x-rayed with 3000 cGray using X-RAD320 (Precision X-Ray Inc.) to prevent cell division. After 5 days, cells were harvested with cell-dissociation medium (Sigma-Aldrich) and used as APCs in assays.

Generation of an MR1 knockout cell line using CRISPR Cas9

The reagents used to mutate the MR1 gene were derived from the toolkit described by (Mali et al., 2013). A codon-optimized synthetic Cas9 cDNA under the control of the CMV promoter (Addgene plasmid #41815) was used in combination with a single guide RNA (sgRNA) comprising a transactivating CRISPR RNA sequence (Mali et al., 2013) as well as the 19-nucleotide protospacer sequence (GATGGGATCCGAAACGCCC) targeting the + strand of exon 3 of the MR1 gene followed by the protospacer associated motif (PAM) AGG. Plasmid DNA serving as template for the transcription of the CRISPR/Cas9 elements were transfected in the carcinomic human alveolar basal epithelial cell line A549 using Lipofectamine 2000 (Invitrogen, Life Technologies, Paisley, UK) according to the manufacturer's instructions. Genomic DNA from A549 cells was isolated with the GenELute mammalian genomic DNA miniprep kit (Sigma-Aldrich, Gillingham, UK). Mutations at the target site were detected using theCEL-I enzyme, as part of the SURVEYOR assay (Transgenomic Ltd, Glasgow, UK), which cleaves DNA duplexes

bearing base pair mismatches, caused by insertions or deletions at proximity of the PAM sequence, within the PCR amplicons generated with primers flanking the genomic target site. The PCR forward primer (5'-GCATGTGTTTGTGTGCCTGT-3') is located in the intron region upstream of the target site and the reverse primer (5'-GGTGCAATTCAGCATCCGC-3') downstream on exon 3. MR1 protein expression at the cell surface was measured by flow cytometry with the anti-MR1 antibody clone 26.5 (a kind gift of Prof Ted Hansen) following overnight stabilization by incubating cells with 50 µg/ml acetyl-6-formylpterin (Schirks Laboratories, Jona, Switzerland). MR1 negative cells were sorted by flow cytometry and single cell clones were derived from the sorted bulk population by limiting dilution (average of 0.3 cells per well). Clonal populations were then screened for MR1 surface expression and DNA indels with the SURVEYOR assay. The region flanking the target site was PCR amplified from the genomic DNA of selected clones using the primers described above fused to restriction sites and the PCR products were cloned into recipient plasmids, which were transfected in chemically competent Top10 *E.coli* bacteria. 10 colonies tested positive for the insert by colony PCR were used to produce plasmid Minipreps, which were sent for Sanger sequencing in order to determine the nature of CRISPR/Cas9 induced mutations. Clone 9 (in the manuscript as MR1-/-) has a 125 bp deletion on one allele and a single bp deletion on the other.

Isolation of TRAV1-2 negative T cell lines and clones

CD8+, CD4-, γδ TCR-, TRAV1-2 negative T cells were FACS sorted from PBMCs, and $1X10^6$ were added to $1X10^5$ irradiated (3500 rad using a Cs source) monocyte-derived autologous dendritic cells infected with *M. smegmatis* (MOI 3) and incubated in RPMI 1640 with 10% human serum, rhIL-2 (2 ng/ml), and rhIL-12 (0.5ng/ml). Seven days later, T cells were removed and stained with CFSE, and then added to $1X10^5$ irradiated (3500 rad) monocyte-derived autologous macrophages infected with *M. smegmatis* (MOI 3) and incubated with rhIL-2 (2 ng/ml) for another week. The resulting T cell line was FACS sorted for CFSE-

diluted cells. The sorted T cells were rested overnight in RPMI 1640 supplemented with 10% human serum, and 0.5ng/ml rhIL-2. The CFSE-sorted T cell line was rapidly expanded using anti-CD3 following the protocol written below, then FACS-purified based on its ability to bind MR1-tetramer loaded with 5-OP-RU(Corbett et al., 2014), rested overnight with rhIL-2 (0.5 ng/ml), and rapidly expanded using anti-CD3 again to generate "D462-E4" T cell clone.

Expansion of T Cell Clones

T cell clones were cultured in the presence of x-rayed (3000 cGray using X-RAD320, Precision X-Ray Inc.) allogeneic PBMCs, x-rayed allogeneic LCL (6000 cGray), and anti-CD3 mAb (20 ng/ml; Orthoclone OKT3, eBioscience) in RPMI 1640 media with 10% human serum in a T-25 upright flask in a total volume of 30 ml. The cultures were supplemented with IL-2 on days 1, 4, 7, and 10 of culture. The cell cultures were washed on day 5 to remove soluble anti-CD3 mAb.

Phosphorylation specific T cell staining for flow cytometry

T cell clones were incubated overnight in RPMI media containing 0.5 ng/ml IL-2 and 2% Human serum. Monocyte-derived dendritic cells were incubated overnight with *S.pyogenes,* or *M. smegmatis* MOI = 10, or nothing, in ultra low adherence culture plates. A three to one ratio of DCs to T cells was co-incubated for 15 minutes at 37°C and then immediately fixed in 2% paraformaldehyde. Following fixation, the cells were permeabilized in ice cold 100% methanol for 30 minutes. Then the cells were washed in FACS buffer to sufficiently remove the methanol and stained with the following antibodies, anti-CD8 (clone RPA-T8, 1:50 dilution, Biolegend), anti-CD3z-pY142 (1:10 dilution, BD), anti-ZAP-70 pY319/Syk pY352 (1:10 dilution BD), or isotype controls (IgG2a for anti-CD3z, IgG1 for anti-ZAP-70, used at a matching concentration to their corresponding phospho-specific antibody), for 45 minutes at 4°C. Isotype controls were used to optimize staining with phospho-specific antibodies. I use the mitogen PHA as a positive

control for TCR stimulation and maximum phosphorylation signal. A minimum of 30,000 $CD8^+$ events were collected for MFI analysis across samples.

Analysis of TCR usage

Amplification and sequencing of TCRB and TCRAD CDR3 regions was performed using the immunoSEQ Platform (Adaptive Biotechnologies™, Seattle, WA). ImmunoSEQ combines multiplex PCR with high throughput sequencing and a bioinformatics pipeline for [TCRB/TCRAD] CDR3 region analysis (Carlson et al., 2013; Robins et al., 2009). The IMTG nomenclature was used throughout the study(Lefranc, 2003).

Flow cytometry staining, sorting, and tetramer staining

Cells (100,000 T cell clones or 1.5 million PBMC) to be analyzed for cell surface marker expression were first incubated at 4°C in a blocking solution of PBS containing 2% normal rabbit serum (Sigma-Aldrich), 2% normal goat serum (Sigma-Aldrich), and 2% human serum to prevent nonspecific binding. Cells were washed in PBS and then incubated with Live/Dead discriminator and surface stains or isotype controls for 20 minutes in the dark at 4°C in a total volume of 50 ul. Cells were then washed and fixed, or fixed and permeabilized (*ex vivo* ICS tests, BD Fix/Perm kit), according to the manufacturer's instructions. Antibodies for intracellular staining were then added for 30 minutes in the dark at 4°C in a total volume of 50ul, and after washing, flow cytometry was performed. Specifically for Figure 3A, $2x10^6$ PBMC from each donor were stained with the MR1-Ag tetramer at 0.3nM in 25ul volume for 45 minutes in PBS buffer containing 2% FBS at room temperature in the dark. Viability and surface stains were added on top of the tetramer stain for another 20 minutes at 4°C. Samples were then washed twice in tetramer staining buffer. All flow cytometry was performed on a Fortessa 18-parameter flow cytometer (BD). All fluorescence activated cell sorting (FACS) was performed using an Influx 11-parameter flow cytometer (BD) by the OHSU flow cytometry core facility. Data were analyzed using FlowJo (v9.8.5). Fluorescence

minus one (FMO) controls were used for optimal gating. Doublets were excluded based on FSC-H and FSC-A, SSC-H and SSC-A, lymphocytes were identified based on FSC-A and SSC-A and CD3 expression, and dead cells were excluded based on Aqua viability dye.

***Ex vivo* Stimulation assay**

To observe the nonclassical T cell response, I used the A549 cell line as APCs because it does not express MHCII and is MHC-I-mismatched to the donor source of T cells. CD8+ T cells were positively selected from healthy donor PBMCs using magnetic bead separation according to the manufacturer's instructions (Miltenyi), added to uninfected WT or *M. smegmatis*-infected (MOI = 3, overnight infection) WT or MR1-/- A549 cells at a ratio of 3:1, and incubated overnight in the presence of Brefeldin A, and 0.5ng/ml rhIL-2 at 37°C. The following day, the cells were stained for surface phenotype markers and Live/Dead discriminator. Following surface staining, cells were fixed and permeabilized using the BD Fix/Perm Kit and stained intracellularly with α-IFN-γ.

