ALTERNATIVE SPLICING OF MR1 REGULATES T CELL RESPONSES TO MYCOBACTERIA

By

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CERTIFICATE OF APPROVAL

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List of Abbreviations

5-OP-RU	5-(2-oxopropylideneamino)-6-D-ribitylaminouracil
6FP	6-formylpterin
BAL	Bronchoalveolar Lavage
β2M	β2-microglobulin
CDR	Complementarity Determining Region
DC	Dendritic Cell
DP	National Broadcasting Corporation
Dox	Doxycycline
ELISPOT	Enzyme-linked ImmunoSpot
FACS	Fluorescence activated cell sorting
GTex	Genotype Tissue Expression project
HLA	Human leukocyte antigen
IFN	Interferon
LAEC	Large Airway Epithelial Cell
MR1T	MR1-restricted T cell
MFI	Mean fluorescence intensity
MHC	Major Histocompatibility Complex
MR1	MHC class I related protein
PBMC	Peripheral blood mononuclear cells

PHA	Phytohemagglutinin
qRT-PCR	Quantitative Real-Time polymerase chain reaction
RNASeq	RNA Sequencing
SNP	Single nucleotide polymorphism
TAP	Transporter associated
TCR	T cell receptor
TNF	Tumor necrosis factor
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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Abstract

MR1-restricted T (MR1T) cells are part of a growing class of unconventional T cells that have emerged as important players in the early response to microbial infection. These cells, like other unconventional T cells, not only emerge from the thymus with immediate effector function, but also recognize non-peptide antigen produced by microbes. Importantly, this represents a paradigm by which unconventional T cells can sample the environment to detect infection, broadening the scope of antigen generated by microbial infection. MR1T cells, in particular, recognize small molecule antigen produced as intermediates in the Vitamin B biosynthesis pathway, introducing an effective means of detecting self-from non-self. The MR1T T cell receptor was originally thought to be semi-invariant and recognize a conserved array of ligands. Recent evidence suggests a wide range of diversity in the MR1T TCR, and this diversity can contribute to detection of a broader range of small molecules, including those distinct from those in the riboflavin synthesis pathway. Additionally, the importance of the MR1T TCRα and TCRβ chains in the recognition of antigen is not known. We show, using a panel of MR1T clones that share TCRα with differing TCRβ chains, that the MR1T TCRβ chain can contribute to recognition of both microbially infected dendritic cells, as well as distinguish between microbial ligand. These results suggest an important role in the TCRβ chain in helping to sense microbial infection. However, given the

wide range of antigen recognized by MR1T cells, as well as their capacity to produce inflammatory cytokines, and their enrichment in mucosal sites, likely in close proximity to commensals also capable of producing MR1T antigen, the question remains on how MR1T cell activation is regulated to prevent unwanted tissue damage or autoimmunity.

MR1T cells recognize antigen presented on the MHC Class I-related molecule, MR1, which is ubiquitously expressed on all cell types. MR1, unlike canonical MHC Class related molecules, is non-polymorphic, and is not expressed on the cell surface in the absence of ligand, suggesting MR1-mediated antigen presentation is tightly regulated to prevent aberrant MR1T expression. Additionally, MR1 undergoes alternative splicing to produce multiple isoforms, of which, only the full length, MR1A, is known to present antigen to MR1T cells. The function of the other splice variants isoforms is not known, and we show, using cell lines lacking endogenous MR1, and plasmids expressing each isoform, that one splice variant, MR1B, can inhibit MR1T activation by mycobacterial ligand presented on MR1A. We also show that transcript expression of MR1A and MR1B vary across tissues and cells from human blood, lung, and small intestine. Finally, we suggest relationship between relative expression of MR1A and MR1B in MR1-expressing thymocytes and frequency of MR1T cells in the thymus. Taken together, we suggest that alternative splicing of MR1 represents a possible means of regulating MR1-mediated antigen presentation to appropriately direct MR1T responses.

Chapter 1: Introduction

The innate and adaptive immune response are required for host defense against pathogens

In addition to physical barrier defense, an intact immune system is a key component in the protection against pathogenic infection. Vertebrates sense foreign microbes and patterns of infection through the host immune system, which have been historically defined as innate and adaptive immunity, based on the mechanisms of sensing self versus non-self.

The innate immune response is the first line of defense against microbial infection, and includes physical defenses, including the epithelial barrier, antimicrobial compounds, and the complement system for opsonization and direct killing of infected cells(Medzhitov and Janeway 1997). Cell types that mediate the innate immune response to pathogens include granulocytes, such as eosinophils and mast cells, neutrophils, and phagocytes. Broadly speaking, the innate immune response mediated by these cell types is characterized by the usage of germline encoded pathogen recognition receptors that are highly conserved and shared across individuals. These receptors recognize general patterns of molecular infection that are highly conserved and invariant across microbes, including lipopolysaccharide (LPS), or lipoteichoic acid (LTA), as well as viral DNA. While these pathogen recognition receptors are highly

limited in diversity, they are expressed by a wide variety of innate immune cells. The wide variety of mechanisms for controlling and killing infectious microbes contributes to the rapid activation of the innate immune response in the early defense against pathogens. Phagocytes, including macrophages and dendritic cells, not only promote direct killing of cells opsonized by the complement system, but also produce a wide range of inflammatory cytokines that serve to amplify the host immune response. Importantly, the innate immune response is not only critical for the direct control of microbial infection, cells mediating the innate immune response also play an important role in recruiting the adaptive immune response to augment the host response to pathogen.

While the innate immune response to pathogen is characterized by a general repertoire of receptors that recognize a broad patterns of microbial infection, the adaptive immune response is defined by the usage of a wide array of highly specific receptors. (Iwasaki and Medzhitov 2015) These receptors are generated through somatic gene rearrangements during B and T lymphocyte development in either the bone marrow or thymus, respectively, and therefore, every cell of the adaptive immune response expresses a unique rearranged antigen receptor. Thus, the antigen receptor repertoire for the adaptive immune response is highly diverse and able to recognize a wide array of specific microbial antigens. The hallmark of adaptive immunity is the clonal expansion of antigen specific B and T lymphocytes following exposure to antigen, which is why the adaptive immune response takes days to mount following infection, in contrast to the almost immediate response generated by the innate immune system. Typically, the adaptive immune response is activated following engulfment of a microbe by a

phagocytic cell, usually a dendritic cell, which then migrates to a lymph node and 'presents' the antigen to a naïve T cell, promotes T cell activation through costimulatory markers, and secretes cytokines to further amplify the immune response. Upon stimulation of naïve CD4+ helper T cells, B lymphocytes are stimulated to clonally expand and produce antigen specific antibodies to continue the adaptive immune response. During this response, antigen-specific naïve B and T lymphocytes clonally expand and differentiate into effector CD4+ or CD8+ T cells, and antibody secreting B cells, which are the major component of humoral immunity and will not be discussed in this thesis. The T cell response to infection is highly complex and consists of a tightly regulated signaling cascade following recognition of microbial antigen through major histocompatibility complex (MHC) molecules on the surface of infected cells. This thesis will focus exclusively on activation of CD8+ T lymphocytes and their roles in antimicrobial immunity.

General T cell biology

T lymphocytes are a key component of the adaptive immune response. Unlike other immune cells, T cell develop in the thymus, and express a rearranged T cell receptor (TCR) that recognizes microbial antigen. The TCR, which is similar in structure to the Ig immunoglobulin family, is expressed on the surface of the T cell and recognizes antigen complexed with an MHC molecule, typically Class I MHC for CD8+ T cells, and Class II MHC for CD4+ T cells (Davis and Bjorkman 1988). Upon recognition of antigen and the MHC complex on the surface of an antigen presenting cell, the TCR initiates a

complex signaling cascade that ultimately leads to activation of the T cell to mediate effector functions, including secretion of cytokine, killing of the target cell, and further amplification of the immune response.

T cell Receptors

T cell receptors can broadly be divided into two subclasses, αβ TCRs, which are more prevalent, and γδ TCRs, which are less abundant, and exhibit features distinct from canonical αβ TCRs. Early studies utilized monoclonal antibodies to block TCR activation and immunoprecipitate a subunit of the TCR, and follow-up studies were performed to further characterize this receptor (Haskins 1983). The T cell antigen receptor was ultimately identified as an 80-90 kD heterodimer on the surface of T lymphocytes (Allison and Lanier 1987). This heterodimer was shown to be an acidic alpha chain with a basic beta chain that were linked by a disulfide bridge. The TCR is not germline encoded. Like immunoglobulin genes secreted by B cells, the TCR consists of separately encoded germline segments that encode variable and constant regions of the gene (Hozumi and Tonegawa 1976, Kurosawa et al. 1981, Tonegawa et al. 1981). These segments are rearranged, as in B cells, by VDJ recombination, utilizing RAG1 and RAG2 enzymes, to generate the α and β chains of the TCR (Oettinger et al. 1990). The α chain is comprised of V and J segments, as well as a Constant (Cα) segment, while the β chain is encoded by V, D, and J segments and two constant (Cβ) segments. This rearrangement of the TCR is a primary contributor of the vast diversity observed in the αβ TCR repertoire, with recent high throughput sequencing analyses estimating greater than 10 million distinct TCR sequences (Qi et al. 2014, Arstila et al. 1999). Additionally, random pairing of αβ

TCRs and N-nucleotide addition following V(D)J recombination contributes to this high TCR diversity.

The T cell receptor, therefore, is a heterodimer of αβ subunits expressed on the surface of T cells. In each TCRα and TCRβ chain variable regions, there are 3 complementarity determining regions (CDRs), which provide interactions with the antigen/MHC complex and can contribute to specificity of antigen recognition (Patten et al. 1984). The CDR1 and CDR2 regions are germline encoded and contact the antigen presentation molecule, while the hypervariable CDR3 region in both the α and β chains are found at the junctional sites of V(D)J recombination and therefore are responsible for the most diversity within a TCR (Hozumi and Tonegawa 1976, Matthyssens, Hozumi, and Tonegawa 1976). This high diversity in the TCR repertoire enables T cells to optimally survey for antigens generated by microbial infection.

T cell coreceptors are essential in efficient recognition of the antigen/MHC complex

In addition to expressing CD3, a defining marker of T lymphocytes, as well as a rearranged TCR, T cells are characterized by the expression of the coreceptors CD4 and/or CD8, which is determined during T cell development in the thymus and contributes to effector T cell fate decisions. Both CD4 and CD8 function to augment activation through TCR engagement with an antigen presenting molecule. While the TCR binds the α1/β1 region of an MHC Class II molecule, CD4 engages with a conserved site the β2 component of MHC Class II (Doyle and Strominger 1987, Konig, Huang, and

Germain 1992). In contrast, CD8, a marker of cytotoxic T cells, engages with the α3 domain of an MHC class I molecule, which presents its ligand on the α1/α2 domain to the TCR (Salter et al. 1990). The engagement of CD4 and CD8 with MHC Class II and MHC Class I molecules functions to stabilizes TCR/MHC/antigen interactions and enable an efficient activation of the immune response.

Other costimulatory molecules have been shown to be essential for TCR recognition of an antigen/MHC complex and downstream signaling to initiate an effector response. The two-signal model of T cell activation establishes the importance of both TCR engagement with ligand/MHC (Signal 1), as well as engagement of TCR costimulatory molecules with their binding partners on the antigen presenting cell (Signal 2) (Mueller, Jenkins, and Schwartz 1989). The lack of costimulatory signals produced by these molecules, even with TCR engagement with an antigen presenting molecule, promotes T cell anergy, rather than stimulation. The most well characterized costimulatory molecule expressed on the surface of T cells is CD28, which engages with B7 on the antigen presenting cell, which triggers signal 2, and promotes T cell activation. However, other B7 binding molecules, such as CTLA-4, can be inhibitory, and function to suppress T cell responses and act as an immune checkpoint (Krummel and Allison 1995, Krummel, Sullivan, and Allison 1996, Engelhardt, Sullivan, and Allison 2006). Other costimulatory molecules that can provide Signal 2 include CD40L (CD154) on T cells that engages with CD40. This engagement, mediated on T follicular helper cell subsets, promotes B cell activation and class switching (Lederman et al. 1992).

T cell signaling

Upon engagement of the TCR with the antigen/MHC complex, a carefully regulated signaling cascade is initiated that amplifies the signal to allow for effector function (Chakraborty and Weiss 2014). This signal propagation is initiated by the TCR, but also requires components of the CD3 complex and other intracellular proteins (Brownlie and Zamoyska 2013). As each T cell expresses one specific TCR, it is capable of surveying for a specific microbial antigen. In addition to the TCR, the CD3 complex is critical in TCR signaling. It is comprised of 2 ε chain, 2 δ chains, and 2 ζ chains. Following initiation of TCR signaling, the SRC family molecule, LCK is recruited to the TCR/CD3 complex by the coreceptors CD4 or CD8 (Purbhoo et al. 2001). LCK phosphorylates Immunoreceptor Tyrosine Activating Motifs (ITAMs), of which there are 10 on the cytoplasmic tails of the various CD3 chains. Upon phosphorylation of the ITAMs, the T cell specific kinase Zap70 is recruited, and then phosphorylated by LCK to be activated (Chan et al. 1992). Activated Zap70 phosphorylates numerous transmembrane proteins to continue propagating the T cell activation signal-these proteins comprise the LAT signalsome complex (Zhang et al. 1998). Phospholipase C is recruited to the complex and then phosphorylated by a Tec kinase. Following activation, phospholipase C then activates the Ras and MAP Kinase pathways (Finco et al. 1998).

Simultaneously, there are numerous costimulatory molecules that also contribute to TCR signaling. CD28 on T cells engages with B7 proteins on antigen presenting cells to activate PI3K signaling to generate the second messengers DAG and IP3. IP3 binds to its receptor in the endoplasmic reticulum to release Ca2+ release, and high

intracellular Ca2+ activates other proteins, including the transcription factor, NFAT. DAG activates protein kinase C as well as the Ras and MAPK signaling pathways. The activation of these pathways ultimately leads to activation of transcription regulators in the nucleus, including AP1, STAT, NFAT, and NFκB, which can then promote T cell effector function by modifying gene expression. These pathways are summarized below (Figure 1.1)

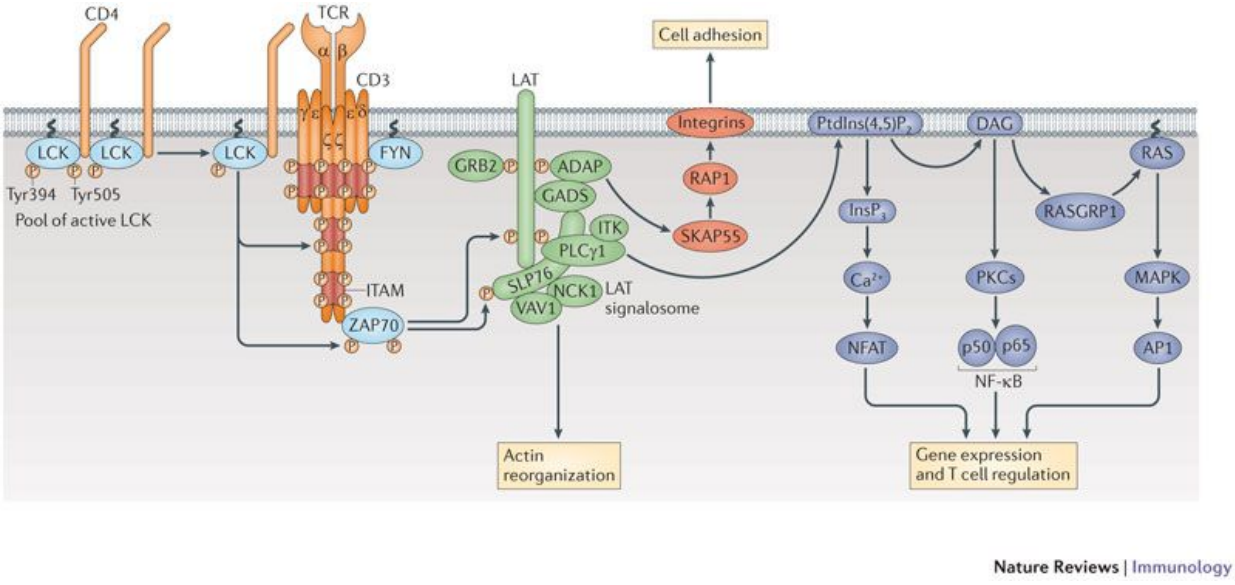

Figure 1.1: General Mechanisms of T cell activation. Reproduced with permission from Brownlie *et al*, *Nature Reviews Immunology* (Brownlie and Zamoyska 2013)

Effector functions of T cells following activation

Upon activation of T cells, a variety of effector functions can be generated that help contain an infection. These functions are typically specific to T cell lineage, either CD4 or CD8, although some overlap is observed. CD4+, or 'helper' T cells, are characterized by generating specific cytokines to further amplify the immune response

to infection. Naïve CD4+ T cells, upon differentiation, can promote antibody production by B cells (T follicular helper cells, T_{FH}), recruit granulocytes to the site of infection by production of IL-17 (Th17), activate an allergic response through the secretion of IL-5 and IL-13 (Th2) , or stimulate cytokine production in response to microbial infection, both through Th2 and Th17 subsets (Zhu and Paul 2010, Paul and Zhu 2010, Zhu, Yamane, and Paul 2010). Additionally, the Th1 subset of CD4+ T cells produces the inflammatory cytokines TNF-α and IFN-γ to promote killing of microbes.

CD8+ effector T cells are characterized by cytotoxic killing of microbially infected cells (Zhang and Bevan 2011). CD8+ T cells have been shown to be critical for the control of intracellular microbes-this was shown by an initial observation that these cells recognized components of Influenza nucleoprotein (Townsend, Gotch, and Davey 1985). CD8+ cells secrete the compounds perforin, granzyme B, and granulysin, which directly kill the infected cell. Granzymes and granulysin are delivered through perforin pores on the infected cell to stimulate cell death through Fas/FasL interactions and activation of caspases (Walch et al. 2015).

Upon differentiation, CD8+ T cells migrate to the site of infection, and can also secrete the Th1 cytokines TNF-α and IFN-γ to amplify immune cell recruitment and killing of infected microbes. These cytokines have been shown to be essential in the response to intracellular microbes. The Th1 cytokines can induce autophagy, a conserved process that coordinates the trafficking of macromolecules and organelles to the lysosome for degradation and can act as a means of cellular survival, particularly in response to intracellular infection (Ni Cheallaigh et al. 2011). Additionally, these cytokines upregulate

MHC protein expression on the surface of antigen presenting cells, which further amplifies the immune response (Skoskiewicz et al. 1985). Th1 cytokine production by CD4+ and CD8+ T cells has been shown to play a critical role in protection against intracellular infection with microbes, including Mtb, *Franciscella tularensis,* and *Salmonella* (Stenger et al. 1997, Flynn et al. 1993, Flynn 1995, Green, Difazio, and Flynn 2013). For my thesis work, I will be using T cell production of IFN-γ as a readout for T cell activation in response to external stimuli or infected antigen presenting cells.