Microorganisms and preparation of antigen presenting cells

M. smegmatis, C.albicans, S.enterica Typhimurium, E.coli, M.tuberculosis, S.pyogenes and *M.avium* were used from frozen glycerol stocks, whereas all other microbes were harvested from overnight growth on agar plates and titered based on OD_{600} readings of a colony suspension. A cell free supernatant was created from an overnight culture of *S.pyogenes* (ATCC 19615) that was sterile filtered and frozen prior to being used in T cell stimulation assays of Fig. 5 C,D,E,F. A549 cells were infected for 2 hours or dendritic cells were infected for 1 hour with microbes at 37C. In figure 4C, A549 cells were used for Yersinia and Shigella infections; dendritic cells were used for all other infections. The MOI and antibiotics used for each microbe was optimized for APC viability and maximal MR1-restricted response: *E.coli* 1, *M. smegmatis* 3, *Shigella flexneri* 1, *Yersinia enterolitica* 1, *C.albicans* 0.1, *M.avium* 30, *Nocardia*

asteroides 1, *Salmonella enterica* Typhimurium 30, *Vibrio parahemolitica* 1, *M.tuberculosis* 30, *N.gonorrhoeae* 1, *P.aeruginosa* 1, *S.aureus* 1, *S.pyogenes* 30, *Enterococcus faecalis* 1 and 10. All infections were performed in the absence of antibiotics. After the indicated infection time, all cells were washed twice in media containing antibiotics and then incubated overnight in an ultra low adherence tissue culture plate before being counted and added to the assay (ELISPOT set-up described below).

Riboflavin dependence growth assay

$5X10^6$ CFU from frozen glycerol stocks of *S.pyogenes* or *E.coli* were added to 10 mL of minimal growth media and cultured at 37°C for 96 hours (*S.pyogenes*) or 12 hours (*E.coli*) in the dark. Minimal growth media was made with M9 salts (BD Difco) supplemented with glucose, $MgSO_4$, $CaCl_2$, as recommended by the manufacturer, and 0.01% w/v amino acids (casein enzymatic digest, Sigma). Riboflavin (Sigma) was added to the growth medium at uM concentrations indicated in Fig. 5. Bacterial growth was measured by absorbance readings at 600 nm.

IFN-γ ELISPOT assay and antibody blocking

A MSHA S4510 96-well nitrocellulose-backed plates (Millipore, bought via Fisher Scientific) was coated overnight at 4°C with 10 μg/ml solution of anti-IFN-γ monoclonal antibody (Mabtech clone 1-D1K) in a buffer solution of 0.1M Na_2CO_3, 0.1M $NaHCO_3$, pH = 9.6). Then the plate was washed three times with sterile PBS and blocked for 1 hour at room temperature with RPMI 1640 media containing 10% heat inactivated human serum pool. Then the antigen presenting cells and T cells were prepared as described below and co-incubated overnight. Briefly, dendritic cells (Fig. 4A, D, Fig. 5), the BEAS2B cell line (Fig. 3D), or the A549 cell line (all other experiments), were used as APCs at $1X10^4$ per well in ELISPOT assays. For all blocking ELISPOT assays, APCs were limited to $5X10^3$ per well. In Figure 4B, C, the A549 cell line was incubated with MAIT antigens over a range of concentrations in the dark for 2 hours. Where stated,

blocking antibodies or antagonists were added for 2 h at 2.5 μg/ml (α-HLA-I clone W6/32, α-CD1a, b, c, d (Branch Moody), 6-formyl pterin (50 μg/ml, Schick Laboratories), and α-MR1 clone 26.5 (Ted Hansen) or appropriate isotype controls). To block the T cell receptor, anti-αβTCR (clone T10B9(Waid et al., 2009), BD Pharmingen), or isotype control, was added to T cell clones at 0.5 μg/ml for 30 minutes at 4°C prior to co-incubation with APCs, T cell clones were added at $1X10^4$ /well. The plate was incubated overnight at 37°C and then washed six times with PBS containing 0.05% tween. The plate was then incubated for two hours at room temperature with a 1 μg/ml solution of anti-IFN-γ-biotin secondary antibody (Mabtech clone 7-B6-1) in 0.5% BSA, 0.05% Tween in PBS. Finally, the plate was washed six times in PBS-tween, then PBS, and developed using an AEC Vectastain kit SK-4200 (Vector labs). I defined a positive response as greater than 25 IFN-γ spot forming units.

Preparation of pulsed fixed antigen presenting cells

Monocyte derived dendritic cells were pulsed for four hours with bacterial culture supernatant from *S.pyogenes* or left untreated and then washed and rested overnight in an ultra-low adherence tissue culture plate. The following day, the pulsed DC conditioned media (Fig. 5C) was collected and added to D462-E4 clone in the ELISPOT plate. Then half of each harvested DC sample was used in the ELISPOT as "unfixed". The other half was fixed by incubating in 0.5% paraformaldehyde (Electron Microscopy Sciences) in PBS for 15 minutes at room temperature. Then, an equal volume of 0.4M Lysine was added to stop the reaction for 5 minutes. The samples were then extensively washed with media, incubated for 1 hour at 37°C, and then washed again before being used in the ELISPOT assay.

Data Analysis

Flow cytometry data were analyzed using FlowJo software 9.8.1 (Tree Star). All statistical analyses were performed using Prism software with nonparametric tests, the Mann-Whitney U test (Fig. 2C).

Nonparametric statistical tests were used for small group sizes (5 donors in this study). In all descriptive statistical analysis, the variance was first confirmed to be similar between groups using standard deviation or standard error of the mean tests, as appropriate and displayed on each graph. p values ≤ 0.05 were considered significant (*$p \leq 0.05$).

Chapter 4: Functional Paralysis of Human T Cells by *Orthopoxvirus* B22R Family Proteins Targets Medial TCR Signal Transduction

Abstract

Orthpoxviridae (OPXV) is a genus of large double-stranded DNA viruses that include the human pathogenic species: Variola, Monkeypox, and Cowpox. T cell mediated immunity is an integral part of the detection and destruction of host cells harboring viruses. However, the relative role of T cell mediated immunity in the immune response to pathogenic OPXV seems to be less important, as it is not required for protection in multiple models. However, Vaccinia is an attenuated virus derived from Cowpox, that was widely used as a vaccine for Smallpox, and currently as a viral vector to elicit T cell responses. This paradox suggests that uniquely, the pathogenic OPXV, but not Vaccinia, species might use immune evasion genes to directly subvert T cell immunity. Here, we use *in vitro* and *in vivo* studies to show that B22R protein and its conserved orthologues is necessary and sufficient to directly cause T cell broad functional paralysis when expressed on the membrane of its host cell. This mechanism does not involve MHC downregulation of the infected APC but instead acted *in trans*, directly on the T cell. Using phospho-flow cytometry, I show that B22R-paralyzed T cells can respond to cytokines, and transmit signals through the TCR, but were unable to flux Ca^{2+} ions upon stimulation. These data reveal that B22R family proteins act through a seemingly novel mechanism proximal to the TCR to directly evade local antiviral T cell responses.

The data presented in Figures 4.1, 4.2, and 4.3 have been published in:

Alzhanova, D., Hammarlund, E., Reed, J., Meermeier, E., Rawlings, S., Ray, C.A., Edwards, D.M., Bimber, B., Legasse, A., Planer, S., et al. (2014). T cell inactivation by poxviral B22 family proteins increases viral virulence. PLoS pathogens 10, e1004123.

The rest of the data is unique to this thesis chapter.

Effort Statement

The work contained in the chapter has been a collaborative project between our lab and Klaus Fruh's Lab. Erin Meermeier performed all of the experiments contained in this chapter with the following exceptions. Dina Alzhanova created the viral protein expression vectors. The Fruh lab performed the *in vivo* rhesus macaque experiment in Figure 4.3 of this chapter.

Chapter 4 Introduction

Orthpoxviridae (OPXV) is a genus of large double-stranded DNA viruses that include pathogenic species like Variola, Monkeypox (MPXV), Cowpox, and Vaccinia, the virus used for vaccination against smallpox (Moss and Shisler, 2001). Variola virus, the causative agent of smallpox, is historically one of the most virulent pathogens known. Although smallpox was eradicated 40 years ago, it is still not clear why mortality rates were so high and what constituted an effective host response against infection in the cases where patients recovered.

Immune response to OPXV

OPXV are zoonotic pathogens that spread through air and direct contact to infect humans. They infect many cell types including epithelial cells, where they disseminate through the infected host by cell-associated viremia and replicate cytosolically (Cho and Wenner, 1973; Downie et al., 1953). Within the genus of OPXV, the viruses are highly conserved (Lefkowitz et al., 2006). This translates to a high degree of shared antigens, which form the basis of cross-protective immunity and thus, the success of using the avirulent Vaccinia virus to immunize for Variola virus (Kennedy et al., 2009).

The adaptive immune system, and $CD4^+$ and $CD8^+$ T cell mediated immunity in particular, are essential for defense against viral infections. OPXV take haven in the cytosol of many cell types that may not have innate mechanisms to control viral infection intrinsically. Therefore, elimination of the reservoir of the infection relies upon T cell-mediated recognition and lysis of the infected cell. Detection of viral-derived peptide-MHC-class I complexes triggers $CD8^+$ T cells to lyse infected cells. Antibodies also play a role in immunity to OPXV infection, presumably in detection of free virions as they spread from cell to cell, in antigen presentation to $CD4^+$ T cells, and in complement activation, (Panchanathan et al., 2006).

However, the specific roles for T and B cells in the response to and in protection from virulent OPXV infection suggest that OPXV have evolved mechanisms to evade T cell immunity. Because smallpox was eradicated in 1977, we rely upon historical studies of the immune response to the avirulent vaccine strain of OPXV, Vaccinia (Kennedy et al., 2009), or newly described animal models, such as Monkeypox infection of rhesus macaques (Cann et al., 2013; Tree et al., 2015). Vaccinia vaccination in animals and humans induces strong humoral and $CD4^+$ and $CD8^+$ T cell responses (Amanna et al., 2006). Vaccinia-induced antibodies are necessary and sufficient for protection against Monkeypox and Mousepox model infections, despite the presence of OPXV-specific T cells (Edghill-Smith et al., 2005; Fogg et al., 2004). In contrast, T cells promote survival of vaccinated mice challenged with lethal doses of Vaccinia. For example, mice can be immunized with an immunodominant MHC-class I-binding peptide from Vaccinia, which then protects the majority of vaccinated mice from a subsequent lethal dose of Vaccinia; in contrast unvaccinated mice which were not protected (Snyder et al., 2004). Moreover, Vaccinia is widely used as T cell-inducing vaccine vector. Thus there seems to be a paradox where the T cell-mediated response should be critical for the clearance of OPXV-infected cells, while it's been observed that they are not necessary for protection. This leads to the hypothesis that certain T cell evasion mechanisms must play a role in virulent OPXV but not avirulent OPXV infections.

OPXV Immune Evasion

The outcome of an infection is determined by a complex interaction between the type of immune response mounted by the host and by evasion mechanisms that the virus has evolved to subvert it. OPXV are known to use a myriad of mechanisms to evade the host immune response. Specifically, OPXV have evolved unique and redundant mechanisms to evade T cell mediated immunity. Published studies show that these mechanisms can impact the ability of a T cell to proliferate, by secretion of suppressive cytokines and blocking inflammatory cytokines and chemokines. OPXV also

directly limit T cell function by increasing pro-apoptotic factors and downregulation of antigen presentation. The plethora of evasion proteins are reviewed in (Bidgood and Mercer, 2015; Seet et al., 2003). For example, evasion of antigen presentation by gene products conserved in Cowpox and Variola occurs via inhibition of TAP and trafficking of MHC class I (Alzhanova et al., 2009; Alzhanova and Fruh, 2010). However, these proteins responsible for inhibition of MHC class I presentation are not conserved in MPXV. Thus, mechanisms underlying MPXV's T cell evasion strategies are less defined.

The first evidence of direct T cell inactivation by OPXV family member MPXV was reported by Dr. Slifka and colleagues were analyzing the first U.S. outbreak of MPXV in 2003. Using blood samples from individuals who recovered from the disease, they discovered that $CD4^+$ and $CD8^+$ T cells from MPV-immune individuals could not respond to MPXV-infected antigen-presenting cells (APC) in *in vitro* experiments, yet they maintained a normal response to Vaccinia-infected cells (Hammarlund et al., 2008). Moreover, virus infected cells maintained normal levels of MHC class II and I expression, indicating that the phenotype was not caused by blocking antigen presentation. Also *in vitro* co-infection experiments showed that MPXV co-infection abrogated specific T cell responses to Vaccinia and Epstein-Barr Virus, suggesting a dominant effect on immune evasion. Active viral expression of an early MPXV gene product was also required for immune evasion. Lastly, Hammarlund and colleagues found that this mechanism did not involve a secreted factor because the evasive phenotype still occurred in the presence of Brefeldin A and the active factor was not found in supernatant of the infected cell. Together, this study observed a novel cell associated mechanism used by OPXV to evade T cell antiviral immunity.

In 2014, we demonstrated that the highly conserved B22R family proteins, encoded by the largest genes of Variola, Monkeypox and Cowpox, render T cells unresponsive to stimulation of the T cell receptor by MHC-dependent antigen presentation or by MHC-independent stimulation (Alzhanova et al., 2014). In contrast, stimuli that bypass TCR-signaling are not inhibited. The gene names of these proteins

are *VARVB22R, MPXV197,* and *CPXV219,* respectively. These proteins are expressed at the surface of the infected cell as an early gene product. MPXV-197 is the largest ORF in the genome of MPXV encoding for 1880 amino-acids (http://www.poxvirus.org/). The protein is predicted to contain a cleavable N-terminal signal peptide (Petersen et al.), multiple N-glycosylation sites, a C-terminal transmembrane (TM) domain, and potentially one or more internal TM domains (Stoffel and Hofmann, 1993).

In the current study I investigated the mechanism of the novel B22R family of proteins in direct functional paralysis of human T cells and their contribution to evasion of the adaptive immune response. This was a collaborative effort between myself and Dina Alzhanova of the Fruh Lab (VGTI, OHSU). I undertook an approach that analyzed the intracellular environment of the paralyzed T cell which is described in this chapter; while the Fruh Lab focused on biochemical protein characteristics and interactions. Investigation of B22R proteins in the context of OPXV infection is key to understanding the pathology of the virus and may give us insight into intracellular regulatory mechanisms of T cell biology.

Chapter 4 Results

To study the characteristics of B22R family putative immune evasion proteins, we needed a way to express them in the absence of other confounding evasion proteins. Therefore, we expressed *VARV B22R, CPXV219,* or *MPXV197* ectopically from an attenuated adenoviral vector in the BEAS-2B human bronchial epithelial cell line. Each protein was designed to also have a C-terminal FLAG tag, to aide in detection of the protein using anti-FLAG antibodies.

The surface expression of MPXV-197-FLAG in BEAS-2B cells was assessed after an infection period of 48 to 72 hours. The adenovirus expressing MPXV-197-FLAG (ad197) was co-transduced with an adenovirus expressing a necessary transactivator protein as originally described in (Streblow et al., 1999). The MOI (ad197 to cell) and timespan to use for optimal surface expression of MPXV-197 was varied and shown in Figure 4.1A. The vast majority of the live cells (84.3%) expressed MPXV197-FLAG after 72 hours when an MOI of 6 was used (Fig. 4.1B). The figures contained in this thesis focus on MPXV197 but we observed similar characteristics with the other two proteins, as published in (Alzhanova et al., 2014). I also repeated experiments in Figures 4.1 and 4.2 with Vaccinia virus engineered to express CPXV-219. Here, CPXV219 expressed from a Vaccinia vector displayed the same cell surface expression and functional paralysis of T cell clones, suggesting that the adenoviral vector was not playing a role in these phenotypes.

MPXV197 is sufficient to interfere with T-cell activation to multiple stimuli

To test whether transiently expressed MPXV197 was capable of inhibiting T cell stimulation I used Ad-197 or control Ad-infected BEAS2B cell line as antigen presenting cells for human T cell clones in an ELISPOT assay (Fig. 4.2). Human T cell clones that are restricted by either classical (HLA-B) or non-classical (HLA-E) MHC class I molecules were used. BEAS2B epithelial cells were infected with either Ad-197 alone or together with Ad-TA to induce MPXV197 expression. These cells, as well as

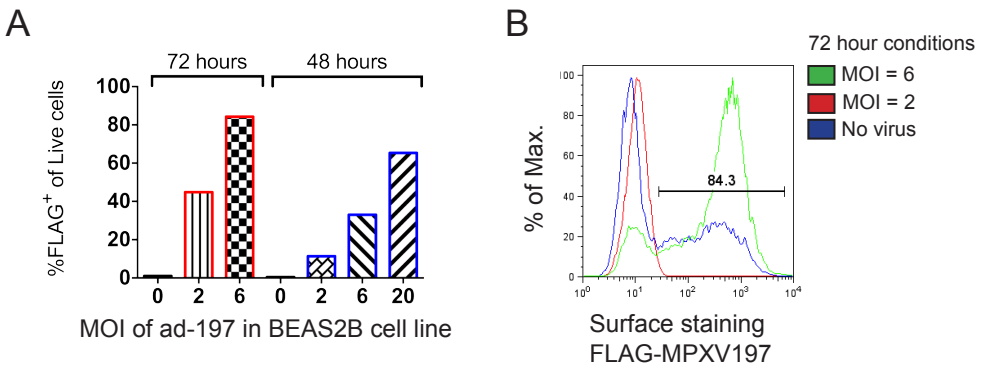


Figure 4.1. Optimization and Quantification of Expression of MPXV-197 in BEAS-2B cell line

(A) Bar graphs depicting staining of MPXV-197-C-terminal-FLAG on the surface of BEAS2B cell line. Hours indicate the time since the cell line was infected with the adenovirus expressing MPXV197 at various multiplicity of infection (MOI) listed on the x-axis.

(B) Histogram overlay of MPXV-197-C-terminal-FLAG surface expression on BEAS-2B cell line after 72 hours post adenoviral transduction (Blue- MPXV-197, Red – MOI 2, Green – MOI 6).

appropriate controls, were then incubated with human *M. tuberculosis*-specific $CD8^+$ T cell clone D466-D6 recognizing peptide CFP_{2-12} peptide presented by HLA-B (Lewinsohn et al., 2007) and clone D160 1-23 which is stimulated by pronase-digested *M. tuberculosis* cell wall in the context of HLA-E (Heinzel et al., 2002). As shown in Fig. 4.2B, stimulation of both clones was inhibited by MPXV197. Moreover, MPXV197 also prevented Ag-independent stimulation of these T cell clones by phytohaemagglutinin (PHA), a lectin that activates the TCR by carbohydrate cross-linking. Additionally, MPXV197 was able to inhibit T cell stimulation even when expressed by tertiary cells not involved in antigen presentation (experiment not shown, set up Fig. 4.2 bottom). Taken together these data demonstrate that MPXV197 is sufficient to recapitulate the T cell unresponsiveness mediated by MPXV for human T cells regardless of the TCR stimulus.