Development of T cells in the thymus

T lymphocytes originate from a common lymphocyte progenitor generated from hematopoietic stem cells in the bone marrow (Stefanova, Dorfman, and Germain 2002, Germain, Stefanova, and Dorfman 2002, Germain 2002). These common lymphocyte progenitors migrate to the thymus, where they further differentiate into thymocytes as they migrate from the thymus cortex to the medulla, and finally, exit to the periphery as naïve T cells. In the thymic cortex, these common lymphocyte progenitors, which have lost the capacity to differentiate into B lymphocytes or NK cells, are now termed double negative (DN) thymocytes, as they lack expression of a TCR, as well as the surface markers, CD4 and CD8. However, they are committed to T cell lineage, which is mediated by Notch1 signaling and homing receptors. DN thymocytes can be further subdivided into sequential subsets, which are characterized by expression or lack of the markers CD25 and CD44 (Stages 1-4). These stages and T cell development are summarized below in Figure 1.2

Stage 3 DN cells express a pre-TCR-α chain, which pairs with a TCRβ chain that has already undergone V(D)J recombination mediated by RAG1 and RAG2. This αβ pair associates with the CD3 complex, and the T cell undergoes multiple further rounds of cell division followed by rearrangement of the TCRα chain to express the mature αβTCR. CD4 and CD8 surface proteins are now expressed, and the T cell is now termed a 'double positive' thymocyte, as it expresses both CD4 and CD8, as well as a rearranged TCR and the CD3 complex.

At this point, T cell selection occurs to generate T cells that can recognize antigen/MHC, migrate to the periphery and clonally expand. 90% of DP thymocytes do not recognize self-ligand/MHC expressed on thymic epithelial cells with enough avidity to be functional in the periphery, and instead, undergo 'death by neglect'. Those DP thymocytes that bind self-ligand/MHC on cortical thymic epithelial cells with abnormally high avidity undergo negative selection and are marked for apoptosis, as these cells could cause autoimmunity if released into the periphery (Anderson et al. 1994). Negative selection, therefore, promotes central tolerance of self-antigens. The remainder of the cells undergo positive selection. Thymocytes that encode a TCR that recognizes self-ligand on MHC Class I molecules express CD8+ T cells, while thymocytes encoding a TCR recognizing ligand on MHC Class II are fated to become CD4+ T cells. Positively selected CD4 or CD8+ thymocytes migrate to the medulla and engage with self-antigen/MHC on the surface of medullary thymic epithelial cells, plasmacytoid dendritic cells, B cells, as well as resident and migratory dendritic cells (Klein et al. 2014). Following this engagement and sampling of self-ligand, selected naïve thymocytes exit

the thymus into the periphery and circulate through the blood and lymph until exposure to foreign antigen on MHC Class I and Class II. The differences between thymic selection in classically restricted T cells versus unconventional T cells is not well understood, and mouse models and *in vitro* experimental systems must be utilized to further explore the similarities and differences.

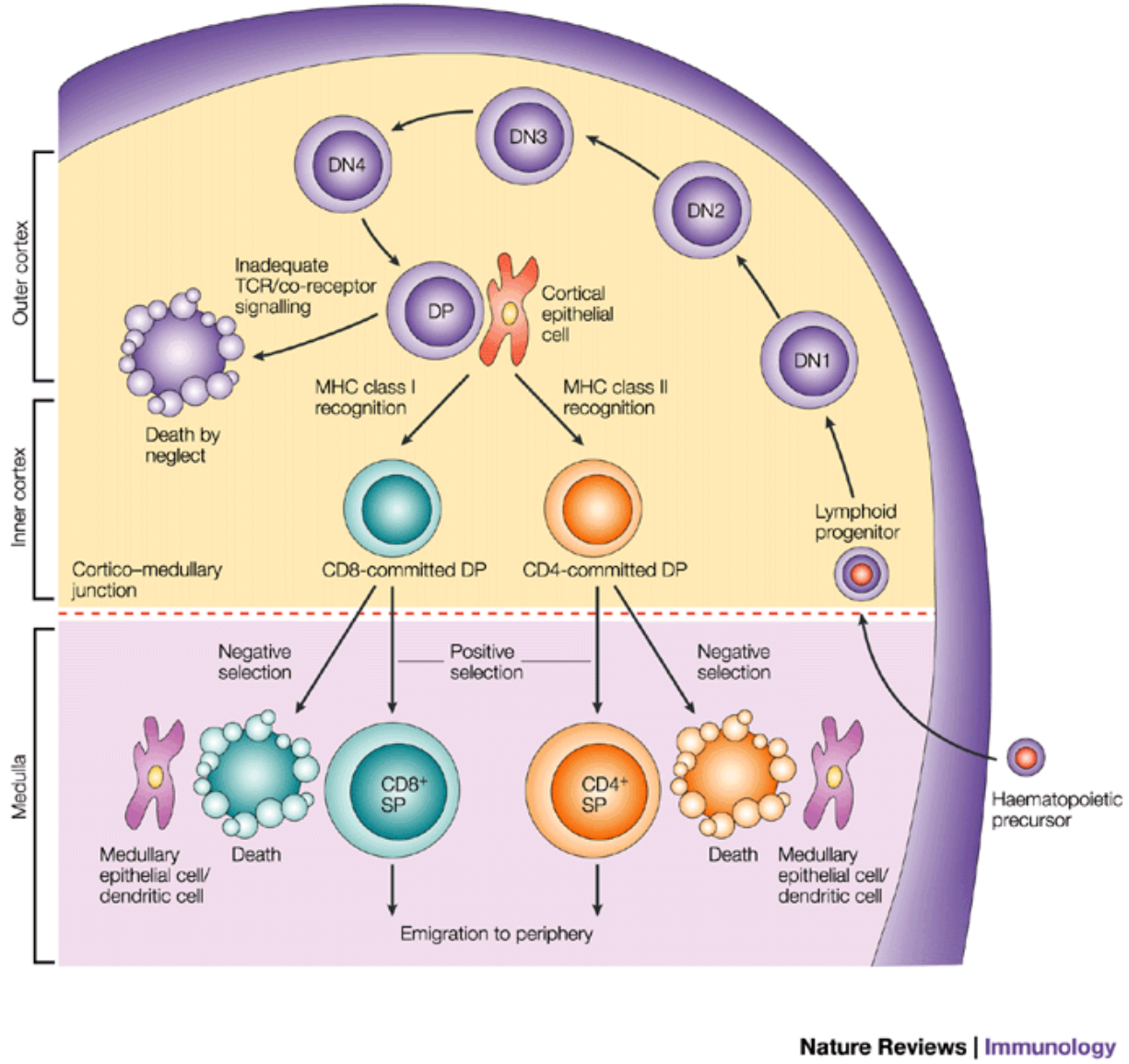

Figure 1.2: T cell development in the thymus. Reproduced with permission from Germain *et al*, *Nature Reviews, Immunology*.

General Antigen Presentation

Structure of MHC Class I molecules

CD4+ T Cells typically recognize antigen presented on MHC Class II, while CD8+ T cells are stimulated by antigen presented on MHC Class I molecules. The Major Histocompatibility Complex, or MHC, is common across all jawed vertebrates, and is essential in stimulating T cell responses upon binding of an antigen generated from a microbe. The MHC, which is expressed on chromosome 6 in humans, encodes genes necessary for antigen presentation. Seminal work showed that showed that the mouse cytotoxic T cell receptor recognized not only peptide antigen from a viral infected cell, but also a molecule of the mouse major histocompatibility complex, either H2-k or H2-d (Zinkernagel 1975). Sequencing of the MHC loci revealed a high degree of polymorphism in the immune genes encoded by the MHC (Parham et al. 1988). The Class I MHC fold for HLA-A, HLA-B, or HLA-C, is derived by a single polypeptide transmembrane heavy chain that associates with β2-microglobulin on the cell surface. The genome organization of the HLA-A showed that MHC Class I molecules encode an α1 and α2 domain, which has been shown to comprise the antigen-binding groove, and is where the majority of MHC polymorphism occurs, an α3 domain that directly engages with β2-microglobulin, and a transmembrane domain (Malissen, Malissen, and Jordan 1982). Crystallography of HLA-A2 showed that the peptide binding groove was comprised of a platform of β-sheets capped by α-helices and could accommodate a peptide of 8-10 amino acids long (Bjorkman et al. 2005). Studies using an OVA peptide and the mouse Class I MHC molecule H-2d showed that OVA323-339 specifically bound

H-2d, but did not bind H-2k, indicating that MHC Class I molecules are highly specific for a ligand, like due to the polymorphism present in the antigen binding groove (Buus et al. 1986, Sette et al. 1987). This, combined with the high level of diversity in the CD8+ TCR repertoire, enables MHC molecules to sample a broad array of peptide antigens generated from microbial infection.

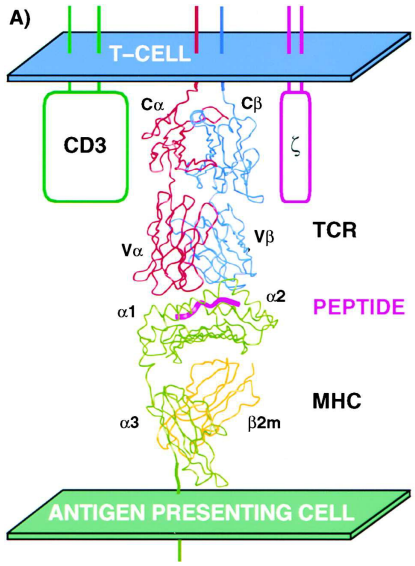

Figure 1.3: Structure of the canonical MHC/peptide/CD8+ TCR complex.
Reproduced with permission from Hennecke, *et al*, *Cell.*

Generation of antigen from microbial infection and loading of MHC molecules

Before a T cell can be stimulated by an antigen/MHC complex, the antigen must be generated by proteolytic processing and degradation of the microbe (Blum, Wearsch, and Cresswell 2013). Generation of peptide from extracellular infection occurs upon internalization of the microbe, followed by trafficking through a vesicular pathway with increasingly acidic compartments, including the late endosome and lysosome, before antigenic peptides are generated through lysosomal proteolysis, and loaded onto MHC Class II molecules for presentation to CD4+ T cells. Intracellular pathogens residing in

the cytosol undergo degradation through the proteasome complex. Following this, these peptide antigens are translocated to the ER through the transporter associated with antigen processing (TAP) for loading onto MHC Class I. Binding of peptide antigen with MHC Class I occurs in concert with assembly of the MHC Class I heavy chain with β2-microglobulin. Following this loading of MHC Class I with peptide in the ER, the complex can then traffic to the cell surface through the Golgi for activation of CD8+ T cells. In addition to TAP, MHC Class I loading with peptide and expression on the cell surface requires the chaperones tapasin, calreticulin, and ERp57, which together, form the peptide loading complex (Flutter and Gao 2004, Herr et al. 2012, Blees et al. 2017). Following expression on the cell surface, the MHC-peptide complex engages with the TCR, stimulating T cell effector function and promoting killing of the infected cell.

Unconventional T cells respond to non-peptide antigen presented on MHC Class Ib molecules

There is a growing body of evidence of the existence of T cells that recognize non-peptide antigen derived from microbial infection. Though these T cells are primarily CD8+ T cells, express a rearranged αβ TCR, and can promote bacterial killing through the production of cytokines and effector molecules, they also share features of innate immune cells (Godfrey et al. 2015). These T cells typically emerge from the thymus with immediate effector function and do not need to undergo priming and clonal expansion upon initial encounter with antigen. Additionally, these cells typically express invariant T cell receptors, with a much more limited repertoire and therefore, are sensors of classes of microbial antigen. The antigens recognized by these cells are also unconventional,

presented on separate and distinct MHC Class Ib, or class I like, molecules, and comprise of leader peptides, lipids and small molecules generated by microbes. These T cells have been shown to play a critical role in the response to microbial infection and can act as sensors of foreign antigens that are not recognized by canonical CD8+ T cells, thereby broadening the scope of the immune system to detect and respond to pathogen. Importantly, though innate and adaptive immunity have typically categorized as separate spheres of the immune response, these T cells bridge the gap, and are also classified as innate-like T cells (Margulies 2014). Below, I will summarize recent findings about specific classes of human unconventional T cell subsets and their restricting molecules. As my dissertation work has been focused on the role of MR1-restricted T cells, I will provide a more thorough introduction to these cells following this summary.

CD1-restricted T cells

The CD1 gene locus is encoded on chromosome 1, separate from the MHC, but encodes antigen presenting molecules that present non-peptide antigen to stimulate T cells. The five CD1 molecules (CD1a, CD1b, CD1c, CD1d, and CD1e) contain hydrophobic grooves to enable binding of lipids, and all but CD1e have been shown to bind these lipid antigens generated from microbial infection, including mycobacteria, to activate CD1-restricted T cells (Scott-Browne et al. 2007, Salio et al. 2014). Each CD1 molecule, however, presents distinct classes of lipid antigens that are specific to the variations in the antigen binding grooves with distinct intracellular trafficking pathways (Porcelli, Morita, and Brenner 1992, Beckman et al. 1994, Brigl and Brenner 2004). Like classical T cells, CD1-restricted T cells can express the CD4 or CD8, or neither, which

corresponds to their effector function, and upon activation, can secrete Th1, Th2, or Th17 cytokines and promote lysis of the infected cell. CD1-restricted T cells express, typically, an $\alpha\beta$ TCR, with a small subset instead expressing the $\gamma\delta$ TCR (Beckman et al. 1994).

A subset of CD1d-restricted T cells, called invariant Natural Killer cells (iNKT), utilize a T cell receptor, that, unlike canonical T cells, is semi-invariant and conserved in humans and mice. These iNKT cells and recognition of CD1d-bound antigen are the most well studied of all the CD1-restricted T cells. Structural analyses of Type I iNKT TCR binding to the CD1d-antigen complex shows that unlike canonical MHC molecules, the iNKT TCRα chain dominates in recognition of the antigen/CD1d complex, potentially in an induced-fit model due to the flexibility in the CD1d binding domain. CD1d binds and presents glycolipids to stimulate the iNKT TCR, and the prototypical lipid is α-GalCer, (Borg, Wun, et al. 2007, Borg, Kjer-Nielsen, et al. 2007). While canonical CD8+ T cells require thymic epithelium for their selection and development, iNKT cells are selected on double positive cortical thymocytes expressing CD1d (Gapin et al. 2001). Additionally, iNKT cells are hypothesized to act as immediate effector cells *in vivo*, suggesting that they play a key role in early detection of microbial infection. iNKT cells have been shown to play an important role in the response to mycobacterial infection, including Mtb, which can generate an abundance of glycolipids that can be presented on CD1 molecules to activate T cell responses (Cerundolo et al. 2009). As CD1-restricted T cells are hypothesized to be immediate effectors and resident at mucosal sites, their ability to recognize non-peptide microbial antigen broadens the scope by which the immune system can sense infection.

HLA-E, HLA-F, and HLA-G-restricted T cells

In addition to encoding canonical MHC-I and MHC-II molecules, the MHC locus also encodes a group of MHC-like molecules (human HLA-E, HLA-F, HLA-G, HFE, mouse H2-M, H2-T, H2-Q) that are similar in structure to class I MHC, but don't necessarily function as antigen presenting molecules. HLA-G has been well studied in its role in promoting maternal tolerance of the developing fetus, and soluble alleles of HLA-G are associated with markers of fetal health (Wiendl et al. 2003). HLA-G has been shown to downregulate cytotoxic responses mediated by HLA-A and HLA-B, and its expression in the placenta suggests that this immunomodulatory role may be of importance in pregnancy. HLA-G is a ligand for inhibitory receptors on NK cells, again supporting the hypothesis that HLA-G is crucial in immune tolerance (Rajagopalan and Long 1999).

HLA-F has recently been shown to present peptide antigen to inhibitory NK receptors (Goodridge et al. 2013, Dulberger et al. 2017). The HLA-F peptide binding groove is unusual compared to canonical Class I MHC, as it has the capacity to present peptides of longer length that can extend beyond the groove. A diverse array of peptides presented on HLA-F are recognized by the inhibitory NK receptor, LIR1, and this likely exerts an immunomodulatory role in downregulating NK responses and inflammation. HLA-F has also been thought to play a protective role in pregnancy, and the potential of HLA-F to present antigen to a non T cell subset expands the role of MHC and MHC-like molecules in the regulation of immune responses.

HLA-E, and its mouse counterpart, Qa-1, present leader sequences of MHC Class I (Adams and Luoma 2013). The hydrophobic binding pocket in the monomorphic HLA-E molecule is highly conserved, and optimally suited to bind and engage with leader peptides. Upon binding leader peptides, HLA-E engages with NKG2 receptors on the surface of NK cells and promotes an inhibitory signal to combat immune evasion strategies of microbes, which typically stimulate Class I downregulation (Braud et al. 1998, Braud and McMichael 1999). However, HLA-E also has the capacity to act as a traditional MHC molecule and activate CD8+ T cells upon exposure to microbial antigen, including glycopeptide antigen derived from mycobacteria, but also from *Salmonella typhimurium,* and viruses, including HIV, LCMV, CMV, HBV, and SIV (Harriff et al. 2017, Joosten, Sullivan, and Ottenhoff 2016, Heinzel et al. 2002). Recently, rhesus macaques mounted a CD8+ T cell response following exposure to SIV peptides presented on HLA-E (Hansen et al. 2016). These SIV peptides consisted of a broad array of antigens, thereby suggesting a mechanism by which diverse HLA-E-restricted T cell responses can be stimulated by antigen presentation. The dual role of HLA-E in regulating NK cell activity and the stimulation of CD8+ T cells highlights the potential of MHC-like molecules in having a wide range of functions in the regulation of the immune response.

MAIT cells and MR1

Though the majority of work on characterizing $\alpha\beta$ T cell receptors focused on CD4+ or CD8+ T cells, the question remained about the nature of TCR receptors expressed by T cells negative for the CD4 and CD8 coreceptors (DN T cells). Murine and human DN T cells had previously been shown to express $\alpha\beta$ TCRs, as well as the

CD3 coreceptor (Londei et al. 1989, Miescher et al. 1988). As DN αβ T cells had recently been shown to recognize members of the non-classical CD1b family of antigen presentation molecules, the possibility remained that these DN T cells could recognize distinct non-classical MHC and antigen (Porcelli, Morita, and Brenner 1992). DN T cells were isolated from human peripheral blood lymphocytes (PBMC) and the sequence of expressed TCR α chains was determined using a combination of RACE single-sided PCR amplification and quantitative PCR (Porcelli et al. 1993). These methods revealed that clones isolated from multiple donors expressed a novel TCRα chain: Vα7.2, that appeared to be joined predominantly to a Jα14 segment, with a conserved joining region between the V and J segments. Further analysis showed that the Vα7.2 chain was relatively abundant, comprising anywhere from 3-10% of total human TCR cDNA. Quantitative PCR analysis of Vβ usage from DN αβ T cells showed an enrichment of Vβ2, Vβ8, Vβ11, and Vβ13 genes, suggesting that DN T cells display preferential usage of TCR chains, and the Vβ repertoire was both oligoclonal and limited. This early work by Porcelli and colleagues also introduced the possibility that the ligand for this TCR was non-polymorphic with a limited repertoire, but remained unknown.

Analysis of T cell subsets isolated from human, bovine, or murine blood showed that the transcript encoding Vα7.2-Jα33 (human) and Vα19-Jα33 (bovine, murine) TCRs were enriched in the DN population of all three species, as well as in human CD8+ T cells (Tilloy et al. 1999). Limiting dilution analysis and sequencing in human DN and CD8+ T cells revealed the presence of Vα7.2-Jα33 TCRs at approximately 13% (DN) and 2% (CD8+) frequency of all TCRs. In this study, Tilloy et al characterized this TCR

as an 'invariant' TCR, due the lack of heterogeneity observed in the VJ segment and no nucleotide trimming or N-addition normally observed in αβ TCRs, and also observed that the α chain preferentially was expressed with a limited Vβ repertoire. Tilloy also speculated about the selecting ligand for these cells, which had not been elucidated. Mouse models deficient for MHC showed that the selecting ligand was dependent on β2-microglobulin, but appeared to be independent of TAP, and likely not a MHC Class Ia or Class II molecule, as the frequency of Vα19-Jα33 cells was higher in $K^{b-/-}/D^{b-/-}$ mice. Additionally, as these cells were not dependent on the nonclassical MHC molecule, CD1, it was speculated that the selecting ligand had an immune function that was distinct from already described MHC and MHC-like molecules.

Further PCR analysis of the α chain TCR confirmed that Vα7.2-Jα33 transcripts were enriched in gut lamina propria (Treiner et al. 2003). Mouse models in this study confirmed that these cells, termed mucosal associated invariant T (MAIT) cells, were also localized to the murine lamina propria and lung, and dependent on both an intact immune system, particularly, B cells for their development. Subsequent studies using MAIT cell hybridomas, cell lines overexpressing MR1, and TCR blocking antibody showed that MAIT cells recognized the recently described non-classical MHC-related molecule, MR1. MR1-knockout mice showed that MAIT cells were absent in MR1-deficient mice, further confirming that MAIT cells were restricted by MR1. As MAIT cells were found to also be dependent on the host microbiota, it was thought that the ligand bound to MR1 was of microbial origin, either peptide or non-peptide, and distinct from those that bound MHC Class Ia, HLA-E, and CD1.

MR1 is a non-classical MHC like molecule

The gene for MHC-related protein 1 (*MR1*) was first reported in 1995 by Hashimoto, who used a PCR based approach to amplify the conserved α3 domain upon screening a human thymus cDNA library to identify other MHC like molecules (Hashimoto, Hirai, and Kurosawa 1995). The gene amplified using this approach was mapped to chromosome 1, not chromosome 6 like canonical MHC, however, the predicted gene and domain organization was thought to be similar to MHC Ia molecules. Subsequent cloning and sequencing revealed murine and human *MR1* displayed a high degree of sequence homology, with nearly 90% sequence similarity between murine and human α1 and α2 domains (Yamaguchi et al. 1997, Yamaguchi, Kurosawa, and Hashimoto 1998). Due to the high degree of homology between classical MHC and *MR1*, it was thought that MR1 functioned to present antigen to stimulate T cells, although the candidate T cells had not yet been identified. Upon further examination of the *MR1* gene, it was found that MR1 was organized in a manner similar to canonical MHC, with a leader peptide, and exons encoding α1, α2, and α3 domains, as well as a transmembrane domain (Riegert, Wanner, and Bahram 1998). Additionally, *MR1* was ubiquitously expressed in human and murine tissues and cell lines. Importantly, unlike MHC Class Ia molecules, which are characterized by polymorphism, *MR1* was found to be non-polymorphic, and indeed, displayed the highest degree of conservation across species, but the significance of this finding, or that of MR1 in immune activation, was unknown (Parra-Cuadrado et al. 2000, Parra-Cuadrado et al. 2001). It was clear, however, that

MR1 was one of many “MHC-like” molecules, including CD1, Zap70, and HLA-E, that had features distinct from canonical MHC Class I.

In addition to the high degree of conservation of MR1 and lack of polymorphism, the gene organization of *MR1* displayed features distinct from class I MHC (Riegert, Wanner, and Bahram 1998). *MR1* was shown to encode a notably long intron 1 and a variable length 3’ UTR, the importance of which are not elucidated. However, more recent reports on non-coding RNA function have highlighted that intronic sequences and UTRs play an essential role in the regulation of gene expression, either by modifying recruitment of the post-transcriptional machinery, or through small nucleotide polymorphisms that impact gene and protein expression (Mercer et al. 2011).

Intriguingly, *MR1* pre-mRNA undergoes alternative splicing to produce multiple isoforms, and these isoforms were detectable in tissues and cell lines by both Northern blot and PCR (Hashimoto 2016). The mechanism of alternative splicing is generated through canonical AT/CG exon skipping. These splice variants of MR1 are characterized by a lack of the α3 domain, but the significance of this is not known, as whether CD8 actually engages with the MR1 α3 domain is of debate (Riegert, Wanner, and Bahram 1998). These isoforms have not been well characterized, although MR1A, the full length isoform, is known to stimulate MR1T cells. My dissertation work has been focused on understanding the function these isoforms in T cell activation and presentation of mycobacterial ligand.

Prior to the discovery that the function of MR1 was to restrict MAIT, or MR1-restricted T cells, a biochemical approach was taken to understand the potential role of

MR1 (Miley et al. 2003). An epitope tagging strategy to bind classical and non-classical MHC was used in combination with baculovirus expression systems. This system enabled detection of the ectodomain of MR1, and revealed that MR1 failed to refold in the presence of components that promote refolding of MHC Class Ia, suggesting that the ligand was not of peptide origin. MR1 was shown to engage with β2-microglobulin, however, it was unclear whether MR1 interacted with the peptide loading complex, or whether MR1 could present antigen(Huang et al. 2005).

MR1 was soon revealed to restrict MAIT cells, both in mouse and human cells, thereby functioning as an antigen presentation molecule (Treiner et al. 2003). However, the nature of the antigen, as well as whether MR1 could present antigen from microbial infection was still yet to be elucidated.

MR1 presents microbial antigen to stimulate MR1-restricted T cells

The discovery that, MAIT cells, or MR1-restricted T (MR1T) cells engaged with the non-classical molecule MR1 gave credence to the hypothesis that MR1T cells played a role in recognizing microbial infection, which was supported by the observation that MR1T cells were absent in the periphery of germ-free mice (Treiner et al. 2003). The physiological role for MR1T cells in immunity was described in two seminal studies that showed that MR1T cells recognized MR1-bound antigen derived from microbes, including bacteria and fungi(Gold et al. 2013, Le Bourhis et al. 2010)

Our laboratory was focused on the role of human non-classical T cells in the response to *M. tuberculosis* infection. An initial finding showed that T cells isolated from the periphery of individuals naïve for exposure to *M. tuberculosis* were still capable of

responding to *M. tuberculosis* infection *ex vivo*, by production of the Th1 cytokines, TNFα and IFNγ (Lewinsohn et al. 2000). As these T cells did not require classical antigen presentation mechanisms to respond to *M. tuberculosis* infection, they were thought to be non-classically restricted by a then-unknown MHC. Subsequently, a population of 'innate-like' *M. tuberculosis*-reactive CD8+ $\alpha\beta$ T cells were isolated from human thymus that were also capable of producing Th1 pro-inflammatory cytokines without the need for classical antigen presentation (Gold et al. 2008). Based on these prior studies, Gold performed limiting dilution analysis to clone T cells from the blood of *M. tuberculosis* infected and uninfected individuals (Gold et al. 2010). Analysis of the antimycobacterial responses of each T cell clone showed that they were restricted by MR1 and not only produced inflammatory cytokines in response to *M. tuberculosis*-infected lung epithelial cells *ex vivo,* but also to infection by *S. typhimurium, Candida albicans, Escherischa coli, Staphylococcus aureus, M. bovis* BCG, and *M. smegmatis*, but not *Listeria monocytogenes*, suggesting that MR1T cells played an important role in recognizing a broad array of microbial infections.

A separate study by Le Bourhis and colleagues purified TRAV1-2+ CD161+ T cells from humans and showed that these cells were produced IL2 in response to MR1-mediated presentation of ligand generated by *Mycobacterium abseccus* or *E. coli*-infection of monocytes (Le Bourhis et al. 2010). Transgenic mouse models expressing the TRAV1/TRBV6 TCR were used to show that murine MR1T cells were activated by a broad array of microbes, including *C. albicans, E. coli, Candida glabrata, Klebsiella pneumoniae, Lactobacillus acidophilus, Pseudomonas aeruginosa, Staphylococcus*

epidermitis, and *S. aureus*. concurring with the report by Gold *et al*. However, not all microbes tested were capable of activating MR1T cells-including the bacteria *Enterococcus faecalis,* and *Streptococcus pyogenes*, as well as viruses.

These two studies, in mouse and humans, provided definitive early evidence that MR1T cells produced inflammatory cytokine in response to infection by a broad array of bacteria and fungi, but not viruses and that this response was dependent on the non-classical antigen presenting molecule, MR1. The antigen detected was still unknown, but it was postulated that this antigen likely was common to the microbes that were capable of stimulating MR1T cell responses.

MR1T cells and the response to infection

The previously described early studies confirmed that MR1T cells played a key role in the detection of a broad array of microbial infection. Mouse models lacking MR1 lacked MR1T cells and those mice were susceptible to infection by mycobacteria and increased bacterial burden (Chua et al. 2012). Mycobacteria-responsive MR1T cells from mouse were shown to have a similar effector phenotype as shown by Le Bourhis *et al* and Gold *et al*, but also produced the cytokine IL-17, and produced the antimicrobial compound, nitric oxide (NO). MR1T cells preferentially expanded in the lung during acute *F. tularensis* infection by day 14 of infection and produced the cytokines IFN-γ and TNF-α, as well as IL-17 (Meierovics, Yankelevich, and Cowley 2013). Importantly, mice lacking MR1 displayed impaired production of inflammatory cytokine and iNOS, as well as a higher bacterial burden. Additionally, this study was the first to implicate MR1T cells

in the recruitment of the adaptive T cell response to the site of infection, as MR1-deficient mice infected with *F. tularensis* showed decreased recruitment of CD4+ and CD8+ T cells to the lung.

MR1-deficient mice were also shown to be more susceptible to infection by *Klebsiella pneumoniae*, with an impaired cytokine response, but paradoxically, these mice were not shown to be impaired in clearance of *E. coli, Shigella dysentarie,* or *Yersenia enterocolitica,* but cytokine production in response to these bacteria was not shown *(Georgel et al. 2011).* This apparent paradox may indicate that MR1T cells responding at differing tissue sites (pulmonary versus gut or oral mucosa), may have different effector functions that are specific for the resident tissue site. These murine studies again suggest an important role for MR1 and MR1T cells in the control of bacterial infection.

Murine models support the hypothesis that MR1T cells not only are critical components of the immune response to infection, but also are likely playing an important role in the early detection of infection. A recent study on the effects of BCG vaccination in rhesus macaques showed that the frequency of MR1/5OP-Ru tetramer positive MR1T cells increased following vaccination and these cells were licensed to produce granzyme B (Greene et al. 2016). In humans, MR1T cells are decreased in peripheral blood of individuals with active tuberculosis, as compared to individuals with latent disease or uninfected, again suggesting that MR1T cells can be recruited to the site of infection (Gold et al. 2010). These cells are enriched in the lung, and can efficiently respond to

Mtb-infected lung epithelial cells, suggesting that they represent an important means of defense against Mtb-infection in humans (Gold and Lewinsohn 2011, Harriff et al. 2014).

The practicality of studying pathogenic infection in humans has limited our current understanding of MR1T responses to microbial challenge. Our laboratory and others utilize *ex vivo* PBMC and lung tissue, combined with molecular approaches to model MR1T function. Vaccination challenges in humans have also revealed MR1T responses against pathogens. Infection of monocytes with *Shigella flexneri* resulted in an MR1-dependnet activation of MR1T responses, as measured by expression of the activation markers CD69 and CD25 by flow cytometry, and promoted lysis of infected cells (Le Bourhis et al. 2010). Additionally, human volunteers vaccinated with an attenuated strain of *S. dysentarie 1* showed an increased activation of MR1T cells, measured by HLA-DR, although this study did not examine functional responses of MR1T cells from peripheral blood. A more recent study showed that in human volunteers challenged with *Salmonella paratyphi A*, MR1T frequencies were increased, along with skewing of the MR1T TCRβ repertoire (Howson et al. 2018). Thus, it is important to note that not only are MR1T cells activated during bacterial infection and are likely playing a critical role in the response to a wide range of infections, exposure to microbial antigen is likely impacting the MR1T TCR repertoire. How that changes MR1T memory and responses to subsequent bacterial challenges is as yet unknown.

MR1T cells are depleted in HIV infection

MR1T cells appear to be implicated in the response to infection with HIV-1. Similar to the findings in individuals with active tuberculosis, CD161+ CD8+ T cells were

depleted in the blood in early HIV infection, expressed tissue homing markers, and were enriched in the gut (Cosgrove et al. 2013). HAART did not restore the frequency of CD161+ CD8+ MR1T cells.

More robust analyses following up on these earlier findings confirmed that Vα7.2+ MR1T cells were persistently depleted in the blood of individuals with chronic untreated HIV infection (Leeansyah et al. 2013). MR1T cells from individuals with chronic HIV infection exposed to fixed *E. coli* displayed a decreased activation (as measured by CD69), as well as impaired production of IL-17, TNF-α and IFN-γ, which was dependent on MR1 and partially restored by treatment. Depletion of MR1T cells and subsequent restoration following early antiretroviral therapy was also observed in HIV+ children (Khaitan et al. 2016). These findings suggest that MR1T cells in HIV infection may be impaired in defense against common pathogens, and may implicate MR1T cell defects in the acquisition of opportunistic infection. HIV infection is known to cause defects in the gut mucosal immune response, which is confirmed by the observations that MR1T cells from HIV-infected individuals display a weakened response to fixed *E.coli.* Intriguingly, MR1T cells also were observed to have an activated effector phenotype in HIV+ individuals, expressing high levels of the surface activation markers CD127, CD27, and CD38, HLA-DR, Tim3, and CD57, but did lose CD161 expression over time. MR1T cells in the rectal mucosa of HIV+ individuals were not significantly decreased, perhaps suggesting that these cells are recruited to the gut and rectal mucosa during HIV-1 infection, similar to the findings in active tuberculosis. A separate study confirmed these findings and showed that MR1T cells were depleted not only from the blood of HIV-

infected individuals, but also the lymph nodes (Eberhard et al. 2014). It is not yet clear whether earlier detection of HIV and initiation of HAART can restore MR1T frequencies and effector phenotype, but follow up studies utilizing the MR1/5-OP-RU tetramer and more physiologically relevant animal models can further define the importance of MR1T cells in HIV infection.

Co infection with HIV and tuberculosis is common in HIV and TB-endemic areas. A study examining MR1T cells in humans with HIV and TB coinfection showed a decreased frequency of CD161+ CD8+ T cells in the periphery of individuals with acute HIV infection or HIV and TB coinfection, as compared to HIV and TB negative individuals, with a majority (>80%) of CD161+ CD8+ cells expressing the MR1T Vα7.2 receptor (Wong et al. 2013). Interestingly, supporting the above findings, elevated levels of PD-1, a marker of T cell exhaustion, were observed in the MR1T cells of individuals with HIV/TB coinfection (Saeidi et al. 2015). Additionally, MRT frequencies were observed to irreversibility diminish both in chronic HCV infection, as well as in HIV/HCV coinfection (Eberhard et al. 2014, Eberhard et al. 2016).

Taken together, these and other studies examining the phenotype of MR1T cells following HIV infection indicate that MR1T may be playing an important role in controlling opportunistic infection, and that HIV infection may diminish MR1T function or exhaust MR1T cells, thereby creating an environment more favorable to HIV-associated infections. What is not yet understood is whether an HIV-infected cell is generating a unique ligand on the surface of MR1 to stimulate MR1T cells, or if the consequences of

HIV infection are due to other mechanisms independent of the TCR and antigen presentation.

MR1T cells can also be activated in a TCR-independent mechanism through IL-12 and IL-18

MR1T cells are typically activated by microbial antigen presented by MR1 on the surface of infected cells. However, other CD8+ T cell subtypes, including iNKT cells, can be stimulated to produce cytokine and expand in the absence of MHC-bound antigen, which is primarily driven by the cytokine interleukin 12 (IL-12) (Brigl et al. 2011, Brennan et al. 2011). Chua and colleagues showed that MR1T cells isolated from Vα19 transgenic mice, similarly, were capable of producing the inflammatory cytokine IFNγ in response to IL12 stimulation in the absence of infection, but cells isolated from MR1$^{-/-}$ Vα19 mice were incapable of stimulation and cytokine production(Chua et al. 2012). Moreover, murine MR1T cell activation by mycobacteria was ablated by incubation with an IL12 blocking antibody. MR1T cells express high levels of the IL-12-receptor β2 (IL12β2), again supporting the hypothesis that IL-12 can contribute to TCR-independent cytokine production or augment TCR-dependent activation (Billerbeck et al. 2010) (Erin Meermeier, unpublished observations).

Though initial observations suggested that MR1T cells do not respond to viral antigen, subsequent reports showed MR1T cells could be stimulated by acute and chronic viral infection in an MR1-independent manner (DENV, Influenza A, HCV) (Wilgenburg et al. 2018). These viral infections, instead, stimulated MR1T cell activation through IL-18, IL-12, and IL-15, as well as type I interferons.

Ussher *et al* showed that MR1T cells could be activated in response to *E. faecalis* infection in the absence of MR1-dependent stimulation (Ussher et al. 2014). Instead, these MR1T cell responses were generated through IL-12 and IL-18, similar to the observation that the MR1T response to *M. bovis* BCG was ablated by IL-12 blockade. To elucidate the contribution of TCR-independent activation to MR1T function, CD161+ CD8+ T cells from PBMC were stimulated to produce with recombinant IL12 and IL18 in the presence or absence of MR1 blocking antibodies, and cytokine production by these cells was not dependent on MR1.(Ussher et al. 2014). Additionally, these Vα7.2-expressing MR1T cells expressed high levels of the IL-18 receptor, along with the Il-12 receptor.