Effector function, like production of cytokines, is activated in T cells upon a series of signals. In order to activate effector function in T cell clones, stimulation by peptide-MHC through the TCR is required. Naive T cells require additional costimulatory signaling through CD28 and cytokines, as compared to T cell clones. Following TCR ligation, co-receptors CD4 or CD8 on the T cell associate with MHC for stabilization, and because the cytoplasmic tails of CD4 and CD8 recruit Lck, this results in a stable association of Lck and its CD3 chain substrate within the TCR complex. Lck is a protein tyrosine kinase that can phosphorylate the ITAMs within the CD3 cytoplasmic tails. Phosphorylation of the CD3 chain creates binding sites for ZAP-70 in the TCR complex and activates it. ZAP-70 is then responsible for phosphorylating and activating transmembrane protein LAT (linker for activation of T cells) and then SLP-76 (SH2-domain containing protein with a role in cytoskeletal rearrangements). Activated LAT attracts phospholipase C, which begins classical signaling through the phosphoinositol pathway and Ca^{2+}, and also Grb2, which begins signaling through the Ras/MAPK pathway. Costimulation on the T cell's surface activates protein kinase C (PKC) which can be further activated by diacylglycerol from

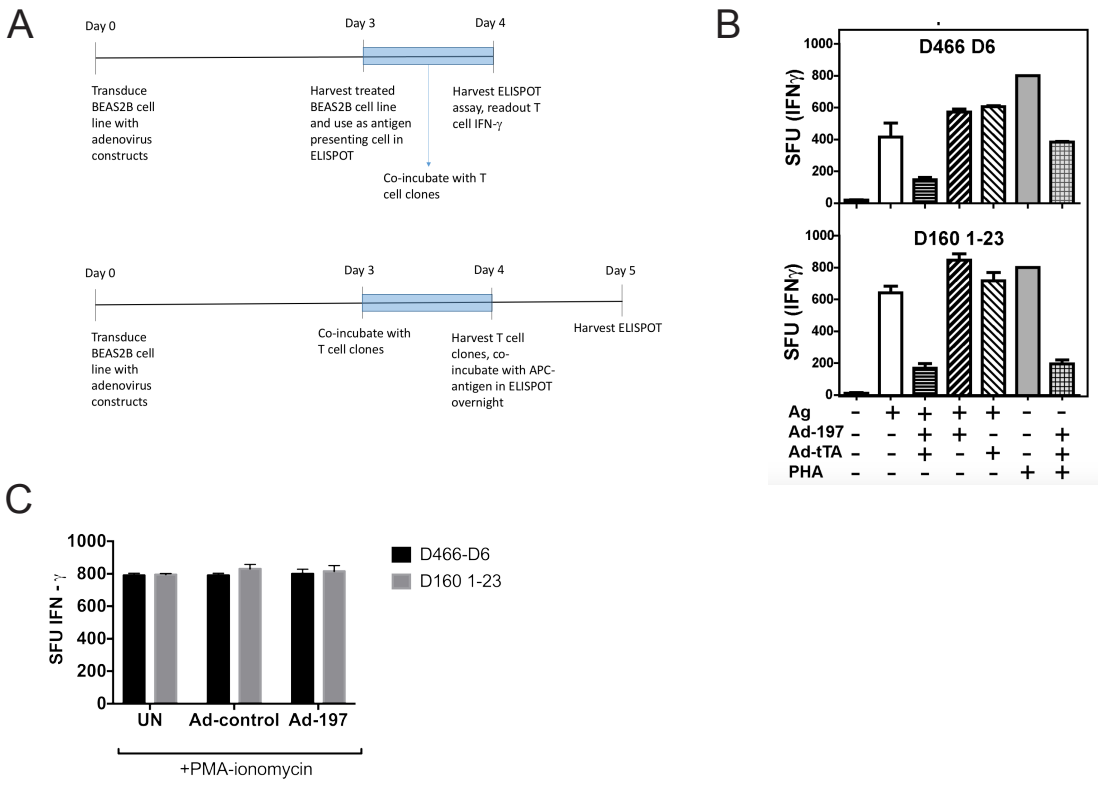


Figure 4.2. MPXV197 inhibits TCR-dependent T cell stimulation (A) Schematic of experimental set-up. The top experiment set-up was used to generate the data shown here. The bottom experimental set-up (uses an APC that did not express MPXV197) yielded similar results. (B) Human Mtb-specific $CD8^+$ T cell clones D466-D6 and D160-1–23 were stimulated with BEAS-2B cells transduced with Ad-MPXV-197/Ad-TA or Ad-TA only in the presence of $CFP10_{2-12}$ peptide or pronase digested Mtb cell wall, respectively. For MHC-independent stimulation, both clones were incubated with PHA. The number of $IFN\gamma^+$ T cells was measured by ELISPOT. Experiment was performed three times with representative results. Error bars refer to the SEM of two replicate wells. (C) Human Mtb-specific $CD8^+$ T cell clones D466-D6 and D160-1–23 were treated with nothing (UN), ad-control (inactive MPXV197), or ad-MPXV197, and then stimulated with PMA-ionomycin. The number of IFNγ+ T cells was measured by ELISPOT. Experiment was performed three times with representative results. Error bars are the SEM of two replicate wells.

phospholipase C by TCR ligation. These pathways culminate in activation of a range of T cell transcription factors: Ap-1, NFAT, and NFkB.

To further determine whether T cells were only unresponsive to TCR-proximal stimuli TCR stimulation was by-passed by activating T cell clones with phorbol myristate acetate (PMA) which activates PKC and the Ca^{2+} ionophore ionomycin to mimic co-stimulation and TCR ligation, respectively. In contrast to antigen- and TCR-specific stimulation from the previous panel, MPXV-197 did not interfere with T cell stimulation by PMA-ionomycin (Fig. 4.2C). These data suggest that later stages of intracellular T cell signaling remains viable after exposure to MPXV197, and that MPXV197 counteracts TCR-dependent signal transduction upstream of PKC and calcium flux receptors/channels.

Due to the observation that B22R family proteins are able to functionally paralyze T cell clones *in vitro*, we hypothesized that they might act the same way *in vivo*. The function of B22R family proteins seems to be specific to non-human primate and human T cells, therefore the Fruh Lab tested the *in vivo* effects in rhesus macaques infected intrabronchially with a non-lethal dose of WT MPXV (strain derived from the 2003 U.S. outbreak) or MPXV Δ197. Blood and bronchoalveolar lavage (BAL) fluid samples were collected at defined days post infection to determine the kinetics of virus replication (Figure 4.3). All rhesus macaques developed a fever, pock lesions, detectable lung viremia, and virus specific CD4 and CD8 T cells. Two of four animals infected with WT MPXV had to be euthanized before the end of the study due to severity of symptoms. The lung viremia in each animal was comparable, indicating similar viral inoculum. By the end of the study, animals infected with WT MPXV had higher lung viremia than MPXVΔ197. In stark contrast, viremia was not detectable in the blood of MPXV Δ197 infected animals, indicating that it was controlled at the site of infection. Animals with WT MPXV infections had viral loads in the blood starting ~ day 7 and resolving around ~ day 17 (Alzhanova et al., 2014).

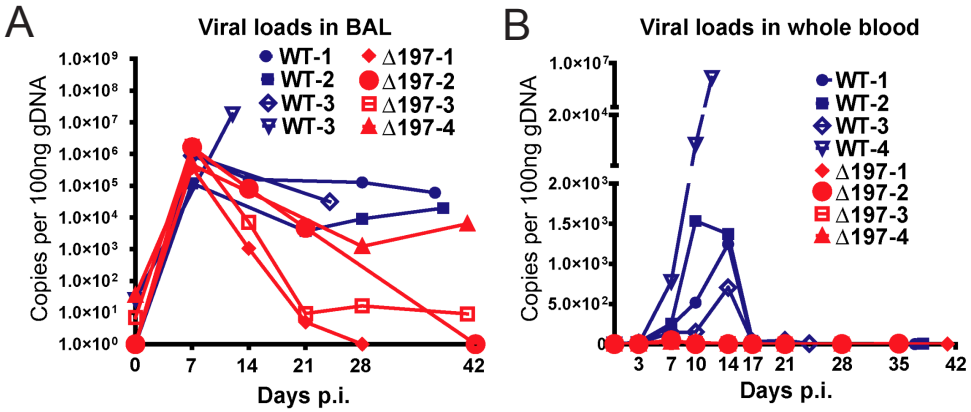


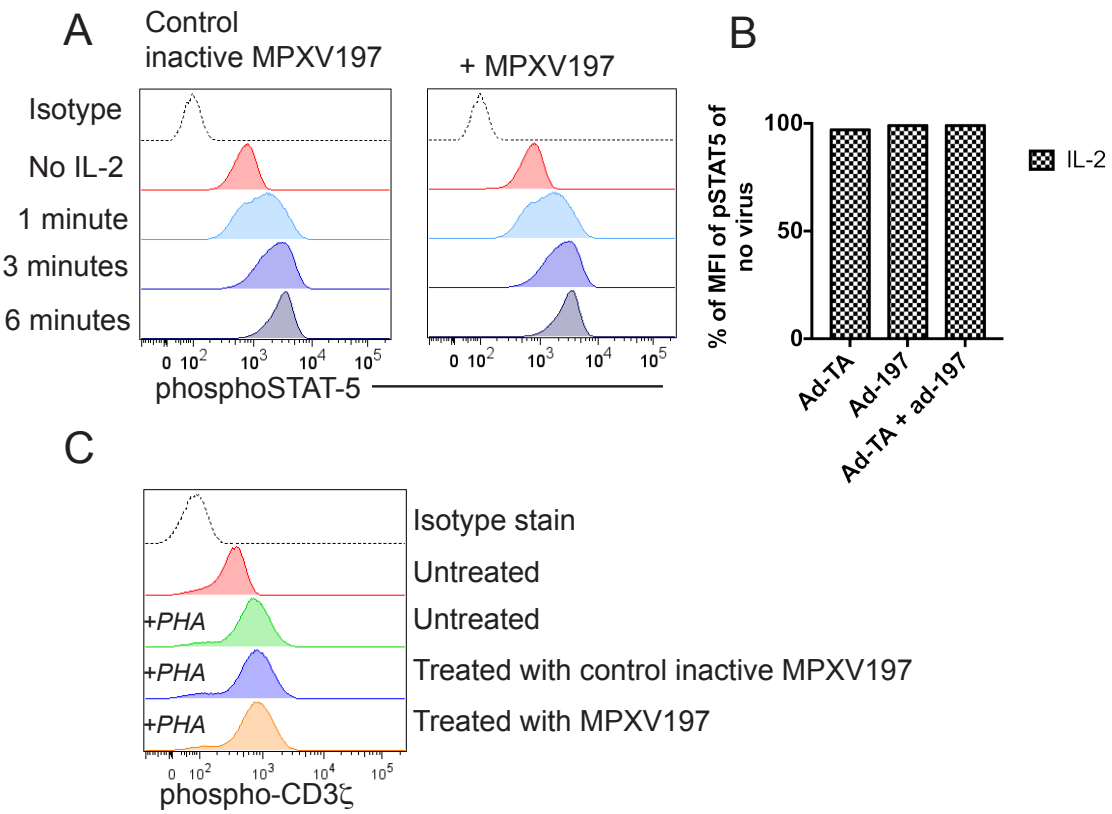
Figure 4.3. MPXVΔ197 is attenuated *in vivo* [Performed by Fruh Laboratory]

Four female rhesus macaques (RM) were inoculated intrabronchially with 2e5 PFU of MPXV US2003 (WT) or MPXVΔ197 on day 0. Whole blood, BAL, and PBMC samples were taken on indicated dpi. Viral loads determined by qPCR in BAL (A) and whole blood (B) of WT (blue) or MPXVΔ197 (red)-infected RM.

With regard to the immune response similar or higher T cell responses were induced in MPXVΔ197-infections compared to WT (Alzhanova et al., 2014). T cells from the blood of MPXVΔ197-infected animals were also able to respond better *ex vivo* to polyclonal anti-CD3 stimulation than those from WT MPXV infections (Alzhanova et al., 2014). These data suggests that MPXV197 is able to induce systemic T cell functional paralysis *in vivo* and that the lack of this protein attenuates MPXV virulence.

We showed that MPXV197, like its CPXV and VARV orthologues, is sufficient to abrogate human $CD8^+$ T cell clone activation in an MHC-dependent and MHC-independent manner, we wanted to know how. Non-specific T cell stimulation that bypasses the TCR, circumvented the functional paralysis elicited by MPXV197, suggesting that it abrogates signaling nearby to the TCR. In order to determine the molecular mechanism of MPXV197's inhibition of T cell activation, I analyzed the intracellular signaling environment through phospho-flow cytometry. To perform this analysis, T cell clone D466-D6 was pre-incubated overnight in the presence of BEAS2B cells expressing MPXV-197 or controls. The controls we used were BEAS2B and T cell only and BEAS2B expressing an inactive MPXV-197. The inactive MPXV-197 does not contain a transmembrane domain. First, it was determined whether a phosphorylation cascade unique to T cells but unrelated to the TCR, IL-2/IL-2 receptor/STAT5 was interrupted by MPXV 197. Normally, IL-2 binds to IL-2 receptor on the surface of T cells and transduces a signal intracellularly to activate and phosphorylate the transcription factor STAT5. As shown in Figure 4.4A, STAT5 is progressively phosphorylated at a amino acid single site in T cells over 6 minutes after the addition of IL-2. The right panel, and 4.4B, shows that this phosphorylation is not affected by MPXV-197 or any of the control proteins. This suggested to us that MPXV-197 does not target cytokine signaling in the target T cell. Due to this signaling cascade being intact in the presence of MPXV-197, this data suggests that the effect of MPXV197 is to more directly inhibit TCR signaling instead of generally inhibiting kinase activity.

One of the first steps of the TCR signaling cascade upon recognition of antigen MHC is the phosphorylation of CD3ζ as part of the T cell receptor complex. To determine whether this step is


A
Control
inactive MPXV197
+ MPXV197
Isotype
No IL-2
1 minute
3 minutes
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0 10^2 10^3 10^4 10^5
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% of MFI of pSTAT5 of no virus
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+PHA
Untreated
+PHA
Treated with control inactive MPXV197
+PHA
Treated with MPXV197
0 10^2 10^3 10^4 10^5
phospho-CD3ζ

Figure 4.4. MPXV-197 does not affect IL-2 receptor or CD3 signaling

(A) Histogram overlay of phosphorylated tyrosine 694 of STAT-5 in T cell clone D466-D6 after treatment with recombinant human IL-2 over a timespan of one to six minutes (y-axis). The left panel shows the response by D466-D6 that had been incubated overnight in the presence of control inactive MPXV197, whereas the right panel shows D466-D6 post-treatment with MPXV197. Isotype control antibody staining is shown on top with dashed lines; resting pSTAT5 (red); IL-2 treatment (blue). Experiment was performed three times with similar results.

(B) Bar graphs depicting the geometric mean intensity (MFI) of pSTAT5 in D466-D6 given IL-2 that had been pre-incubated with BEAS2B cell line expressing transactivator (TA), MPXV197 (ad-197), or both, compared to untreated T cell clone.

(C) Histogram overlay of phosphorylated tyrosine 142 of CD3ζ after treatments listed on the y-axis (right). Isotype control antibody staining is shown on top with dashed line; resting pCD3ζ (red); PHA treatment (blue). Experiment was performed two times with similar results.

targeted by MPXV197, T cell clone D466-D6 was stimulated with PHA and the phosphorylation of tyrosine 142 in an ITAM of CD3ζ after 10 minutes was quantified by flow cytometry (Figure 4.4C). Upon stimulation, there was not a difference between T cell clones treated with no virus, control virus, or MPXV-197 indicating that this step of the signaling cascade was not targeted by MPXV197.

The phosphorylation cascade triggered by a TCR engagement of MHC-antigen culminates in calcium ion flux into the cytosol of a T cell. Prolonged elevation of intracellular Ca^{2+} is important for signal transduction into the nucleus by NFAT transcription factor activation and cytoskeletal rearrangements. NFAT activates the transcription of pro-inflammatory genes on its own and in synergy with other transcription factors, for example, genes that encode for cytokines like IL-2, TNF-α, and IFN-γ. The intracellular Ca^{2+} concentration in resting T cells is maintained at 100nM, whereas the extracellular Ca^{2+} concentration is 1mM, resulting in a large concentration gradient of ion across the plasma membrane (Lewis, 2001). Following the engagement of the TCR with a pMHC on an APC, the intracellular Ca^{2+} concentration can increase to 1uM through the sequential operation of two interdependent processes: (1) Release of phospholipase C (PLC)-dependent intracellular Ca^{2+} stores, and (2) extracellular Ca^{2+} influx through plasma membrane Ca^{2+} channels (Lewis, 2001).

To determine whether MPXV197 affects a T cell's ability to modulate Ca^{2+} , T cell clones were pre-treated overnight with MPXV197 or controls, then loaded with a fluorescent dye that indicates cytosolic Ca^{2+}. Using a flow cytometer, resting levels of Ca^{2+} (0-60 seconds) were compared to PHA-stimulated levels of Ca^{2+}. A representative dot plot in Figure 4.5A reveals the rapid elevation of ion levels upon TCR stimulation of T cell clone D466-D6. MPXV-197, but not a control inactive MPXV197, blocked the influx of Ca^{2+} upon TCR dependent stimulation (Fig. 4.5B, left two panels). The results from multiple experiments are summarized in Figure 4.5C. In contrast TCR independent ionomycin stimulation of Ca^{2+} was not affected (Fig. 4.5B, right two panels). Ionomycin is an ionophore that is used as a reagent to force calcium ions into the cytosol; it does so by acting as an ion shuttle that is permeable to cell