Though the majority of T cell responses to infection or stimuli occur through a TCR-dependent mechanism and antigen presentation, most T cells are capable of stimulation through cytokines. This IL-12/IL-18 mediated responsiveness of MR1T cells may be playing a role to augment TCR-dependent activation or broaden the role of MR1T cells in the response to infection. The implications of cytokine mediated and antigen independent MR1T activation, particularly in the setting of autoimmunity, are as yet unknown, but are an important consideration of MR1T cells as therapeutic targets.

The role of MR1 and MR1T cells in disease

Though there is significant experimental evidence that MR1T cells are important sensors of early microbial infection, the question remains of the significance of MR1T

cells in disease, both infectious and non-infectious. As MR1T cells are highly enriched at mucosal sites where there is an abundance of commensal species that can produce stimulatory ligands, there remains a potential implication in autoimmune disease. As our understanding of MR1T cells and MR1 has expanded, there is increasing evidence for MR1T cells in a wide range of disease pathologies. I have previously summarized the evidence for a protective role of MR1T cells in infection, and therefore, will be focusing this following section on other diseases where MR1T cells may be implicated.

MR1T cells and MR1 in pulmonary disease

MR1T cells have been shown by our laboratory and other groups to be highly enriched in the lung mucosa, where they are likely playing an important role in early human defense. There is broad experimental evidence for the importance of MR1T cells in sensing and responding to pulmonary infection, including mycobacteria, *Franciscella,* and *Klebsiella pneumoniae,* among others. It is also increasingly clear that the MR1T TCR repertoire, as well as the range of ligands that can bind MR1 to stimulate MR1T cells is much more diverse than previously thought, and thus, the scope of MR1T cells in the lung to respond to pulmonary pathogens is large. We and others have shown that in individuals with active tuberculosis, MR1T cells were depleted in the blood, but restored following anti-tuberculosis treatment (Sharma et al. 2015). Additionally, while the MR1 coding sequence is known to be nonpolymorphic, a polymorphism in an intron of the *MR1* gene was associated with increased susceptibility to tuberculosis, and an increased risk of death from tuberculosis-associated meningeal disease in a cohort of individuals from Vietnam (Seshadri et al. 2017). Whether other SNPs and genetic

variants, either in MR1 or in proteins associated with MR1 or MR1T cells, can be associated with pulmonary disease in humans is not yet known, but represents an intriguing area of research.

In murine models challenged with *Legionella longbeachae* infection, a sustained expansion of pulmonary MR1T cells was observed, with increased inflammatory cytokine production, and decreased bacterial burden (Wang et al. 2018). This was not observed in MR1-deficient mice, indicating a role for MR1-mediated presentation of microbial antigen, but was enhanced when MR1T responses were stimulated by prior boosting with exogenous antigen, suggesting a potential target for vaccine development against *Legionella*. Dendritic cells infected with clinical isolates of *Streptococcus pneumoniae* serotype 19A, a cause of invasive pneumococcal disease, were shown to stimulate MR1T responses, but the magnitude of these responses were linked to genetic differences in the riboflavin synthesis pathway, suggesting that genetic variation in microbes can also impact MR1T activation (Hartmann et al. 2018).

As MR1T cells are capable of producing inflammatory cytokines and are enriched in the lung, there has been interest in their implication in non-infectious pulmonary disease. MR1T cells were observed to be decreased in the blood and sputum of patients with asthma treated with inhaled corticosteroids, but the mechanism is not understood (Hinks 2016). A recent analysis of MR1T cells in children enrolled in the Urban Environment and Childhood Asthma (URECA) study showed that the frequency of MR1T cells was observed to be significantly lower in 1-year old children who subsequently developed asthma by age 7, and correlated with the frequency of CD4+ IFN-γ producing

T cells, suggesting an important protective role for early MR1T cell expansion in asthma (Chandra et al. 2018). A separate study showed that in children with asthma exacerbations, the frequency of IL-17 producing MR1T cells was increased (Lezmi et al. 2018). Whether distinct subsets of MR1T cells play opposing roles in asthma, or the age and timing of MR1T expansion in the lung mucosa impacts disease onset and severity is not yet known, but it is clear that MR1T cells may be contributing to disease severity in asthma and allergic disease.

In addition to directly compromising pulmonary function, chronic lung diseases are also associated with susceptibility to bacterial infections. For example, nontypeable *Hemophilus influenza* A (NTHI), an airway colonizing microbe, is frequently implicated in lower airway infection in COPD patients (Sriram et al. 2018). A study of patients with COPD that were being treated with inhaled corticosteroids showed that MR1T cells responsive to NTHI were slightly decreased in the BAL or endobronchial biopsy of individuals with COPD treated with corticosteroids (Hinks et al. 2016). Additionally, reduced frequencies of MR1T cells in patients with cystic fibrosis were associated not only with disease severity, but also with treatment for cystic fibrosis, and notably, infection with *Pseudomonas aeruginosa (Pincikova et al. 2018, Smith et al. 2014)*. Whether decreased MR1T cells are associated with increased susceptibility to chronic lung disease, or whether chronic lung disease leads to a decrease in MR1T cells is not yet known, but raises the intriguing therapeutic potential for MR1T cells in these diseases.

MR1T cells in inflammatory diseases

MR1T cells have been implicated in a number of autoimmune diseases. This corresponds with their enrichment in mucosal sites and proinflammatory phenotype-as dysregulated MR1T activation can lead to unwanted tissue damage and autoinflammation. Early clonotypic analysis of Vα7.2-Jα33 expressing cells in autopsy of individuals with from multiple sclerosis (MS) showed that MR1T cells were enriched in central lesions of these individuals (Illes et al. 2004). A more recent study suggested that MR1T cells were reduced in the blood of individuals with MS, enriched in brain tissue, but not in CSF, and this was linked to an increased serum production of IL-18, a cytokine known to be increased in patients with MS (Willing et al. 2014). Antibody based detection showed reduced frequencies of MR1T cells in the blood of relapsing-remitting MS patients, which was restored following clinical recovery, and reflected disease severity (Miyazaki et al. 2011). However, these MR1T cells were thought to play an immunomodulatory role *in vitro*, again highlighting the potential for context-specific MR1T responses in disease, but it is not clear if other markers of disease can impact MR1T function. While it is established that MR1T cells are enriched in the CNS of individuals with MS, their mechanism of trafficking and their role and frequencies in peripheral blood is controversial and not well understood. Whether cytokine production and immune cell activation of and by MR1T cells is due to unexpected bystander effects or a direct response to disease has not yet been established. Additionally, discordant observations may be explained by variations in patient cohorts, either age, gender, environmental factors, or genetic variants not fully elucidated.

Patients with inflammatory bowel disease also displayed a decreased frequency of MR1T cells in peripheral blood in both ulcerative colitis and Crohn's disease, and a lower abundance of MR1T cells in the gut mucosa of both patient cohorts (Hiejima et al. 2015). Additionally, peripheral blood MR1T cells were found to express proapoptotic factors, suggesting that the decreased frequency may be related to cell death. Conversely, a separate study indicated an increase in MR1T cells in inflamed mucosa of patients with inflammatory bowel disease, as compared to the healthy mucosa (Haga et al. 2016). Additionally, these cells expressed an activated phenotype, and *in vitro* analyses showed that MR1T cells from IBD patients produced increased IL-17, with reduced IFN-γ production. Whether MR1T cells play a pathogenic or protective role in IBD remains to be understood, as does the role of MR1 and MR1T cells in early inflammatory disease, as well as chronic inflammation.

MR1T frequencies have also been shown to be reduced in the periphery of individuals with ankylosing spondylitis (Hayashi et al. 2016). An inverse correlation was observed between clinical score and MR1T frequency in patients with systemic lupus erythematous (Cho et al. 2014). The authors of this study also noted an increase in MR1T frequency in the synovial fluid of patients with rheumatoid arthritis, and in both cases, MR1T cells were shown to express high levels of PD-1, a marker of T cell exhaustion. These data suggest that activated MR1T cells are recruited to the site of inflammation, where they can potentially secrete cytokines to contribute to the pathogenesis of autoimmune diseases.

Reduction of blood MR1T frequency has also been observed in obese adults (Magalhaes et al. 2015, Carolan et al. 2015). Moreover, obesity is associated with the onset and development of Type 2 diabetes, and in childhood obesity, reduced MR1T frequency was correlated to insulin resistance. In both studies, peripheral blood MR1T cells exhibited an activated phenotype, with increased production of IL-17. The link between MR1T cells, adipose cell inflammation, and promotion of type 2 diabetes is not yet understood.

Taken together, these observations suggest that MR1T cells can contribute to the onset of autoimmune disease pathology. The decreased MR1T frequency in the periphery of patients with individuals with autoimmune disease suggest that these cells are activated and recruited to the site of inflammation, similar to MR1T recruitment in during infection at mucosal sites. In this situation, however, MR1T cells are aberrantly activated-and whether this is mediated by an as yet unknown self-ligand, an inappropriate response to ligand produced by commensal bacteria, or occurs through an antigen and MR1-independent mechanism is not yet understood, and further studies, both in animal models of disease and in patient cohorts, are necessary to further our understanding of the role of MR1T cells in autoimmunity.

Development and characterization of MR1T cells

MR1T cells emerge from the thymus with effector function

The ontogeny of MR1T cells is not yet well understood, nor are the differences in MR1T development from other CD8+ T cell subsets. Classical CD8+ T cells, as described earlier, require thymic epithelial cells for their selection and development, emerging from the thymus with a naïve phenotype (Anderson et al. 1994). However, murine MHC Ib-restricted CD8+ T cells reactive to *Listeria monocytogenes* were shown to undergo positive selection on MHC Class I expressing hematopoietic cells, and these cells emerged from the thymus with an activated effector phenotype (Urdahl, Sun, and Bevan 2002). Murine CD1-restricted NKT cells expressing the NK1.1 receptor were selected on hematopoietic cells expressing MHC Class I (Bendelac 1995, Bendelac et al. 1994). Further studies looking at liver-resident murine NKT cells showed that these cells were absent in β2-microglobulin-deficient mice, but did depend on MHC I expression on hematopoietic cells (Ohteki and MacDonald 1994). We showed that human αβ thymocytes stimulated *ex* vivo with allogeneic Mtb-infected dendritic cells produced granzyme B, and IFN-γ, and were non-classically restricted, but later shown to be MR1-restricted (Gold et al. 2008, Gold et al. 2013).

MR1T cells were shown to require an intact thymus for development and expansion (Tilloy et al. 1999). Early observations on MR1T cell development using knockout murine models showed that MR1T development was independent of TAP, but required β2-microglobulin (Treiner et al. 2003). Moreover, fetal liver chimeras generated from β2-microglobulin-deficient and wild type C57/B6 showed that MR1T selection and

expansion required β2-microglobulin expression on hematopoietic cells, similar to the mechanism proposed for NKT cell development. Furthermore, both murine and human MR1T cells appeared to require MR1 expression on B cells, indicating a potential requirement for their selection in the thymus, but this finding has not been fully validated (Martin et al. 2009). Therefore, it is clear that MR1T development requires MR1 expression on a hematopoietic cell. An open question remains about the ligand required for the development of MR1T cells-whether MR1 presents a self-ligand to promote MR1T development is unknown, but has been suggested (Huang et al. 2009). Alternatively, it has been proposed that transcription factors involved in T cell development, such as PLZF, which is expressed on MR1T cells and required for iNKT development, may also contribute to MR1T thymic development, at least, in murine models (Koay et al. 2016). More recently, MR1T cells, as well as NKT cells, have been shown to express the transcription factors RORγt and T-bet as early as the thymus, and this may play a role in tissue homing and maturation (Salou et al. 2019).

MR1T cells, defined by the expression of the canonical TCRα chain (Vα7.2) and an integrin marker, CD161, were shown to be present at low (approximately 0.5%), but detectable frequencies in human CD8+ thymocytes, with a higher frequency detected in peripheral blood (Martin et al. 2009). Murine models to further understand the MR1T ontogeny were used to show that murine MR1T thymocytes were found in fetal thymic organ culture of iVα19 transgenic mice, and likely depended on MR1 for their selection (Martin et al. 2009).

Our laboratory sought to further understand how thymic MR1T cells function in response to Mtb-derived antigen. Using flow cytometric analysis of MR1T TCR (Vα7.2) expressing CD8+ cells isolated from human thymus, as compared to MR1T cells from cord blood and from adult PBMC, Gold and colleagues showed that MR1T cells were detectable in all three compartments, albeit with lower frequencies in the human thymus, concurring with previous findings (Martin et al. 2009, Gold et al. 2013). An HLA-mismatch assay that was previously developed was used where CD4-depleted (CD4-CD8+, CD4-CD8-) cells from human thymus, cord blood, and PBMC were incubated with alveolar epithelial cells (A549) infected with Mtb as antigen presenting cells in order to elucidate the functional capacity of MR1T cells, as measured by intracellular production of TNF-α and surface staining for the MR1T TCR (Vα7.2) (Gold et al. 2010). This assay showed that, as expected, there were Mtb-reactive MR1T cells present in cord blood and PBMC. Intriguingly, a population of Mtb-reactive MR1-restricted thymocytes were detectable in the thymus as well, suggesting, like other MHC class Ib-restricted T cells, antigen-inexperienced MR1T cells may emerge from the thymus with immediate effector function. MR1T thymocytes were assayed for naïve or activated phenotype and were shown to express the surface markers, CD45RA, an isoform of CD45 that broadly characterizes naïve T cells. as well as CD62L and CCR7. Moreover, MR1T cells from the thymus also expressed detectable levels of sjTRECs, DNA products generated from VDJ receptor gene rearrangement, again indicating that MR1T thymocytes are naïve with effector function, but then further expand in the periphery.

The selecting cell for MR1T cells is not known, but is likely of hematopoietic origin and probably requires expression of MR1. Gold and colleagues showed that while the majority of cells do not express detectable levels of surface MR1, a population of CD45+ CD3+ double positive (CD4+ CD8+) thymocytes were shown to express endogenous surface MR1 expression. Further characterization of these double positive MR1-expressing thymocytes showed that they are capable of presenting antigen to stimulate MR1T clones (Erin Meermeier, unpublished observations). This is similar to NK1.1 T cells, which require CD1d expression on double positive (CD4+ CD8+) thymocytes for their selection.

MicroRNA, short non-coding RNA of about 22 nucleotides, have recently been shown to augment T cell development in the thymus. A specific microRNA, miR181 was shown to play a key role in the development of NK cells from hematopoietic precursors, potentially through the regulation of the Notch signaling pathway (Cichocki et al. 2011). miR181a/b1 were subsequently shown to be a measure of TCR signal strength and abrogation of this microRNA impaired iNKT development in mice (Zietara et al. 2013). The absence of miR181a/b1 in mice also arrested MR1T development in the thymus and blocked functional maturation and acquisition of the transcription factors PLZF, RORγt, and T-bet (Winter et al. 2018). miR181a/b1 has also been shown to promote regulatory T cell development, and therefore, may serve as a general mechanism of T cell development in the thymus (Lyszkiewicz et al. 2019). Though it is not known how microRNA fits into the paradigm of MR1T development, it is likely that non-coding RNA

may be important in the development of MR1T cells in the thymus, likely in conjunction with double positive MR1 expressing thymocytes and an as yet known self-ligand.

A recent study using the MR1/5-OP-Ru tetramer to understand MR1T development in the thymus proposed a 3 stage thymic development pathway for murine MR1T cells, as defined by the expression or absence of CD24 and CD44 (Stage 1: CD24+ CD44-, Stage 2: CD24- CD44-, Stage 3: CD24- CD44+). (Koay et al. 2016) Stage 1 MR1T thymocytes were described as small immature thymocytes that appeared to resemble the earliest stage of iNKT development in the thymus, while Stage 3 cells were larger, produced cytokines in response to stimulation, and more closely resembled peripheral CD8+ MR1T cells. Ontogeny studies performed using human thymus, cord blood, and blood from young and adult donors suggested a similar intrathymic development pathway in humans, with an increase in mature Stage 3 MR1T cells with age. Furthermore, functional maturation and acquisition of cytokine effector function was shown to occur at the last stage of MR1T development. However, it is still not known what stimuli are required for MR1T development and expansion, as well as how the microbiome, and variations across tissues, may impact the subsequent development and tissue homing of MR1T cells. Additionally, genetic variations between individuals may play a key role in not only the frequency of MR1T cells, double positive MR1T cells, and MR1T development, but also in acquisition of effector function.

Phenotypic analysis of MR1T cells

Prior to the development of the MR1/5-OP-RU tetramer, which detects MR1T cells specifically through their TCR that can engage with 5-OP-RU bound to MR1,

identification of MR1T cells relied on cell surface markers. Most notably, the development of an antibody to the human MR1T TCRα chain TRAV1-2 (Vα7.2, clone 3C10), enabled detection of MR1T cells (Reantragoon et al. 2013, Gherardin et al. 2018). However, as shown by Meermeier *et al*, identification of MR1T cells purely by TRAV1-2 staining did not encompass the full repertoire of MR1T cells, which could be better identified utilizing the MR1/5-OP-RU tetramer (Meermeier et al. 2016). MR1T cells, were also shown to express high levels of IL18Rα, and as shown earlier, are responsive to IL-12 and IL-18-mediated stimulation (Ussher et al. 2014, Billerbeck et al. 2010). Additionally, MR1T cells are also characterized by the expression of CD161, a member of the C-type lectin superfamily (Dusseaux et al. 2011). CD161 expression, in conjunction with surface staining of Vα7.2 (TRAV1-2), prior to the development of the MR1T tetramer, has been used in the majority of studies to define MR1T cells. Our laboratory sought to further characterize surface markers that defined MR1T cells in the blood, and showed that CD161 lacked sensitivity in defining pathogen-reactive MR1T cells, as some MR1T cells reactive to microbes expressed low levels of CD161. Additionally, we show that a unique marker, CD26, or dipeptidyl peptidase 4, was also strongly associated with MR1T cells in peripheral blood (Sharma et al. 2015). We, therefore, define 'phenotypic MR1T cells' as those that express the MR1T TCR, Vα7.2, as well as high levels of CD161 and CD26, as measured by flow cytometry staining. However, we also utilize the 5-OP-RU tetramer to also define MR1T cells, as the tetramer directly engages with the MR1T TCR through MR1 bound ligand.

MR1T cells were shown to produce the inflammatory cytokines IFNγ and TNF-α in response to stimulation, either by cytokine or microbial infection (Le Bourhis et al. 2010, Le Bourhis et al. 2013, Gold et al. 2010, Gold et al. 2013, Harriff et al. 2014). Additionally, other groups have shown that MR1T cells also produce the cytokine Interleukin-17 in response to microbial infection (Dusseaux et al. 2011, Jo et al. 2014, Rahimpour et al. 2015). Work from our laboratory showed that though MR1T cells did not produce IL-17 in response to *M. smegmatis*-infected APCs, these cells did produce Th1 cytokines, as well as granulysin, to promote cell killing (Sharma et al. 2015). Additionally, these cells also expressed a unique and specific array of surface markers, including CD150 (SLAMF1), a molecule that potentiates T cell activation and expansion.

Early characterization of peripheral MR1T cells showed that, in addition to their memory phenotype (CD45RO+ CD45RA-), MR1T cells also displayed an effector memory phenotype, with high expression of CD127 and CD25, NKG2D, and low expression of CD62 ligand, as well as staining for CD150 (Dusseaux et al. 2011). MR1T cells also expressed high levels of CCR6 and CXCR6, and heterogeneous expression of CXCR4, and CCR9. These chemokine markers and receptors are known to play a crucial role in homing to tissue sites, so it was thought that circulating MR1T cells had the ability to track to tissues.

MR1T cells are highly abundant at mucosal sites, including the lung, skin, gut mucosa, and female genital mucosa (Gibbs et al. 2017, Tilloy et al. 1999, Treiner et al. 2003, Park and Kim 2018, Gold et al. 2010). Here, they likely play a key role in the initial response to infection, however, they are in close proximity to commensal bacteria that

can also produce MR1T ligands. Therefore, it is clear that MR1T cells must be tightly regulated to avoid aberrant activation in the absence of pathogenic stimuli.

Bacteria mediate innate immune responses through the sensing of pattern associated molecular patterns (PAMPs), particularly through Toll-like receptors (TLRs) on the surface of immune cells. TLR signaling represents a non-specific mechanism by which the immune system can sense infection. Though the significance of TLR signaling in generating MR1T responses is not known, two studies implicated TLR8 and TLR9 in MR1T activation. Activation with an array of TLR agonists, particularly TLR1 and TLR6 promoted MR1T production of IFN-γ (Ussher et al. 2016). TLR8 agonists induced an IL-12/IL-18 mediated stimulation of hepatic MR1T cells to produce cytokine (Jo et al. 2014).

MR1T cells are also highly abundant in the liver (Tang et al. 2013). As the liver is a highly immunologically active site, MR1T cells are likely sensing and sampling the microbial environment to detect infection. Intriguingly, hepatic MR1T cells are activated by IL-7, which promoted their production of inflammatory cytokines and IL-17 in response to TCR stimulation. IL-7 treatment has been shown to restore defective MR1T function in HIV infection following HAART, and may be playing a specific role in the cytolytic activity of MR1T cells *in* vivo (Kurioka et al. 2014). Observations from our laboratory show that MR1T cells isolated from human lung express markers poising them for bacterial killing (Erin Meermeier, unpublished observations). It remains to be seen in humans, however, how MR1T phenotype changes depending on infection status and tissue sites sampled, and whether there are broad subsets of MR1T cells that are poised for specific functions at tissue sites.

MR1 presents small molecules derived from the riboflavin biosynthesis pathway to stimulate MR1T cells

Though the role of MR1T cells in recognition of microbial antigen was well established, the specific nature of the antigen presented by MR1 was not known. Due to the high degree of conservation of MR1 across species and individuals, and that MR1T cells were believed to express an invariant TCRα chain with a limited repertoire of TCRβ chains, it was hypothesized that the antigen that activated MR1T cells was of a conserved nature. Additionally, it was thought that this antigen was of microbial origin, as MR1T cells appeared to require commensal species for development, and that these cells were activated by a wide range of bacteria and fungi.

Seminal work by Kjer-Nielsen and colleagues provided the first conclusive evidence to identify the MR1 ligand (Kjer-Nielsen et al. 2012). Their work was based on the incidental finding that folic acid containing media established more efficient refolds of the MR1/β2-microglobulin complex. Mass spectrometry and time of flight analyses identified the putative ligand to be a photodegradation product of folic acid, 6-formylpterin (6FP), which was subsequently shown to promote the surface expression of MR1 on C1R cells.

Though 6FP was shown to upregulate surface expression of MR1, it did not stimulate MR1T cell upregulation of CD69, a measure of T cell activation. Therefore, Kjer-Nielsen and colleagues wished to find an antigen isolated from microbes that could stimulate MR1T cells. They utilized supernatant from *S. typhimurium* grown in minimal media to identify a class of antigens called ribityllumazines. These ribityllumazines were

derived during the riboflavin biosynthesis pathway. A subsequent study identified other potent activators of MR1T cells- these compounds were shown to be pyrimidine based precursors of riboflavin (Corbett et al. 2014). One of these compounds, 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU), was generated from the reaction of 5-amino-6-D-ribitylaminouracil (5-A-RU) and methylglyoxal (MeG). A tetramer of MR1 loaded with 5-OP-RU was shown to universally detect MR1T cells (Reantragoon et al. 2013). Riboflavin, an essential vitamin, is synthesized by microbes, including those that stimulate MR1T cells, but not by humans, suggesting a role for MR1T cells in distinguishing between self and non-self. As prior studies had demonstrated that microbes with intact riboflavin synthesis pathways efficiently stimulated MR1T cells, this suggested that only bacteria and fungi with the capacity to synthesize riboflavin were capable of activating MR1T cells (Gold et al. 2010).

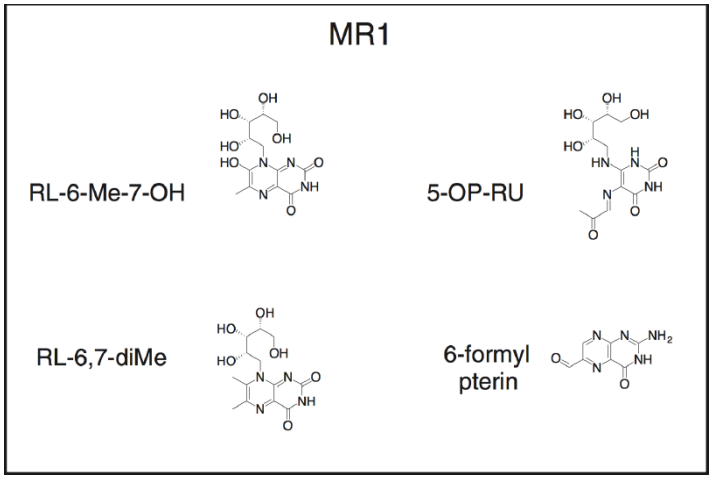

Figure 1.4: Canonical ligands recognized by the MR1T TCR. Reprinted with permission from Godfrey, *et al, Nature Reviews Immunology*

The MR1-ligand-TCR complex

The identification of 6FP as a ligand for MR1 yielded insight on the structure of the MR1 binding pocket and the engagement with the MR1T TCR (Kjer-Nielsen et al. 2012). Work performed simultaneously by the Adams and Rossjohn groups yielded important insight into the structure of the MR1T/ligand/MR1 complex. The ligand binding region, encoded by the α1 and α2 domains, appeared to more closely resemble the HLA-A2 binding pocket than the CD1d binding pocket, with charged and aromatic residues exposed. Unlike the HLA-A2 binding pocket, the ends of the MR1 pocket was closed, and the binding pocket, comprised of two grooves, was smaller than that of HLA-A2, which can accommodate 9-mer peptides (López-Sagaseta, Dulberger, Crooks, et al. 2013, Kjer-Nielsen et al. 2012). The 'A' pocket, which is known to bind antigen, is charged and hydrophobic, and therefore ideally suited to engage with the small molecule ligands, while the 'F' pocket is more flexible and has not yet been shown to engage with ligand (López-Sagaseta, Dulberger, Crooks, et al. 2013, López-Sagaseta, Dulberger, McFedries, et al. 2013). 6FP was shown to sit in the binding pocket centrally, positioned towards the β sheet, and its binding to MR1 was dominated by significant hydrophobic interactions. As MR1 is nonpolymorphic, the flexibility inherent in the MR1 ligand binding groove may be of importance in binding a wide array of ligands (Patel et al. 2013). The Lys43 residue in the MR1 binding pocket made a Schiff Base with the formyl group of 6FP, and has also been shown to make a Schiff base with the ribityl chain of other

stimulatory MR1T ligands, and this interaction has been postulated to be essential for MR1T stimulation by MR1 bound ligand (Kjer-Nielsen et al. 2018). The recent observations that MR1 can bind and present ligand derived outside of the riboflavin synthesis pathway does raise questions on how the MR1 ligand binding groove can accommodate an increasingly diverse array of ligand.

Further crystallography and modeling analyses showed yielding insight into the binding structure. The MR1T TCR was shown to be structurally similar those of MHC Class I, and docked centrally and orthogonally with the MR1 binding cleft (Patel et al. 2013, Reantragoon et al. 2012). The TCR was also shown to interact with conserved amino acids in the MR1 ligand binding domain, and these contacts were predominately hydrophobic and dominated by MR1. The 6FP moiety, though it was shown to sit in the MR1 binding pocket, was inaccessible to the MR1T TCR, suggesting a reason for why 6FP is incapable of stimulating MR1T cells. Concurrent work performed by Erin Adams and colleagues showed that the MR1T TCR was oriented to the A' pocket MR1, poised to make contacts with ligand (López-Sagaseta, Dulberger, Crooks, et al. 2013, López-Sagaseta, Dulberger, McFedries, et al. 2013) Additionally, these studies showed that the binding pocket of MR1 was more flexible than previously thought and could potentially accommodate a range of antigen.

In addition to yielding important insight on the interactions of MR1 and the TCR with microbial antigen, these crystallography and modeling studies suggested that the MR1T TCR antigen was a conserved molecule produced by microbes that stimulate MR1T cells. Microbes that do not encode the riboflavin synthesis pathway, such as

Enterococcus faecalis and *Streptococcus pyogenes*, are incapable of stimulating TRAV1-2+ MR1T cells.

The relative contribution of the MR1T TCRα and TCRβ chains in the recognition of microbial ligand

While canonical CD8+ TCRs required usage of both the TCRα and TCRβ chains in mediating contacts with MHC bound peptide, CD1d-restricted TCR interactions with CD1d and ligand were dominated by the TCRα chain. The question remains about the role of the MR1T TCRα and TCRβ chains in engaging with MR1 and ligand. While Patel et al showed that both the TCRα and TCRβ chains docked with the MR1/ligand complex, the Vβ residues were not ideally situated to mediate recognition of ligand (Patel et al. 2013) Point mutations in the Vβ region were shown not to impact ligand binding, however, switching out the entire TCRβ chain did impact ligand recognition, suggesting a consensus docking mode of TCR to MR1 bound ligand (Reantragoon et al. 2012). This suggested that the TCRα chain predominated in the recognition of ligand.

Concurrent work suggested contradictory roles for TCRα and TCRβ chains (López-Sagaseta, Dulberger, Crooks, et al. 2013, López-Sagaseta, Dulberger, McFedries, et al. 2013). The α chain contacts with MR1 and ligand were shown to be highly conserved, but β chain contacts were more limited and much more diverse. While other groups reported the TCRβ chain as dispensable in mediating contacts with MR1-bound ligand, these studies suggested that the TCRβ chain contacts were necessary to fine tune contacts and enhance ligand recognition. Indeed, contacts with ligand were shown to induce flexibility in the CDR3β region of the TCRβ chain. Subsequent work

confirmed that variations in the TCRβ chain were necessary in fine-tuning the MR1-ligand complex (Eckle et al. 2014). While all of these studies to date have confirmed the importance of the MR1T α chain in the binding and recognition of ligand, the importance of the TCRβ chain is still not understood, but may be playing a key role in fine tuning engagement with MR1-bound ligand.

Diversity in the MR1 ligandome and MR1T TCR repertoire

There has been recent evidence for diversity both in the range of MR1 ligands, as well as in the MR1T TCR repertoire. An MR1T cell that was restricted by MR1 expressed a TCRα chain distinct from canonical MR1T cells (TRAV12-2) that was capable of recognizing *Streptococcus pyogenes*-derived ligand (Meermeier et al. 2016). Additionally, as *S. pyogenes* is incapable of synthesizing riboflavin, this provided evidence for MR1 binding ligands produced outside of the riboflavin biosynthesis pathway. These newly described MR1T cells also displayed distinct responses to previously described exogenous ligand as compared to TRAV1-2 expressing MR1T cells, suggesting that the MR1T TCR can distinguish between ligand. *In silico* approaches were used to identify a broader range of MR1T antigen (Keller et al. 2017). These approaches identified ligands that could upregulate MR1 surface expression on C1R cells, and stimulate MR1T activation, but also identified distinct non-activating ligands. Importantly, these new ligands identified were distinct from intermediates in the riboflavin synthesis pathway, but instead included common pharmacologic agents. These data suggested that MR1 could bind a more diverse array of ligands, but, did raise the question of aberrant drug interactions.

Our laboratory recently explored the landscape of the MR1 ligandome derived from *M. smegmatis* and *E.coli*. Intriguingly, mass spectrometry analysis showed that the repertoire of ligands generated from the two microbes were distinct (Harriff et al. 2018). Additionally, molecular networking analysis showed small molecule MR1 ligands distinct from the riboflavin biosynthesis pathway. These photolumazine ligands (PLI, PLIII), were potent activators of MR1T cells, and distinct MR1T TCRs could distinguish between these novel ligands. Taken together, these data highlight the potential for diversity in the range of antigens that are recognized by MR1T cells.

Concurrent with these reports about the diversity in the MR1T ligandome, there has been increasing evidence that the MR1T TCR repertoire is broader than previously described. Though initial studies suggested that the MR1T TCR was semi-invariant, studies utilizing MR1T tetramers loaded with antigen showed unexpected heterogeneity in the TCR repertoire, including TCRβ chains distinct from the typical TRBV6-4 and TRBV20-1 (Reantragoon et al. 2013). Our laboratory sequenced the TCRs of microbially reactive CD8+ T cells isolated from human donors and showed the high degree of heterogeneity in the MR1T TCR repertoire across individuals, both in the TCRα and TCRβ chains (Gold et al. 2014). Additionally, distinct TCR usage was observed between microbial species, suggesting that the MR1T TCR repertoire could be shaped by exposure to microbe, and these distinct TCRs.

Contrary to these findings, deep sequencing and cloning of the MR1T TCR repertoire suggested that only a small number of clonotypes were responsible for the majority of MR1T cells in blood (Lepore, Kalinichenko, Kalinicenko, et al. 2014). Though

these findings suggested a limited an oligoclonal TCRβ chain repertoire, they were identified through a relatively small number of T cell clones (200), versus sequencing of all MR1-restricted CD8+ T cells.

Gherardin and colleagues identified atypical TRAV1-2- TCRs that recognized MR1 bound ligand, concurring with the observation that TRAV12-2 T cells could be stimulated by MR1 bound antigen (Gherardin, Keller, Woolley, Le Nours, et al. 2016). This study showed that MR1T cells were capable of reacting to folate based antigen that was based on discrete TCR usage, with a hypervariable CDR3β region that was potentially contributing to recognition. Usage of the MR1/5-OP-RU tetramer identified MR1T cells that displayed a high degree of variation in the TCRα chain usage with a potentially broad CDR3β repertoire (Gherardin et al. 2018). More recently, a study of healthy human volunteers infected with *Salmonella paratyphi A* demonstrated skewing of the TCRβ repertoire, indicating that the TCRβ repertoire was not only shaped by microbial exposure, but also could govern recognition of MR1-bound ligand (Howson et al. 2018).

Taken together, there is a broad array of evidence of increased diversity in the MR1T TCR repertoire and in the range of activating ligands. This supports the hypothesis that MR1 and MR1T cells play an important role in surveying the microbial metabolome in the response to infection.

Pathways of MR1 antigen processing

The mechanism by which MR1 engages with microbial ligand and is trafficked to the cell surface is not yet elucidated, however, it is known that the pathway is distinct from Class I MHC molecules. The pathways for MR1 presentation of MR1T antigen are crucial in surveying the environment and detecting microbial infection through the binding of small molecule ligands.

The differences with Class I MHC begin at the level of cell surface expression of MR1. Class I MHC molecules, as well as HLA-E, are constitutively expressed on the surface of cells (Hansen and Bouvier 2009). However, MR1 expression is not detectable on the surface of cells in the absence of infection or exogenous ligand (Chua et al. 2011, Miley et al. 2003). MR1 expression is upregulated on the surface of cells upon the administration of the exogenous ligand 6FP, and detectable by surface staining for MR1 using an α-MR1 antibody (Clone 26.5) (Kjer-Nielsen et al. 2012). Alveolar epithelial cells (A549) infected with Mtb did display a subtle shift in surface MR1 expression, measured by flow cytometry, and were shown to be capable of stimulating MR1T cell production of IFN-γ (Gold et al. 2010). This absence of MR1 expression on the surface at steady state suggests that perhaps MR1 expression is induced by microbial ligand, following which, the MR1-ligand complex is expressed on the cell surface long enough to be recognized by the MR1T TCR.

Our laboratory has shown, using Beas2B bronchial epithelial cell lines overexpressing MR1, that MR1 resides in intracellular vesicles that are LAMP1+ and express Rab6 (Harriff et al. 2014, Harriff et al. 2016). These Beas2B cells have also been

shown to stimulate MR1T cells following infection with mycobacteria, including Mtb, *M. smegmatis*, and *M. bovis* BCG, and this is a system I will be describing in subsequent chapters. Using a lentivirally mediated shRNA knockdown screen followed by validation using siRNA and plasmid overexpression systems, vesicular trafficking proteins were identified to impact MR1 trafficking and presentation of Mtb antigen to MR1T cells. These trafficking proteins included Syntaxin18, an ER-localized SNARE protein, as well as VAMP4, which is thought to play a role in trafficking through the trans-Golgi network, and Rab6, a protein that is involved in retrograde Golgi trafficking (Harriff et al. 2016).

However, an alternate hypothesis proposes that MR1 resides primarily as an immature protein in the endoplasmic reticulum, with no protein observed in endosomal vesicles (McWilliam et al. 2016). These observations were seen in studies utilizing the exogenous stimulatory ligand 5-OP-RU and an inhibitory ligand Ac-6-FP in C1R cells overexpressing MR1. Additionally, in the absence of MR1 ligand, MR1 was shown to be sensitive to endoglycosidase-H, confirming prior findings, but was EndoH resistant following addition of exogenous ligand (Yamaguchi and Hashimoto 2002). In this model, immature MR1 resides in the ER as a presynthesized pool, and upon exposure to exogenous ligand, can complete folding, engage with β2-microglobulin, and be trafficked to the cell surface to activate MR1T cells. It was hypothesized that the loss of positive charge on Lys43, which occurs upon formation of a Schiff base with the exogenous stimulatory ligand, 5-OP-RU, is required for MR1 egress through the ER and Golgi to the cell surface. However, new ligands have been identified that do not neutralize the Lys43 positive charge in MR1, but stimulate MR1T cells, and therefore, further characterization

of MR1 trafficking following endogenous ligand is necessary. It is possible that the flexibility in the MR1 binding pocket could generate as yet unknown contacts with these novel ligands that may also enhance MR1 stability and folding. McWilliam also proposes that the MR1-ligand complex is expressed on the cell surface for a short duration of time before being endocytosed and recycled, perhaps suggesting a mechanism by which MR1 quickly samples the environment to stimulate MR1T activation.