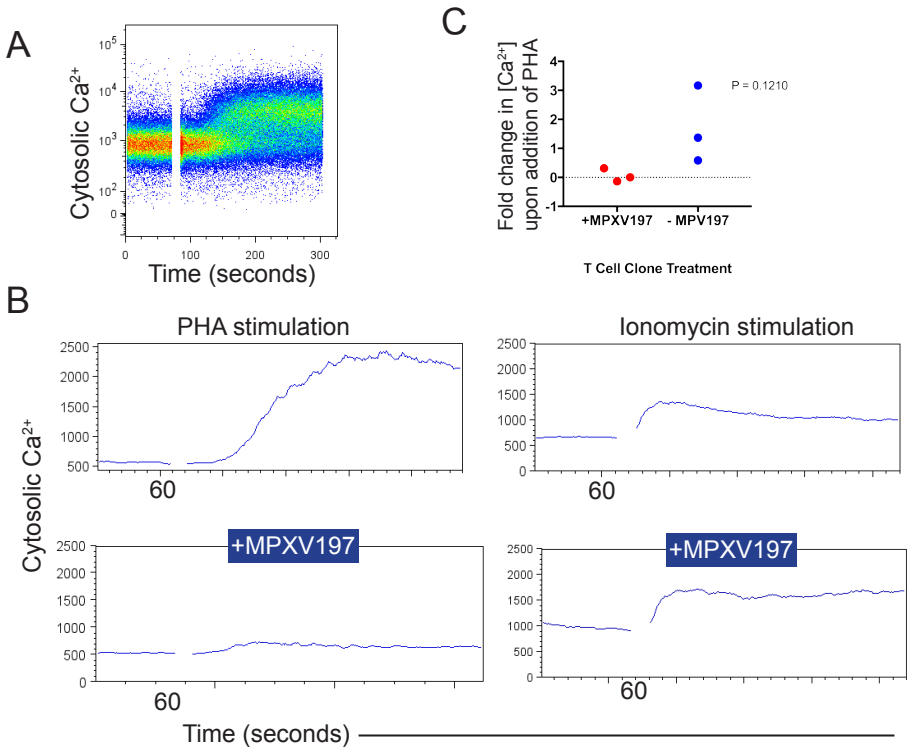


Figure 4.5. MPXV-197 blocks intracellular Ca^{2+} flux

(A) Flow cytometry dot plot showing representative cytosolic calcium levels in D466-D6 T cell clone during an initial 60 second resting period, followed by addition of PHA stimulus at 60 second time point. Experiment is representative of three different T cell clones analyzed over three independent experiments (HLA-B restricted D466-D6 T cell clone; MR1-restricted T cell clone D426-B1; HLA-E restricted clone D160 1-23). (B) Kinetics analysis of MFI of cytosolic Ca^{2+} over time in D466-D6 T cell clone. The left two panels show T cell clone response to PHA stimulation, whereas the right panels are representative of the response to ionomycin treatment. The top panels show the response by D466-D6 pre-incubated with BEAS2B cells that express inactive MPXV-197, and the bottom panels show the response by D466-D6 pre-incubated with BEAS2B cells that express MPXV-197. Experiment was performed three times with similar results as summarized in (C), which shows the fold change in ion concentration upon addition of ionomycin stimulus across three

membranes. These results indicate that MPXV197 most likely exerts it effect on T cell activation at the stage of or upstream of PLC activation.

Chapter 4 Discussion

The goal of this project was to further define the repertoire of proteins OPXV used for T cell evasion and understand specific suppressive mechanisms used to directly inhibit human T cell activation. *In vivo* studies using MPXV show that MPXV197 is crucial for the *in vivo* virulence of this virus in a rhesus macaque model. The deletion mutant MPXVΔ197 did not establish blood viral titers above the limit of detection in any of the challenges. We hypothesized that OPVX B22R family proteins, specifically MPXV197, are able to cause inhibit T cell activation through directly interacting with the T cell and experiments were performed to elucidate the mechanism.

The data presented in this thesis indicate that the mechanism of B22R proteins is likely focused at proximal TCR signaling and does not involve apoptosis. T cells were also tracked for apoptosis over a 24-hour time span and their viability was not affected. Moreover, antigen specific T cells were still able to bind relevant tetramers (Alzhanova et al., 2014) and transduce a signal immediately intracellularly from the TCR in the presence of MPXV197. This suggests that the structure of the TCR and co-receptor are not affected by MPXV197. However, MPXV197 reduced the TCR signal cascade from reaching the point of Ca^{2+} release. These results indicate that the evasion mechanism likely occurs after TCR engagement with peptide MHC and inhibits TCR signal transduction proximal to the TCR.

$CD8^+$ T cells detect and lyse virus infected cells through recognition of viral peptides displayed on MHC class I directly or indirectly by cross presentation. The conclusion that B22R-family proteins inhibit T cell function directly was unexpected as many dsDNA viruses inhibit antigen presentation and induce the secretion of T cell suppressive factors. Nevertheless, there are some viral proteins that directly interact with T cells. For example, the human cytomegalovirus (CMV) protein UL11 is expressed

from the surface of the infected cell where it can interact *in trans* with T cell CD45, a large phosphatase molecule (Gabaev et al., 2011). Upon binding, UL11 alters the structure and inactivates CD45 so that it cannot perform its function in the TCR signaling cascade. Specifically, when the T cell interacts with an APC, CD45 activates the Src family kinase Lck. Based on our data that show that phosphorylation of the TCR complex was still intact, B22R family proteins most likely do not operate through this mechanism. CMV can also upregulate pro-apoptotic ligands on the surface of infected dendritic cells that can induce apoptosis in activated T cells (Raftery et al., 2001). B22R family proteins most likely do not operate through this mechanism, as the treated T cells are functionally paralyzed but do not apoptose. Lastly, Kaposi's sarcoma-associated herpesvirus encodes a homolog of human CD200 (called vOX2) which is a potent inhibitor of T cell activation by binding to CD200 receptor on the surface of T cells (Misstear et al., 2012). T cells uniquely express the CD200R which is a transmembrane negative regulatory protein. Signaling through CD200R activates cytosolic Ras-GAP which prevents activation of PI3K, Akt, and Erk, kinases necessary for TCR signal transduction. B22R family proteins could act through CD200R and this represents a logical target. Overall, B22R family proteins do not share any homologous domains with other viral so I hypothesize that they use a novel mechanism.

The exact mechanism through which B22R family proteins act remains elusive. Based on our data presented in this chapter, I would hypothesize that B22R family proteins target and inactivate a transmembrane T cell protein with function in regulating TCR signal transduction. There are many candidate proteins expressed on both $CD8^+$ and $CD4^+$ that have been reviewed recently for the regulatory T cell field and cancer immune checkpoint field (Chen and Flies, 2013; Pardoll, 2012). Candidate proteins that act in concert with the TCR but oppose co-stimulation include CTLA-4, PD-1, BTLA-4, TIM3, TIGIT, CD160, LAG-3, LAIR-1, B7-1, B7-H1. However, the majority of these receptors are not constitutively expressed on resting T cells, but are upregulated after activation or during exhaustion. Therefore, it is less likely that B22R family proteins target these T cell proteins, as the mechanism of

action occurs in less than one hour and acts on T cells regardless of their activation state. It would be more likely that B22R family proteins target a T cell surface protein with an essential role in T cell activation leading to cytokine production. Direct cell contact was required for suppression of TCR signaling in T cells, yet the inhibited state of T cells was sustained after removal of MPXV infected cells from the co-culture. Therefore, B22R family proteins likely alter a cell surface protein in a long-lasting fashion or in a way that triggers an alteration of the intracellular environment.

MPXV-mediated suppression might involve a parallel mechanism to what is used by T-regulatory T cells. $CD4^+$ T-regulatory cells inhibit conventional T cell activation through many mechanisms, in part they directly inhibit TCR signaling. Recently Peter Krammer's group has been investigating a phenomenon whereby T-regulatory cells rapidly suppress TCR-induced signaling events and Th1 cytokine production within 1 hour of co-culture (Schmidt et al., 2011). This mechanism requires cell-contact and Ca^{2+} influx is blocked immediately after TCR stimulation. The exact mechanism has not been discovered yet, but might inform us to a possible mechanism for B22R family proteins. q

Taken together, the most logical targets of B22R family proteins are CD200-receptor (discussed above), linker of activation (LAT), plasma membrane $Ca2^+$ channels, or T cell immunoreceptor with Ig and ITIM domains (TIGIT) or the TIGIT-related molecule CD96. B22R family proteins could interact with LAT on the T cell surface. This large transmembrane protein localizes in lipid rafts and is the main scaffold for kinases that mediate TCR signal transduction. LAT is activated by phosphorylation by ZAP-70. LAT is responsible for bringing key kinases within the proximity of the TCR; therefore, if it is excluded from the area of the plasma membrane where it localizes upon TCR activation or if its phosphorylation is reduced, then B22R could hinder signal transduction. B22R family proteins might also target a T cell's essential plasma membrane Ca^{2+} channels to prevent calcium influx that is necessary for full signal transduction. In the presence of MPXV197, we observed that TCR stimulation was unable to cause an influx of Ca^{2+}. In contrast, when ionomycin was added as a Ca^{2+} membrane shuttle or positive control,

the T cell's intracellular Ca^{2+} levels were elevated. This phenotype could be caused by B22R if it blocks the Ca^{2+} channel in the plasma membrane. Lastly, B22R could engage TIGIT or TIGIT-related protein CD96 that are expressed on the T cell and NK cell plasma membrane, more constitutively than other inhibitory receptors. TIGIT and CD96 are transmembrane proteins that are characterized by their immunoreceptor tyrosine-based inhibitory motif (ITIM) domains in the cytoplasmic tail (Chan et al., 2014). The natural ligands for TIGIT and CD96 are nectin-like proteins, including the poliovirus receptor, CD155. After ITIM-possessing inhibitory receptors interact with their ligand, their ITIM motif becomes phosphorylated by enzymes of the Src kinase family, allowing them to recruit the phosphatases SHP-1 and SHP-2, or the inositol-phosphatase called SHIP. This mechanism would act to shut off TCR signaling quickly as it does not require new protein synthesis and phosphatases inhibit the activation of molecules involved in TCR transduction. If B22R family proteins do target TIGIT or CD96 the mechanism might be able to inhibit NK cells as well. While each of these hypotheses could be tested individually, I would suggest a future experiment in the form of a screen to directly test which T cell surface proteins can interact with MPXV197. A co-immunoprecipitation procedure, similar to the one employed in (Gabaev et al., 2011) would suit this question well. Because the study of membrane proteins is notoriously difficult, I would propose to stabilize recombinant MPXV197 on a planar lipid bilayer and then incubate it with the membrane fraction of proteins derived from T cell clones. Then protein complexes could be analyzed by a co-immunoprecipitation process.