These two models propose alternative mechanisms by which preformed MR1 samples the intracellular environment and can be loaded with ligand and traffic to the surface to stimulate MR1T cells. However, it should be noted that these two findings are generated under very different conditions. In the model proposed by Harriff *et al*, lung epithelial cells are infected with mycobacteria, while, in the second model, C1R immortalized B cells are exposed to synthetic ligand, therefore, the nature of ligand exposure may impact trafficking and folding. The possibility remains that different cell and tissue types may have different intracellular distributions of MR1-suggsting a context-specific mechanism by which redundant pathways exist by which MR1 engages with ligand and is expressed on the cell surface.

Work in our laboratory suggests potential chaperone proteins required for MR1 trafficking to and from the cell surface. Though it has long been established that MR1 trafficking and expression, unlike canonical Class I, does not require TAP, the proteasome, or the peptide loading complex (Huang et al. 2008, Gold et al. 2010). However, MR1-mediated antigen presentation does require the MHC Class II-associated invariant chain and HLA-DM (Blum, Wearsch, and Cresswell 2013). These proteins

chaperone MHC II molecules through an endosomal pathway, and work from our laboratory suggest that an endosomal pathway is utilized by MR1 to sample ligand and traffic to the cell surface. However, epithelial cells do not utilize the MHC II antigen presentation pathway, but a similar endosomal trafficking mechanism may occur through the use of Syntaxin18, VAMP4, and Rab6 (Harriff et al. 2016).

A recent study by Karamooz and colleagues suggests distinct trafficking pathways for MR1 when exposed to intracellular or extracellular antigen (Karamooz et al. 2019). siRNA-mediated knockdown of endosomal trafficking proteins in MR1GFP expressing cells identified in a prior lentiviral-mediated screen showed that Syntaxin 4 was required for presentation of *M. smegmatis*-derived supernatant, or exogenous antigen, but dispensable for presentation of Mtb-derived antigen, which is a exclusively intracellular infection (Harriff et al. 2014, Harriff et al. 2016). Knockdown of other trafficking proteins, VAMP2, and Syntaxin16, resulted in impaired antigen presentation of both intracellular Mtb-derived and extracellular *M.smegmatis*-derived ligand, suggesting that there are both common and distinct chaperone proteins required for MR1-mediated antigen presentation. Additionally, using a doxycycline-inducible MR1AGFP expression system, which allows for the detection of preformed MR1, Karamooz *et al* show that a preformed pool of MR1 pretreated overnight with 6-FP, a non-stimulatory MR1T antigen, can boost the presentation of exogenously synthesized antigen to MR1T clones. In addition to stabilizing MR1 on the cell surface, 6-FP was also shown to rescue MR1 from degradation, which was measured by increased detection of MR1GFP by flow cytometry.

While short term exposure to 6-FP appears to inhibit MR1T antigen presentation, a longer exposure may promote MR1 recycling and reuse.

Taken together, these data suggest multiple and potentially context-specific mechanisms by which MR1 can be loaded with ligand and trafficked to and from the cell surface to stimulate MR1T cells. 6-FP, a non-stimulatory MR1 ligand, appears to be required for egress of MR1 from the ER to the cell surface, and the importance of the Schiff base contact with Lys43 is not known. These models all suggest an important role for MR1 in sampling both intracellular and extracellular ligands in the detection of infection. Different trafficking pathways may exist in order to broaden the ability of MR1 to present microbial ligand, both endogenously derived and exogenous to stimulate MR1T activation. The importance of different trafficking pathways or chaperone proteins in different tissue types, particularly those exposed to commensal bacteria, is not yet known. Nor is it known whether two separate pools of MR1 exist to sample intracellular or extracellular ligand.

Therefore, MR1 and MR1T cells represent an important means of host surveillance of the environment for self versus nonself. The differences in MR1 and MR1T cells from their canonical counterparts broadens the scope by which the immune system is capable of recognizing and responding to foreign antigen.

Conclusions

My dissertation focuses on broadening our understanding of MR1T cell detection of microbial antigen through MR1 and how this recognition and responsiveness can be modulated by regulation of MR1 gene expression. In the next Chapter, I will briefly review common methods and techniques used to study T cell biology. In Chapter 3, I will focus on the MR1T TCR and ask the question of whether the TCRα or TCRβ chain can mediate differences in recognition of microbial infection, as well as of exogenous microbial antigen. In Chapter 4, I will focus on understanding how alternative splicing of MR1 represents a potential novel mechanism by which MR1T activation can be regulated. Using molecular biology approaches, I will explore the function and expression of the MR1 isoforms. I will then discuss possible physiological roles for the MR1 splice variants, using cell lines, as well an intriguing role for human thymocytes, suggesting, perhaps, that alternative splicing of MR1 may impact thymic development of MR1T cells.

Chapter 2: General methods for studying MR1T cells

Though I will further expand on all methods used for experiments in this thesis, here, I will describe common methods I used to study MR1 and MR1-restricted T cells. Recent important advances in techniques and reagents to study MR1T cells have enabled more detailed and mechanistic analyses of these cells and their function in both mouse and human.

ELISPOT

The ELISPOT assay, a derivative of the ELISA assay, is a means of measuring T cell responses (here, cytokine production of IFN-γ) to a given stimuli. In this thesis, I will be describing T cell production of IFN-γ to infected antigen presenting cells, or antigen presenting cells exposed to exogenous ligand. The technique was developed in the 1980s (Sedgwick 2005) and has been adapted and frequently utilized since then. Briefly, an ELISPOT utilizes a 96 well plate coated with an antibody for a T cell effector molecule, here, IFN-γ, but TNF-α or Granzyme B responses can also be measured. Following this, different stimulation conditions can be tested throughout the plate, and the antigen presenting cells, antigen, and T cells are incubated overnight. Cytokines produced by

the T cell are ‘captured’ by the plated antibody, and following incubation, the plate is washed and a secondary antibody to the cytokine is added, which is conjugated to a fluorescent molecule. An enzyme-linked reaction is generated and the cytokine is detect as colored spots, which can be measured and counted on an AID ELISPOT reader. This assay is a highly quantitative and sensitive method to enumerate antigen-specific T cell responses. While most of the ELISPOT assays I describe in my dissertation are measuring T cell responses to an antigen presenting cell exposed to exogenous antigen or infected with a microbe, I also utilize a modified version of an ELISPOT assay that our lab has developed (Harriff et al. 2018). In this assay, antigen loaded MR1 tetramer (described below) is plated along with the coating antibody, and T cell responses to the antigen/MR1 complex are directly measured without the need for antigen presenting cells.

Flow Cytometry

Flow cytometry is utilized to quantify protein expression of markers at the single cell level following staining of cells with monoclonal antibodies conjugated to fluorophores. In my thesis, I describe two uses for flow cytometry, to measure surface expression of proteins on various cell types following antibody staining, and also to directly measure fluorescent protein expression in cells that are expressing recombinant DNA conjugated to either GFP or RFP.

Surface staining and subsequent flow cytometric analysis enables specific detection of cell populations within a heterogeneous mixture, such as peripheral blood mononuclear cells, lung cell suspensions, small intestine cell suspensions, or thymocytes. Originally, identification of MR1T cells was performed through staining for the canonical MR1T TRAV1-2 TCRα chain (Vα7.2), using an antibody generated to be specific for this TCR (Le Bourhis et al. 2010, Gold et al. 2010). However, the Vα7.2 TCR is also expressed on other T cell subsets, and therefore, expression of the TCR, while sufficient for MR1T clones, is not specific for MR1T cells. Therefore, detection of MR1T cells also requires staining for surface markers that are associated with these cells, including CD161 and CD26 (Sharma et al. 2015). However, the limitations of relying on the semi-invariant TCR, even with these other markers, for detection of MR1T cells is that non-TRAV1-2+ MR1T cells have recently been described in the literature (Gherardin et al. 2018, Meermeier et al. 2016). Therefore, an approach that can detect all MR1-restricted T cells must be utilize to fully understand MR1T biology.

A modification of flow cytometry, fluorescence activated cell sorting, is utilized for much of my dissertation. Here, I will stain cells with antibodies to surface proteins, and sort cells based on surface markers in order to isolate specific cell subsets.

Tetramers

The tetramer, first described in 1996, is a powerful tool used to study T cells in an unbiased manner (Altman et al. 1996). Tetramer staining is the definitive standard for the detection of T cells, as it is much more sensitive than prior approaches, and, in the case of tetramer based staining for MR1T cells, can stain all MR1T cells in a population, not just those expressing the originally described semi-invariant TCR (Reantragoon et al. 2013). Briefly, a tetramer is comprised of four recombinant antigen-presenting molecules, each loaded with an antigen, and bound with biotin/streptavidin. This tetramer is also conjugated to a fluorophore to enable detection by flow cytometry. A tetramer used to detect specifically MR1T cells was generated by tetramerizing 4 molecules of recombinant MR1 with the *Salmonella*-derived antigen, 5-OP-RU. While the tetramer is a useful and powerful tool to study and quantify T cell subsets, one limitation is that the antigen must be known to be loaded on to the MHC molecule. However, recently, MR1 tetramers that were folded in the presence of bacterial supernatant (*E. coli*, or *M. smegmatis*) are loaded with a wide variety of bacterial MR1 ligand (Harriff et al. 2018). In order to measure functionality of T cells that engage with tetramer, we utilize a modified ELISPOT assay recently developed by Harriff and colleagues using the MR1/5-OP-RU tetramer to stimulate MR1T production of IFN-γ (Harriff et al. 2018).

Chapter 3: The MAIT TCRβ chain contributes to discrimination of microbial ligand

Abstract

MR1-restricted T (MR1T) cells are key players in the immune response against microbial infection. The MR1T T cell receptor (TCR) recognizes a diverse array of microbial ligands and recent reports have highlighted the variability in the MR1T TCR that could further contribute to discrimination of ligand. The MR1T TCR CDR3β sequence displays a high level of diversity across individuals and clonotype usage appears to be dependent on antigenic exposure. However, the relative contribution of the CDR3α and CDR3β sequences in the recognition of MR1-bound ligand is not well elucidated. To address the relationship between the MR1T TCR and microbial ligand, we utilized a previously defined panel of MR1T clones that demonstrated variability in responses against differing microbial infections. Sequencing of these clones revealed four pairs, each with shared CDR3α and differing CDR3β sequences. These pairs demonstrated varied responses against microbially infected dendritic cells, as well as to *Salmonella typhimurum*-derived ligand: 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil, suggesting that the CDR3β contributes to differences in ligand discrimination. Taken together, these results highlight a key role for the MR1T CDR3β region in distinguishing between MR1-bound antigen.

Introduction

MHC-related protein 1 (MR1)-restricted T (MR1T) cells are key players in the immune response against microbial infection (Le Bourhis et al. 2010, Gold et al. 2010) In humans, MR1T represent 1-10% of all circulating T cells and are activated by microbial antigen presented by MR1 on the surface of infected cells.(Treiner et al. 2003, Porcelli et al. 1993). MR1T cells play an important role in containment of bacterial infection as they are highly abundant at mucosal sites and exit the thymus with innate effector functional capacity (Gold et al. 2013, Treiner et al. 2003). Indeed, mice lacking functional MR1T cells have displayed decreased bacterial control as well as impaired recruitment of the adaptive immune response (Meierovics, Yankelevich, and Cowley 2013, Georgel et al. 2011).

Human MAIT cells, a subset of MR1T cells, have been defined by the expression of a semi-invariant T cell receptor (TCR) comprising a *TRAV1-2*$^{+}$ TCRα (TRA) chain, encoding a limited repertoire of *TRAJ* genes, paired to a TCRβ (TRB) chain with varied *TRBV* usage (Tilloy et al. 1999, Porcelli et al. 1993). The first description of activating, MR1 T cell-specific ligands were biosynthetic intermediates in the riboflavin synthesis pathway (Patel et al. 2013, Corbett et al. 2014, Kjer-Nielsen et al. 2012). These compounds are produced by microbes that stimulate MR1T cell responses, thereby introducing a mechanism of self vs. non-self-recognition by MR1T cells.

Recent reports have demonstrated that the repertoire of ligands capable of stimulating MR1T cells is broader than previously thought and includes ligands generated from non-riboflavin synthesizing microbes, such as *Streptococcus pyogenes,*

as well as ligands derived from cultured mouse tumor cells and human monocyte cell lines (Lepore, Kalinichenko, Colone, et al. 2014, Meermeier et al. 2016). Keller utilized *in silico* binding assays to explore the landscape of potential MR1T cell reactive antigens and showed that compounds lacking the ribityl tail of canonical MR1-restricted ligands could stimulate MR1T cells. These ligands were not only bacterial-derived metabolites, but also comprised of a variety of synthetic compounds, including pharmacologic agents (Keller et al. 2017). Recently, metabolomic analyses revealed the identity of shared or distinct non-riboflavin derived antigens from *Escherichia coli (E. coli)* and *Mycobacterium smegmatis (M. smegmatis)* to activate MR1T cells (Harriff et al. 2018).

The relative contribution of MR1T cell TRA and TRB chains in ligand recognition is incompletely understood. Biochemical analysis suggested a critical role for the CDR3α loop in antigen recognition, with Tyr95α predicted to mediate hydrogen bond contacts with the ribityl chain of riboflavin-derived ligands (López-Sagaseta, Dulberger, Crooks, et al. 2013, Kjer-Nielsen et al. 2012). While mutagenesis of single residues within the MR1T CDR3β loop had no discernable effect on ligand recognition, exchange of the entire MR1T CDR3β region for a non-MR1T cell derived CDR3β sequence ablated antigen recognition (Reantragoon et al. 2012). However, Lopez-Sagaseta and colleagues used recombinant humanized forms of bovine MR1 exposed to ligands present in *E. coli* culture supernatant to demonstrate that the MR1T CDR3β loop is important for contact with MR1-bound antigens.(López-Sagaseta, Dulberger, McFedries, et al. 2013) While these data suggest that both TRA and TRB chains contribute to ligand

recognition, the relative contribution of individual TCR chains to the recognition of microbial infections remains to be elucidated.

Increasing evidence suggests that the MR1T TCR repertoire is shaped by exposure to microbial ligands. Analyses of TCR repertoire patterns in circulating, microbially reactive MR1T cells demonstrated diversity in the CDR3β chain usage (Gold et al. 2014). For a given individual, CDR3α usage was limited and could be linked to a specific microbe. Furthermore, Dias et al have reported that the *TRBV* usage in MR1T cell-specific TCRs contributes to responses to MR1-bound antigen and the magnitude of this usage appears to be microbe-dependent (Dias, Leeansyah, and Sandberg 2017). In contrast, Lepore and colleagues found that the CDR3β region is of limited diversity and is shared across individuals (Lepore, Kalinichenko, Colone, et al. 2014). More recently, it has been shown experimentally that vaccination with *Salmonella enterica serovar paratyphi A* resulted in the clonal expansion of MR1T cells, leading to a CDR3β repertoire that is both biased and oligoclonal.(Howson et al. 2018) This raises the intriguing possibility of the MR1T TCR repertoire in an individual adapts to microbial exposure.

Here, we show that a number of MR1T cell clones sharing identical CDR3α regions display a diverse response profile to microbially infected dendritic cells (DCs). Selective recognition of distinct microbes and the *Salmonella enterica* serovar *typhimurium* (*S. typhimurium)*-derived antigen, 5-OP-RU, was clonotypically linked to the individual CDR3β region in each responding clone. Taken together, these results

indicate a greater role than previously described for the MR1T CDR3β in discriminating between bacterial ligands.

Results

MR1T cell clones display diverse responses to microbially infected DCs

We have previously utilized limiting dilution analysis to generate a panel of *Mycobacterium tuberculosis* (Mtb) reactive CD8+ T cell clones from PBMC of healthy or Mtb-infected individuals. These clones all are *TRAV1-2*$^{+}$ and recognize microbial antigen bound to MR1, as measured using a blocking antibody to MR1 (Gold et al. 2010). 120 MR1T cell clones were subsequently tested for production of IFN-γ in response to DCs infected with Mtb, *Mycobacterium smegmatis* (*M. smegmatis*), *Mycobacterium marinum* (*M. marinum*), or *Escherichia coli* (*E. coli*). As shown in Fig 1a, MR1T cell responses were quantitatively diverse. To understand whether or not these responses could be grouped functionally, linear regression analysis was performed and correlation between microbial responses was analyzed. As demonstrated in Fig 1b, responses to *M. smegmatis* and *E. coli* were similar (Pearson's r = 0.74, n =86), while Mtb and *M. marinum* were highly correlated (Pearson's r = 0.74, n = 42). These data suggest that *M.smegmatis* and *E.coli* share a similar ligand repertoire, as do Mtb and *M.marinum*.

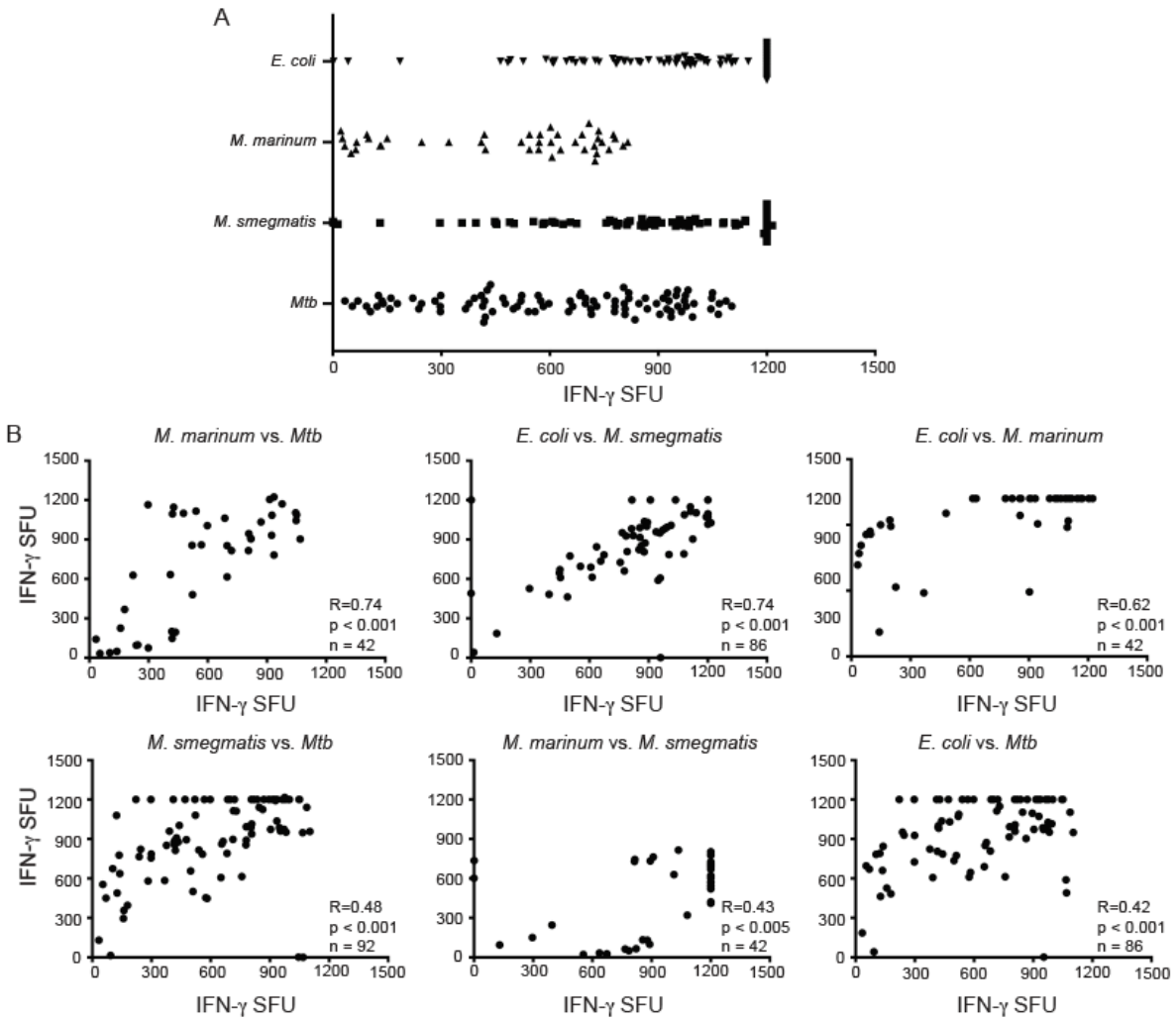

Figure 3.1: Diversity of MR1T responses to microbially infected DCs

MR1T clones generated by limiting dilution analysis from human PBMC were stimulated overnight with dendritic cells infected with Mtb, *M. marinum*, *E.coli*, or *M. smegmatis*. IFN-γ ELISPOT analysis was performed to measure cytokine production by MR1T clones (20000 MR1T cells per well, 20000 APC per well). (A). IFN-γ production by MR1T clones in response to all microbial infections, with each spot representing one clone (n = 42 for *M. marinum*, n = 86 for *E.coli*, n = 92 for *M. smegmatis* and Mtb). (B) Scatter plot of comparison of IFN-γ production by MR1T clones from (Fig 1A) between microbial infections. Linear regression analysis was performed and Pearson's correlation coefficient (R) with sample size (n) was calculated and reported for each comparison

MR1T TCR sequencing reveal clones sharing identical CDR3α regions but distinct CDR3β sequences

To establish the relationship between TCR clonotype and microbial discrimination, we sought to characterize the clonotypic repertoire in our panel of MR1T cell clones. *TRAV1-2*$^{+}$ CD8^{+} MR1T cell clones (Fig 2a) were evaluated for MR1-dependent IFN-γ production in response to *M. smegmatis* infected A549 cells (Fig 2b). In addition, we included a panel of MR1T cell clones isolated from bronchoalveolar lavage of Mtb-infected individuals. The TCR sequence of each validated MR1T clone was determined using an unbiased template switch anchored RT-PCR approach, as previously described (Quigley et al. 2011).

Name	TRAV	CDR3α	TRAJ	MAIT Match Score	TRBV	CDR3β	TRBJ
D0033A2	1-2	CAVTDSNYQLI	33	0.9583	3-1	CASSQAETELNTGELF	2-2
D0033A10					3-1	CASSSGLEVTGELF	2-2
D0033A6	1-2	CAVVDSNYQLI	33	0.9587	4-2	CASSHSSGTGGNEQF	2-1
403A9					20-1	CSARDGGEAYNEQF	2-1
450A9	1-2	CAVRDSNYQLI	33	0.9567	20-1	CSAREVEGTYEQY	2-7
571F3-2					6-4	CASSEASGGTDTQY	2-3
450B9	1-2	CAVMDSNYQLI	33	0.9573	6-1	CASTPSGEFSEAF	1-1
427D8-2					5-1	CASSLLRQGTEKLF	1-4

Table 3.1: Paired MR1T clones with the same TCRα chain but differing TCRβ chains

The majority of sequenced MR1T cell clones expressed *TRAV1-2/TRAJ33* encoded TRA chains that were paired to *TRBV6* or *TRBV20-1* TRB chains, although exceptions were observed where other *TRBV* genes were encoded (e.g. *TRBV3-1, TRBV4-2, TRBV19*; Supplementary Table 1). All sequences were analyzed for similarity to previously reported MR1T CDR3α sequences using an algorithm based on the method described by Shen et al, 'MAIT Match', where reported MR1T CDR3α sequences are analyzed for similarity to published sequences (Shen W.-J. 2012). Based on our prior analyses of MR1T CDR3α chains using this tool, we chose a MAIT Match score of 0.95 as a conservative threshold to define MR1T consistent TCRs. Using this analysis, we show that the majority of the clones were verified to display a high degree of similarity to previously described MR1T cell specific TCRs (Supplementary table 1). Despite evidential publicity in the CDR3α region of sequenced MR1T cell clones, it was apparent that there was no clonotypic sharing of CDR3β regions across donors. Of the 35 clones sequenced, we identified four "pairs" of MR1T cell clones that shared an identical CDR3α sequence with differing CDR3β sequences (Table 2). To our surprise, one pair arose from the same donor (e.g. D0033A2 and D0033A10) and exhibited distinct CDR3β sequences despite both utilizing *TRBV3-1* gene usage. This indicated that there is no clonotypic sharing in CDR3β region of MR1T TCRs across individuals.

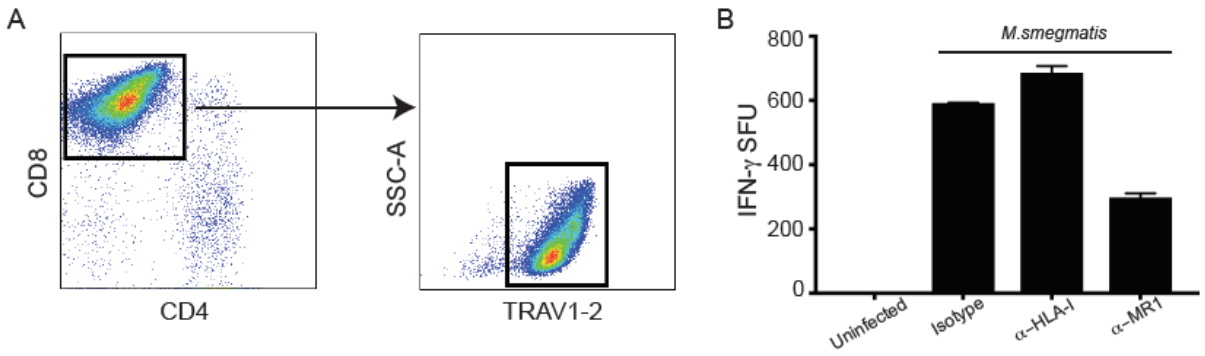

Figure 3.2: Screening of MR1T clones for TRAV1-2 expression and reactivity to *M. smegmatis*

Example plots (571F3-2) of flow cytometric and IFN-γ ELISPOT analysis of MR1T clones sent for sequencing of the MR1T TCR (A): MR1T clones were stained with antibodies to CD3, CD4, CD8, TRAV1-2, and viability stain. CD8+ TRAV1-2+ clones were sorted for sequencing of the MR1T TCR. Plots are gated on live CD3+, CD4- cells. (B): Responsiveness of MR1T clones (5 x 10^3/well) to A549 cells (5 x 10^3/well), incubated 1h with blocking antibodies (5 μg ml^{-1}) to MR1 (α-MR1, Clone 26.5), pan-Class I (α-HLA-I, Clone W6-32), or an anti-mouse IgG2a isotype, and infected overnight with *M. smegmatis* (MOI = 3). IFN-γ production was measured by ELISPOT and reported as IFN-γ spot forming units/5000 T cells (IFN-γ SFU). Error bars represent mean and standard error from duplicate wells.

Paired MR1T clones differentially recognize microbially infected dendritic cells

While the role of the CDR3β in recognition of peptide ligand presented on classical MHC is well defined, it is not clear the extent to which the MR1T TCRβ chain contributes to recognition of microbial antigens. Consequently, we tested each paired MR1T for responsiveness to dendritic cells infected with *Salmonella enterica* serovar Typhimurium *(S. typhimurium)*, *C. albicans*, or *M. bovis* BCG (BCG*)*.

As shown in Figure 3, the overall pattern of microbial responsiveness was similar for clone pair 450B9 and 427D8-2. While 427D8-2 responded to *S.typhimurium*, *C. albicans*, and BCG at magnitudes comparable to the MR1T control clone, 426G11, the magnitude of 450B9 responses to these infections was 2-fold weaker for BCG ($p = 0.0008$), and nearly 3-fold weaker for both *S. typhimurium* ($p < 0.0001$)and *C. albicans* ($p < 0.0001$). This suggests while 450B9 appears to have diminished functional avidity, it seems to be independent of the TCRβ.

In contrast, the clone pair 403A9 and D0033A6 yielded a very different result. Specifically, responses by 403A9 and D0033A6 were similar in magnitude to *BCG* infection. However, while 403A9 responded robustly to *S. typhimurium* infection, the D0033A6 response was 2-fold weaker ($p = 0.016$). The D0033A6 response to *C. albicans* was slightly, but not significantly weaker than the response by 403A9. These results indicate that the differences in the CDR3β between D0033A6 and 403A9 could contribute to the recognition of *S. typhimurium* derived antigens, but not to antigens derived from BCG or *C. albicans*.

The clone 450A9 displayed a slightly, but not significantly, weaker response to *S. typhimurium* than 571F3-2. Responses of 450A9 and 571F3-2 to *C. albicans* were similar in magnitude, but 450A9 responses to BCG were significantly weaker than 571F3-2 ($p = 0.039$).

The clone pair D0033A2 and D0033A10, however, displayed no differences in magnitude when responding to all three infections, suggesting that the differences in the TRB chain did not confer specificity for these clones in recognizing antigen.

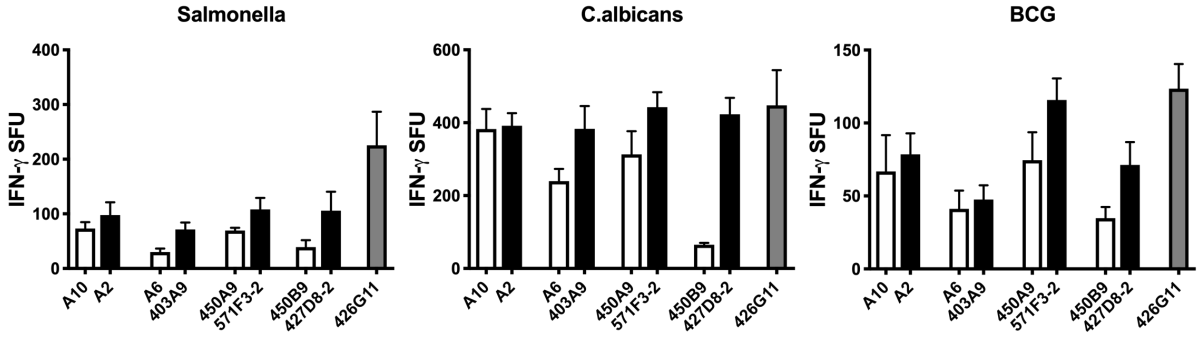

Figure 3.3: IFN-γ production by paired MR1T clones in response to microbial infection

MR1T cell clone pairs sharing the same CDR3α and different CDR3β sequences were stimulated with DCs infected with *C. albicans* (MOI = 3), *S. enterica* serovar typhimurium (MOI = 30), or *M. bovis* BCG (MOI = 15). IFN-γ ELISPOT was performed as described in (Fig 2b). Data are aggregated from 3 independent experiments, which were each performed in duplicate. Error bars represent mean and standard error. Student's t-test was performed to compare IFN-γ responses between paired MR1T clones and p-values < 0.05 were considered significant (*$P \leq 0.05$; ** $P \leq 0.01$, *** $P \leq 0.005$).

To extend these observations further, we sought to determine whether differences in MR1T cell CDR3β region confer selectivity against the predominant, MR1-restricted antigen synthesized by *S. typhimurium,* 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) (Corbett et al. 2014). Accordingly, we utilized a plate-bound MR1/5-OP-RU tetramer ELISPOT (tetraSPOT) assay, which we have previously demonstrated will activate MR1T cell clones (Harriff et al. 2018), to evaluate MR1/5-OP-RU specific responses in each pair of MR1T cell clones (Figure 3.4). Here, the MR1/5-OP-RU tetramer was plated over a range of concentrations to assess the functional avidity (EC_{50}) of each clone, as established previously (Ioannidou et al. 2017). All MR1T cell clones responded to the plate bound MR1/5-OP-RU tetramer, and each response profile and avidity measurement largely mirrored that observed in functional assays with *S. typhimurium* infected DCs (Figure 3). Most notably, the 403A9 clone had an EC_{50} that was 4-fold higher than D0033A6. In contrast, the responses between the other paired clones were similar, both in magnitude and in EC_{50}, again confirming the responses of MR1T clones to *S. typhimurium*. The responses of the paired clones to a MR1/6FP control tetramer was zero (data not shown). Taken together, these findings implicate the MR1T CDR3β loop as an important component for ligand discrimination during microbial infections, notably *S. typhimurium.*

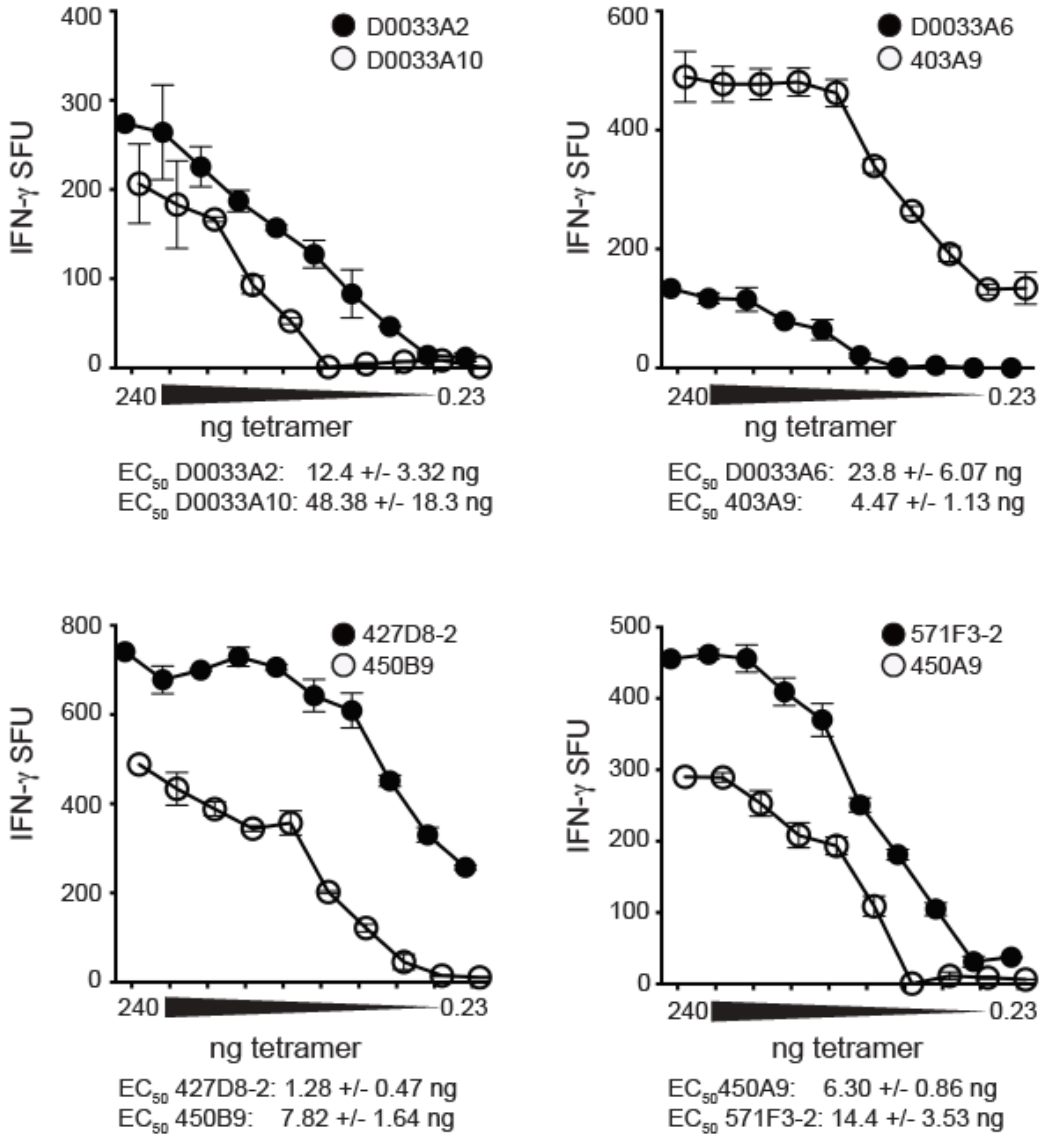

Figure 3.4: Responses of paired MR1T clones to plate bound 5-OP-RU MR1 tetramer

Paired MR1T clones were stimulated with plate-bound MR1/5-OP-RU tetramer at a range of concentrations as indicated. Modified ELISPOT analysis was performed as described previously (Harriff et al. 2018) to assess IFN-γ production by MR1T clones in response to plate bound tetramer. Error bars represent means and standard error of technical replicates. Results are representative of 3 independent experiments. EC50 was calculated and reported for each clone.

Discussion

To explore the relationship of ligand discrimination and TCR usage, we sequenced TCRs from a panel of *TRAV1-2*$^{+}$ MR1T cell clones. The diversity in the CDR3α and CDR3β chains was consistent with prior reports (Lepore, Kalinichenko, Colone, et al. 2014, Lepore et al. 2017, Gold et al. 2014). Through the identification of clone pairs that shared identical CDR3α loops but possessed unique CDR3β regions, we were able to assess the degree to which the CDR3β chain contributed to selective microbial recognition. As we found that the TCRβ chain was important for the selective recognition of *S. typhimurium*, but less so for *C. albicans* or BCG, these data would support the presence of ligand diversity between these microbial species. This conclusion is also supported by the observation that MR1T responses to *E. coli* and *M. smegmatis* are correlated, as are responses to Mtb and *M. marinum*. Furthermore, recent studies have demonstrated that the repertoire of activating ligands for MR1T cells varies between *E.coli* and *M.smegmatis* (Harriff et al. 2018).