How the B22R family proteins engage the target T cell is still unknown. In all, deciphering the mechanism of action of B22R-family proteins, a conserved large protein expressed by virulent OPXV, will give us information about novel direct methods to inhibit TCR activation and represent possible therapeutic avenues for modulating T cell function.

Chapter 4 Materials and Methods

Cells and Cell Lines

BEAS-2B human bronchial epithelium cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (FBS, Hyclone Laboratories, Inc, Logan, UT). *M. tuberculosis*-specific T cell clones were rapid expanded and maintained as previously described (Lewinsohn et al., 2007).

***In vitro* Expression of OPXV Immunomodulatory Proteins**

To study T cell responses to MPXV 197, BEAS-2B cells were infected with Ad-tTA (8 MOI) or Ad-197 (6 MOI) and Ad-tTA (1.7 MOI) for 72h. Alternatively, to study T cells responses to CPXV 219 the cells were pretreated with 10 nM ST246 kindly provided by SIGA Technologies (Corvallis, OR) and infected with VACV wild type or VACV-219 recombinant virus (2 MOI, 10 M ST246) for 2h. The ST246 drug was included at all other stages of the experiments utilizing VACV infected BEAS-2B cells.

Adenoviral vectors and tags (Created by the Fruh Lab)

Protein expression vectors: Codon-optimized sequences of the C-terminal 3xFlag (DYKDHDGDYKDHDIDYKDDDDK) fusions of MPXV197 and VARV B22R proteins were synthesized at GenScript (Piscataway, NJ). All coding sequences were cloned into pCDNA3.1 vector (Life Technologies). The resulting plasmids were used for construction of the recombinant adenoviruses viruses. To achieve the protein expression these viruses were co-infected with Ad-TA virus expressing transactivator (TA) protein. We used an inactive form of MPXV197 as a negative control in our experiments. This protein did not contain a transmembrane domain and was validated to not cause the inactive T cell phenotype.

Flow cytometry

All flow cytometry was performed on a Fortessa 18-parameter flow cytometer (BD). Fluorescence minus one (FMO) and isotype controls were used for optimal gating. Doublets were excluded based on FSC-H and FSC-A, SSC-H and SSC-A, lymphocytes were identified based on FSC-A and SSC-A, then CD8 and CD3 expression. **Surface staining for FLAG:** BEAS2B cell line was analyzed for expression of MPXV197 on its surface by staining for the C-terminal FLAG tag. Briefly, polyclonal anti-FLAG or isotype control was added to harvested BEAS2B cells at 1 μg/ml for 15 minutes at 4C, then washed and fixed. **Staining for Phosphorylation:** Treated T cell clones were immediately fixed in 2% paraformaldehyde. Following fixation, the cells were permeabilized in ice cold 100% methanol for 30 minutes. Then the cells were washed in FACS buffer to sufficiently remove the methanol and stained with the following antibodies, anti-CD8 (clone RPA-T8, 1:50 dilution, Biolegend), anti-CD3 (OKT3, 1:50 dilution, Biolegend), anti-CD3z-pY142 (1:10 dilution, BD), or anti-STAT5-pY694 (1:20 dilution, BD), or isotype controls (used at a matching concentration to their corresponding phospho-specific antibody), for 45 minutes at 4°C. Isotype controls were used to optimize staining with phospho-specific antibodies. A minimum of 30,000 $CD8^+$ events were collected for MFI analysis across samples. Data was analyzed using FlowJo Version 9.9.3.

ELISPOT with D466-D6 and D160 1-23

To study T cell responses to MPXV 197, BEAS-2B cells were infected with Ad-TA (8 MOI) or Ad-197 (6 MOI) and Ad-TA (1.7 MOI) for 72h. Alternatively, to study T cells responses to CPXV 219 the cells were pretreated with 10 M ST246 kindly provided by SIGA Technologies (Corvallis, OR) and infected with VACV wild type or VACV-219 recombinant virus (2 MOI, 10 M ST246) for 2h. The ST246 drug was included at all other stages of the experiments utilizing VACV infected BEAS-2B cells. Indicated Mtb-specific T cell clones were co-incubated with infected BEAS-2B cells for 3h or overnight and then stimulated with peptide CFP_{2-12} (clone 466-D6), pronase digested Mtb cell wall (clone D160-23), or

phytohemagglutinin (PHA) in IFN-γ Ab (clone 1-D1K, MABTECH AB, Nacka Strand, Sweden) coated ELISPOT plates as previously described (Lewinsohn et al., 2007). The staining was detected with IFN-γ mAb conjugated to horseradish peroxidase (HRP) (clone 7-B6-1, MABTECH) and developed using AEC Vectastain-Elite kit (Vector Laboratories, Burlingame, CA).

MPXV Animal challenge [Performed by the Fruh Lab]

All animal experiments were reviewed and approved by the OHSU Institutional Animal Care and Use Committee. Eight adult female RM animals were utilized for *in-vivo* studies of the T cell responses to MPXV US2003 wild type (WT) and MPXV 197 mutant. Cohort 1 (WT) included animals 29437 (WT-1; 7 year-old), 29785 (WT-2; 10-year-old), 21111 (WT-3; 13-year-old), and 28689 (WT-4; 13-year-old). Cohort 2 (KO-197) included animals 29792 (197-1; 8-year-old), 29398 (197-2; 11-year-old), 29424 (197-3; 13-year-old), 28664 (197-4; 10-year-old). The animals were infected intrabronchially with $5x10^5$ PFU/animal of WT and the mutant viruses delivered in 1ml of phosphate-buffered saline (PBS). Blood and bronchoalveolar lavage (BAL) samples were collected on the day of infection (day 0) and later on indicated days post infection. Peripheral blood mononuclear cells (PBMC) were isolated from blood by centrifugation via Histopaque gradient.

Stimulation and Quantification of IL-2 Signaling

T cell clone was maintained in a rapid expansion flask with low dose IL-2 (0.5ng/ml) for 24 hours. Then, the T cell clone D466-D6 was co-incubated overnight in the presence of the BEAS2B cell line expressing nothing (empty vector), transcriptional activator (adTA), adTA and a control inactive version of MPXV197, or adTA and MPXV197. Treated T cell clones were then harvested from the adherent BEAS2Bs, washed, and stimulated with recombinant human IL-2 (50 ng/ml Immunex) for fifteen minutes at 37C. The cells were then fixed, permeabilized, and stained as described above. Specifically, the level

of phosphorylated STAT5 was quantified by staining with the antibody specific to the phosphorylated transcription factor (1/50 dilution, BD PhosFlow) or the appropriate isotype control.

Stimulation and Quantification of phospho-CD3ζ

T cell clone D466-D6 was co-incubated overnight in the presence of the BEAS2B cell line expressing nothing (empty vector), transcriptional activator (adTA), adTA and a control inactive version of MPXV197, or adTA and MPXV197. Treated T cell clones were then harvested from the adherent BEAS2Bs, washed, and stimulated with phytohemagglutinin (PHA, 10 μg/ml) for ten minutes at 37C. The cells were then fixed, permeabilized, and stained as described above. Specifically, the level of phosphorylated CD3-zeta was quantified by staining with the antibody specific to the phosphorylated tyrosine 142 (1/10 dilution, BD PhosFlow).

Stimulation and Quantification of Cytosolic Ca^{2+}

T cell clone D466-D6 was harvest from its rapid expansion flask on day 14, washed, and rested overnight in low dose IL-2 (0.5ng/ml) with or without MPXV treatments. The next day, the cells were loaded with 0.5uM cytosolic Calcium dye eFluor 514 (eBioscience) for 30 minutes at 37C, by following the manuf. instructions. Then the cells were washed twice in media, suspended at 1e6 cells/mL on ice, and transported to the flow cytometer. There, each sample was warmed to 37C in a water bath, PI was added to measure viability of cells, and a 60 second resting measurement of cytosolic calcium was taken. Then, stimulus was added (5 μg/ml PHA, or 1 ug/ml ionomycin) and a subsequent five-minute measurement was taken. Data was analyzed using the FlowJo kinetics software on version 9.9.3. The 60 second resting measurement (geometric mean of channel measuring cytosolic Ca) was used to set the threshold value for each sample. The moving smoothed average cytosolic Ca level for all live $CD3^{+}CD8^{+}$ cells in the sample is presented.

Chapter 5 Thesis Summary and Conclusions

MR1 and the T cells it activates provide the field of immunology with a new paradigm whereby a conserved antigen presentation molecule can present small metabolites that are essential to microbes, as patterns of infection to activate a family of semi-invariant TCRs. Our focus has been to determine the role of diversity in MR1-restricted TCRs to detection of microbial infection and whether or not this process allows for selectivity in antigen recognition. The results presented in Chapter 3 of this thesis formally demonstrate the existence of MR1-restricted T cells that use a non-TRAV1-2 semi-invariant TCR. While these cells are much rarer than MR1-restricted T cells expressing the TRAV1-2 TCR, this finding demonstrates that assumptions about the use of a single semi-invariant TCR by MR1-restricted T cells must be reassessed. The definition of MAIT cells as a discrete family of T cells that use a semi-invariant TCR, are enriched at mucosal sites, and have innate like qualities is not questioned by the data in this thesis. It remains to be determined whether non-TRAV1-2 MR1-restricted TCRs share these qualities and the unique developmental pathway in the thymus.

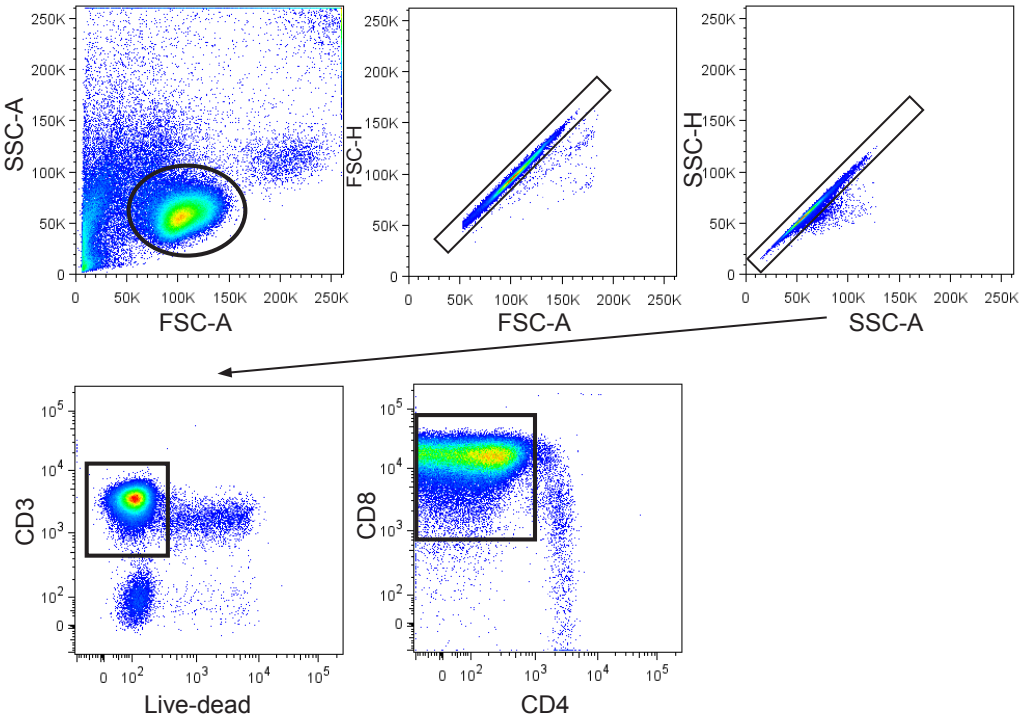
Pertaining to the thymic development of MR1-restricted T cells, the data from Chapter 3 of this thesis led us to ask whether MR1-restricted T cells that used non-TRAV1-2^{+} TCRs were more frequent during T cell development in the thymus, prior to exposure to microbial antigens. Through T cell cloning of MR1-restricted thymocytes identified by a tetramer, we discovered all of them to express a TRAV1-2^{+} TCR. This data supports a scenario where TRAV1-2^{+} T cell selection in the thymus is favored over other TCRs. Further research into how MAIT cells are selected on MR1^{+} hematopoietic cells in the thymus, and whether a ligand is involved, will give insight into the relationship between TRAV1-2^{+} TCRs and MR1 during development.

The ability of the MR1-restricted TRAV12-2^{+} TCR to recognize *S. pyogenes* infection necessitates a reevaluation of the family of ligands that MR1 can present, as *S. pyogenes* does not create the

previously defined ligands. While the nature of the MR1-ligands that alternative MR1-restricted TCRs can recognize remains elusive, it is clear that MR1-restricted TCRs can share cross reactivity to the previously defined Vitamin-B derived ligands, as presented in Chapter 3. Unpublished data from our collaboration with the Rossjohn Laboratory (Monash University), suggests that the TCR of the D462-E4 T cell clone "docks" over the MR1-antigen groove in a distinctive way from MAIT TCRs. This mechanism could allow the D462-E4 TCR to recognize different antigens that may be presented in a different part of the MR1 antigen binding groove. Further research into the nature of the MR1-ligands created by *S. pyogenes* infection will give the field insight into the repertoire of antigens that can be presented by MR1.

Appendix Table 1
Nucleotide sequences of the junctional regions surrounding the CDR3 and T cell receptor of D462-E4

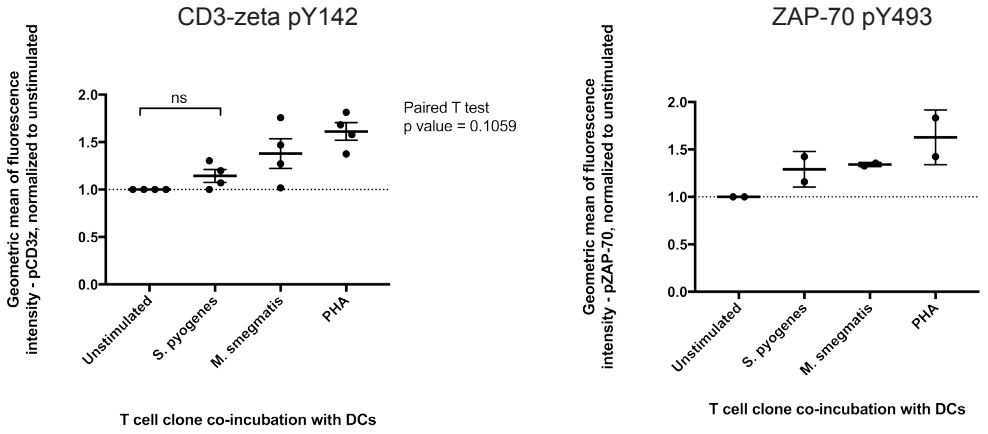
Nucleotide sequence	CDR3 Amino Acid	CDR3 Length	V Gene Name	J Gene Name
CATCAGAGACTCCCAGCCCAGT-GATTCAGCCACCTACCTCTGTGC-CGTGAGAGATGCAGGCAACATGCT-CACCTTTGGAGGG	CAVRDAGNMLTF	36	TCRAV12-02	TCRAJ39-01
AACATGAGCCCTGAAGACAGCAG-CATATATCTCTGCAGCGTGGGGGGGGA-CAGCCTTATAGGCAATCAGCCCCAG-CATTTTGGTGAT	CSVGGDSLIGNQPQHF	48	TCRBV29-01	TCRBJ01-05



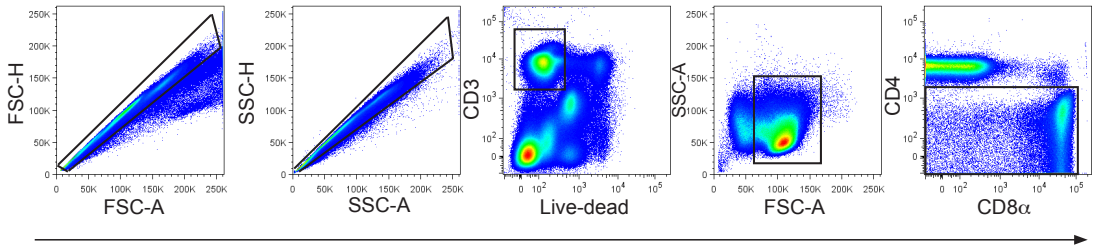
Appendix Figure 1. Flow cytometry gating strategy for the $CD8^+$ PBMC T cell stimulation assay.

All events were first gated on a uniform cell size using area parameters and then for single events using area versus height parameters. Then T cells were selected for expression of CD3 and exclusion of a dye indicating their viability (Live-dead). Finally, T cells that expressed CD8, but not CD4, were analyzed for their intracellular cytokine expression.

Appendix Figure 2. Summary of experiments analyzing phosphorylation states of CD3 and ZAP-70 within treated D462-E4 T cell clone



Appendix Figure 2. Dot plots showing the phospho-specific staining levels of CD3 (left) or ZAP70 (right) of clone D462-E4 across independent experiment repetitions. The Y-axis shows the fold change of the geometric mean fluorescence intensity when the unstimulated T cell measurement was normalized to 1.



Appendix Figure 3. Flow cytometry gating strategy for MR1/Ag tetramer staining.

All events were first gated on single cell events using area and height parameters. Then cells were selected for expression of CD3 and exclusion of a dye indicating their viability (Live-dead). Next, cells were gated on a uniform lymphocyte size. Finally, T cells that did not express CD4 were analyzed for their ability to bind the MR1 tetramer.

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