Prior studies have explored the structural basis by which the MR1T TCR recognizes MR1-bound ligand. However, there is ambiguity about the relative importance of the CDR3α and CDR3β chains in mediating contacts with MR1 and its ligand. The MR1T TCR appears to dock in a conserved mechanism to engage with MR1-bound ligand and the Tyr95α mediates contact with the ribityl tail of canonical ligands (Eckle et al. 2014). However, with recent evidence for non-riboflavin derived ligands binding MR1 to stimulate MR1T cells, the role of other residues in both the TCRα and TCRβ chains remains to be determined (Harriff et al. 2018, Meermeier et al. 2016, Keller

et al. 2017). Though the variability in reported CDR3β chains did not contribute to large structural changes in docking of the MR1T TCR, it is possible that CDR3β hypervariability plays a role in fine-tuning MR1T engagement with antigen, as CDR3β chains enriched in glycine were reported to make fewer contacts with MR1-bound ligand (Reantragoon et al. 2013). The CDR3β chain has been reported to display conformational flexibility in its ability to engage with MR1 and its ligand, with the intriguing possibility that distinct ligands can modulate this fine-tuning of the CDR3β and perhaps increase the potency of TCR contacts with stimulatory ligand (Eckle et al. 2014, López-Sagaseta, Dulberger, Crooks, et al. 2013). Differences in the MR1T TCRβ chain result in varied affinity between the TCR and MR1-bound ligand. This, combined with a more conserved CDR3α that also engages with ligand, indicates that both chains are vital in MR1T cell detection of antigen (López-Sagaseta, Dulberger, McFedries, et al. 2013). Our results support this hypothesis, as all MR1T clones were capable of recognizing and responding to microbial antigen. However, the differences in the CDR3β region did result in changes in magnitudes of responses, to microbial infection, and exogenous ligand.

In recent years, there has been increasing evidence for diversity among the MR1 ligandome, and the ability for individual TCRs to discriminate between these ligands. While MR1-restricted ligands were first identified as metabolites from the riboflavin biosynthesis pathway, recent evidence has shown that ligands can be derived from other pathways or sources and have unique structural features (Meermeier et al. 2016, Gherardin, Keller, Woolley, Le Nours, et al. 2016, Keller et al. 2017, Harriff et al. 2018). Therefore, the MR1T cell specific TCR may recognize a wider array of microbially derived

antigens than previously thought and would suggest the possibility that MR1T cells are selective in the recognition of these ligands. Such selectivity would raise the possibility that MR1T cells have immunologic memory.

Recent studies suggest that the MR1T cell-specific TCR repertoire is shaped in response to the wide range of MR1T antigen produced by microbes (Harriff et al. 2018, Howson et al. 2018, Gold et al. 2014). Our results suggest that in addition to a highly diverse MR1T TCR repertoire, distinct pathways exist between microbes to produce MR1T cell activating ligands. Additionally, the MR1T TCR repertoire is shaped by exposure to microbial infection (Dias, Leeansyah, and Sandberg 2017, Gold et al. 2014, Howson et al. 2018). Taken together, these data support the hypothesis that the MR1T cell-specific TCR repertoire adapts in response to exposure to an increasingly diverse microbial metabolome.

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.

Cell lines

A549 cells (ATCC CCL-185) were used as stimulators for IFN-γ ELISPOT analysis for MR1T clones, and cultured according to recommended guidelines. Cell lines were confirmed to be mycoplasma free.

Expansion of T-cell clones

T-cell clones were cultured in the presence of X-rayed (3,000 cGray using X-RAD320, Precision X-Ray Inc.) allogeneic PBMCs, X-rayed allogeneic LCL (6,000 cGray) and anti-CD3 monoclonal antibody (20 ng ml^{-1}; 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 monoclonal antibodies.

Sorting and expansion of BAL-derived T cell clones

Cells from bronchoalveolar lavage of Mtb-positive individuals were stained with a viability stain, antibodies against CD4, CD8, TCRγδ, and the MR1/5-OP-RU tetramer at optimized concentrations. Cells were sorted on the basis of live CD4- TCRγδ-, CD8+, MR1/5-OP-RU tetramer+ and limiting dilution was performed as previously described with minor modifications (Lewinsohn et al. 2000). Briefly, 3 T cells per well were incubated in a 96 well U-bottom plate with X-rayed allogeneic PBMC and LCL, supplemented with IL-2 (5 ng ml^{-1}), IL-7, IL-12, and IL-15 (0.5 ng ml^{-1}), and anti-CD3 monoclonal antibody (30 ng ml^{-1}) to generate MR1T clones, which were tested in an IFN-γ ELISPOT to confirm responsiveness to Mtb-infected A549 cells, as well as MR1 restriction. Mtb-responsive MR1-restricted clones were subsequently expanded as described above and used for downstream experiments.

Monocyte-derived DCs

PBMCs obtained by apheresis were resuspended in 2% human serum in RPMI and were allowed to adhere to a T-75 flask at 37 °C for 1 h. After gentle washing twice with PBS, nonadherent cells were removed and 10% human serum in RPMI containing 30 ng ml^{-1} of IL-4 (Immunex) and 30 ng ml^{-1} of granulocyte–macrophage colony-stimulating factor (Immunex) was added to the adherent cells. The cells were X-rayed with 3,000 cGray using X-RAD320 (Precision X-Ray Inc.) to prevent cell division. After 5 days, cells were harvested with cell-dissociation medium (Sigma-Aldrich, Gillingham, UK) and used as APCs in assays.

Microorganisms and preparation of APCs

M. smegmatis, C. albicans, S. Typhimurium, E. coli, M. tuberculosis, M.marinum, and *M. bovis* BCG were utilized from frozen glycerol stock. A549 cells were infected overnight with *M. smegmatis* at a multiplicity of infection (MOI) of 3 at 37 C. DCs were infected either 2h (*C. albicans, S. Typhimurium, M. bovis* BCG) or overnight (*M. smegmatis, M. tuberculosis, E. coli, M. marinum*) at 37 C. The MOI and antibiotics used were optimized for APC viability and maximal MR1-restricted response. All infections were performed in the absence of antibiotics, and following the indicated infection time, cells were washed twice in media containing antibiotics, counted, and added to the ELISPOT assay.

Staining and sorting of CD8+ TRAV1-2+ MR1T clones

MR1T clones to be sorted were first incubated in a blocking solution of PBS supplemented with 5% heat inactivated goat serum, 5% heat inactivated pooled human serum, and 0.5% heat inactivated fetal-bovine serum. 1 x 10^6 MR1T cells were stained with propidium iodide viability stain, and antibodies to CD3, CD4, CD8, TRAV1-2 in the dark at 4 °C for 30 minutes. Samples were then washed with PBS. Live, CD3+, CD4-, CD8+, TRAV1-2+ cells were sorted on an InFlux 11 parameter cell sorter (Becton Dickinson, NJ, USA) with the Oregon Health and Science University flow cytometry core facility. Cells were sorted into RNALater (Invitrogen) for further RNA isolation and analysis. Data were analyzed using FlowJo software (TreeStar).

Molecular analysis of TCR usage.

Clonotypic analysis of sorted cell populations was 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 IMGT nomenclature is used in this study (Lefranc et al. 2003).

CDR3α sequence similarity

Similarity between CDR3β sequences was calculated as described previously (Gold et al. 2014). This method allows similarities to be assigned between sequences of different length in an alignment-free manner. An implementation of the similarity matching between CDR3α sequences is publicly available at http://www.cbs.dtu.dk/services/MAIT_Match. The server takes as input a list of CDR3α sequences, and returns for each a score based on the maximal sequence similarity with a reference database of MAIT cell CDR3α sequences. A perfect match has a similarity score of 1, and a perfect mismatch a similarity score of 0.

IFN-γ ELISPOT

A MSHA S4510 96 well nitrocellulose-backed plate (Millipore, bought via Fisher Scientific) was coated overnight at 4 °C with 10 μg ml^{-1}solution of anti-IFN-γ monoclonal antibody (Mabtech clone 1-D1K) in a buffer solution of 0.1 M Na_2CO_3, 0.1 M $NaHCO_3$,

pH=9.6). Then, the plate was washed three times with sterile PBS and blocked for 1 h at room temperature with RPMI 1640 media containing 10% heat-inactivated HS pool. Then, the APCs and T cells were prepared as described above and co-incubated overnight. Briefly, DCs or the A549 cell line (all other experiments) were used as APCs at 5×10^3 or 1×10^4 per well in ELISPOT assays. For all blocking ELISPOT assays, APCs were limited to 5×10^3 per well. Where stated, blocking antibodies or antagonists were added for 2 h at 5 µg ml^{-1} (α-HLA-I clone W6/32, and α-MR1 clone 26.5 (Ted Hansen) or appropriate isotype controls). T-cell clones were added at 5×10^3 per well. The plate was incubated overnight at 37 °C and then washed six times with PBS containing 0.05% Tween. The plate was then developed as previously described and analyzed using an AID ELISPOT reader (Harriff et al. 2018).

Plate bound tetramer ELISPOT (tetraSPOT) assay

Plate-bound tetramer ELISPOT (tetraSPOT) assay was performed as previously described (Harriff et al. 2018). ELISPOT plates were coated with anti-IFN-γ antibody as described above. At the time of coating, MR1 tetramers loaded with either the stimulatory ligand 5-OP-RU or the non-stimulatory ligand 6-FP were also added to wells at amounts between 0 to 0.24 ug ml^{-1} per well. After overnight incubation at 4ºC, ELISPOT plates were washed three times with PBS, then blocked with RPMI+10% human serum for 1 hour. 1×10^4 MAIT cell clones were added to wells overnight. IFN-γ ELISPOTs were enumerated following development as previously described (Meermeier et al. 2016).

Data analysis

Data were analyzed and plotted using Prism 7 GraphPad Software (La Jolla, California). Statistical significance was determined using unpaired Student's two-tailed *t*-test, unless otherwise indicated. For comparisons of MR1T responses between microbial infection, linear regression analysis was performed, and Pearson's correlation coefficient was calculated. For measurements of functional avidity, the curves were transformed to a semilog scale and normalized. Best-fit EC_{50} was calculated by Prism and statistical significance was determined from differences in Log EC_{50}. Error bars in the figures indicate the standard deviation, standard error of the mean, or the data set range as indicated in each figure legend. *P* values ≤ 0.05 were considered significant (* $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Chapter 4: Alternative Splicing of MR1 regulates antigen presentation to MR1T cells

Abstract

MR1 restricted T (MR1T) cells play a key role in early immune defense against a broad array of pathogens. These cells are activated upon encountering microbial antigen bound to MR1 on the surface of an infected cell. As MR1T cells are highly enriched in mucosal sites and respond to microbial infection, presentation of ligand by MR1 must be tightly regulated to prevent aberrant T cell activation. MR1 undergoes alternative splicing. Only the full length isoform, MR1A, can activate MR1T cells. The other isoforms, MR1B and MR1C, are not well characterized and their function remains unknown.

We analyzed splice variant expression across the GTEx RNASeq dataset. We found variation in the relative abundance of MR1A to MR1B transcript, with increased relative MR1A transcript observed in blood and lung. These findings were also observed in antigen presenting cells isolated from human blood and lung, as well as epithelial cell lines derived from a variety of tissues. Intriguingly, we show that lowered relative MR1A/MR1B expression in uterine and breast cancer cell lines appeared to be associated with function, as these cells were less capable of stimulating MR1T responses to antigen.

To test the function of MR1 isoforms, we generated a bronchial epithelial cell line lacking MR1. We show that only MR1A stimulates MR1T cell activation following mycobacterial

infection. However, coexpression of MR1B with MR1A leads to decreased MR1T cell production of IFN-Y in response to mycobacteria. Using a doxycycline-inducible MR1AGFP construct, combined with transient transfection of MR1B either prior to or post MR1A expression, we show that MR1B may be functioning to limit abundance of MR1A protein, thus presenting a potential mechanism of inhibition.

Double positive MR1 expressing thymocytes are the only cell in the body to constitutively express MR1 on the cell surface, and are thought to play a role in the thymic selection of MR1T cells. We show that relative *MR1A/MR1B* gene expression in DP MR1 expressing thymocytes is correlated not only with surface protein expression, but also, intriguingly, with frequency of MR1T cells in the thymus. This suggest an exciting second role for alternative splicing of MR1 in helping regulate thymic development of MR1T cells.

Our results suggest alternative splicing of MR1 represents a potential means of directing appropriate MR1T cell responses to infected cells. These data raise important questions about the role of gene expression and genetic modifications in modifying T cell responses to bacterial antigen.

Introduction

MR1-restricted T (MR1T) cells are important responders in the immune response to microbial infection. A subset of CD8 αβ T cells, MR1T cells are highly abundant-representing 1-10% of circulating T cells and are enriched in mucosal sites, including the skin, intestinal mucosa, and lung (Dusseaux et al. 2011). MR1T cells are stimulated to produce the effector cytokines IFNγ and TNFα following encounter with infected cells (Gold et al. 2010, Le Bourhis et al. 2010). MR1T cells respond to a wide array of microbes-including, but not limited to, Mycobacterium tuberculosis (Mtb), *Escherichia coli (E. coli)*, and *Candida albicans (C. albicans)* (Gold et al. 2010, Le Bourhis et al. 2010). Mice lacking MR1T cells were unable to fully control infection with F. tularemia, displaying a delay in activation of CD4+ and CD8+ T cells and production of IFNγ (Meierovics, Yankelevich, and Cowley 2013). As shown in Chapter 3 of this dissertation, the MR1T T cell receptor is highly diverse and can respond to a wide range of ligands. Therefore, MR1T cells represent an important mechanism by which the immune system senses and responds to microbial infection.

MR1T cells recognize microbial antigen processed and presented by the MHC Class I related molecule, MR1 (Treiner et al. 2003). MR1, though similar in many ways to canonical Class I molecules, has distinct features that render it uniquely suited to present antigen to MR1T cells. Unlike MHC Class I molecules, which are constitutively detected on the cell surface, MR1 resides in the ER and late endosomal vesicles in the absence of infection (Harriff et al. 2014). Following infection, MR1 binds microbial ligand, and this complex traffics to the cell surface to stimulate MR1T cells. We have previously

shown that MR1 trafficking does not utilize canonical Class I trafficking molecules, but instead, is dependent on the vesicular trafficking proteins Syntaxin18 and VAMP4**.** More recently, we have observed that distinct trafficking pathways exist to present endogenous and exogenous mycobacterial antigen by MR1 to stimulate MR1T cells (Harriff et al. 2016).

While MHC Class I molecules traditionally present peptide antigen to stimulate CD8+ T cell responses, MR1 binds and presents microbial small molecule metabolites to MR1T cells. These antigens were first described as intermediates in the riboflavin synthesis pathway, but recent reports have highlighted the increasing diversity of the MR1T ligand repertoire. In addition to presenting antigen from microbes that are capable of synthesizing riboflavin, including mycobacteria, MR1 also binds and presents antigen from *S. pyogenes*, a bacteria that cannot synthesize riboflavin (Meermeier et al. 2016). Keller showed, using an *in silico* screen, that MR1 can bind a range of synthetic compounds, including commonly prescribed pharmaceuticals (Keller et al. 2017). We have recently shown through metabolomics analysis that MR1T stimulatory antigens include ligands distinct from those generated in the riboflavin synthesis pathway (Harriff et al. 2018). These reports raise the intriguing possibility of the role of MR1 in presenting ligands from a wide array of microbes.

The gene organization of MR1 is distinct from canonical MHC molecules. *MR1* is non polymorphic, highly conserved across species and individuals, with the transcript ubiquitously expressed (Hashimoto, Hirai, and Kurosawa 1995, Yamaguchi et al. 1997, Parra-Cuadrado et al. 2000). *MR1* pre-mRNA undergoes alternative splicing to produce

multiple isoforms, which have been demonstrated, at the mRNA level, to be expressed in human tissues and cell lines (Riegert, Wanner, and Bahram 1998). The genomic organization of MR1 is similar to MHC Class I molecules, with α1 and α2 domains that bind ligand, an α3 domain that interacts with β2-microglobulin, and a transmembrane domain for surface expression (Yamaguchi and Hashimoto 2002, Riegert, Wanner, and Bahram 1998). The full length variant, *MR1A*, contains all encoded exons and can stimulate MR1T cells. *MR1B* lacks the α3 domain, but does encode the ligand binding and transmembrane domain. The function of MR1B is not well elucidated. Overexpression of MR1B in a fibrosarcoma model suggested a potential role of MR1B in stimulating MR1T cells following infection with E. coli (Lion et al. 2013). *MR1C* is a putative soluble isoform, lacking both the α3 and transmembrane domains, however, there are no reports on its expression or function.

Here, we sought to determine what role the MR1 isoforms play in the presentation of microbial ligand to activate MR1T cells. We show that *MR1* splice variant transcript are detectable across human tissues, with higher relative *MR1B* mRNA in tissues that are more likely to require tight regulation of T cell activation. We developed a lung epithelial cell line deficient in *MR1* and utilized this system to show that MR1B antagonizes MR1A in presenting mycobacterial antigen to MR1T cells. MR1B appears to remain in intracellular vesicles, and intriguingly, we demonstrate that expression of MR1B may inhibit the abundance or stability of MR1A protein. Finally, we show that surface expression of MR1A on CD4+CD8+ thymocytes is associated with relative mRNA expression of the MR1 splice variants. Taken together, our results in this chapter

indicate a novel mechanism by which MR1T cell activation is potentially regulated for appropriate targeting of immune responses against microbial infection.

Results

MR1A and MR1B are ubiquitously expressed

To more precisely explore the expression of the MR1 splice variants, we utilized an algorithm developed specifically to analyze relative splice variant expression from high-throughput RNA sequencing data. This algorithm (Snaptron) is based on quantifying the unique junctional sequences generated by the inclusion or exclusion of exons from alternative splicing events (Nellore et al. 2016, Wilks et al. 2018). A more detailed description of the algorithm utilized and its parameters is described by Nellore and Wilks (Nellore et al. 2016, Wilks et al. 2018). We queried the Genotype Tissue Expression Project's (GTex) transcriptome of over 10000 samples from 53 non-diseased tissue sites from 700 deceased individuals (Consortium 2013). Using this algorithm, we were able to distinguish *MR1A* from *MR1B* mRNA due to the inclusion of exon 4, which thereby yields unique junctional sites. Here, we show that *MR1A* and *MR1B* transcript are expressed across human tissues, as seen in prior, non-quantitative studies (Figure 1). The ratio of *MR1A* to *MR1B* varied across tissues, with higher relative *MR1A* mRNA observed in blood, bone marrow, liver, and lung, and lower relative *MR1A* observed in uterine cervix, breast, small intestine, and colon. Interestingly, we observed that in all the tissues queried, the *MR1A/MR1B* ratio was consistently less than 0.5, indicating that all the tissues did express higher relative *MR1B* than *MR1A.* However, this analysis does not take into account the individual cell types within a tissue, and therefore, there is likely differential expression of the MR1 splice variants between cell types. Uterus and colon are tissue sites that require very tight regulation of the immune response, therefore, a

lower *MR1A* expression at steady state may be essential to preventing aberrant MR1T activation in the context of commensal microbes or during pregnancy.

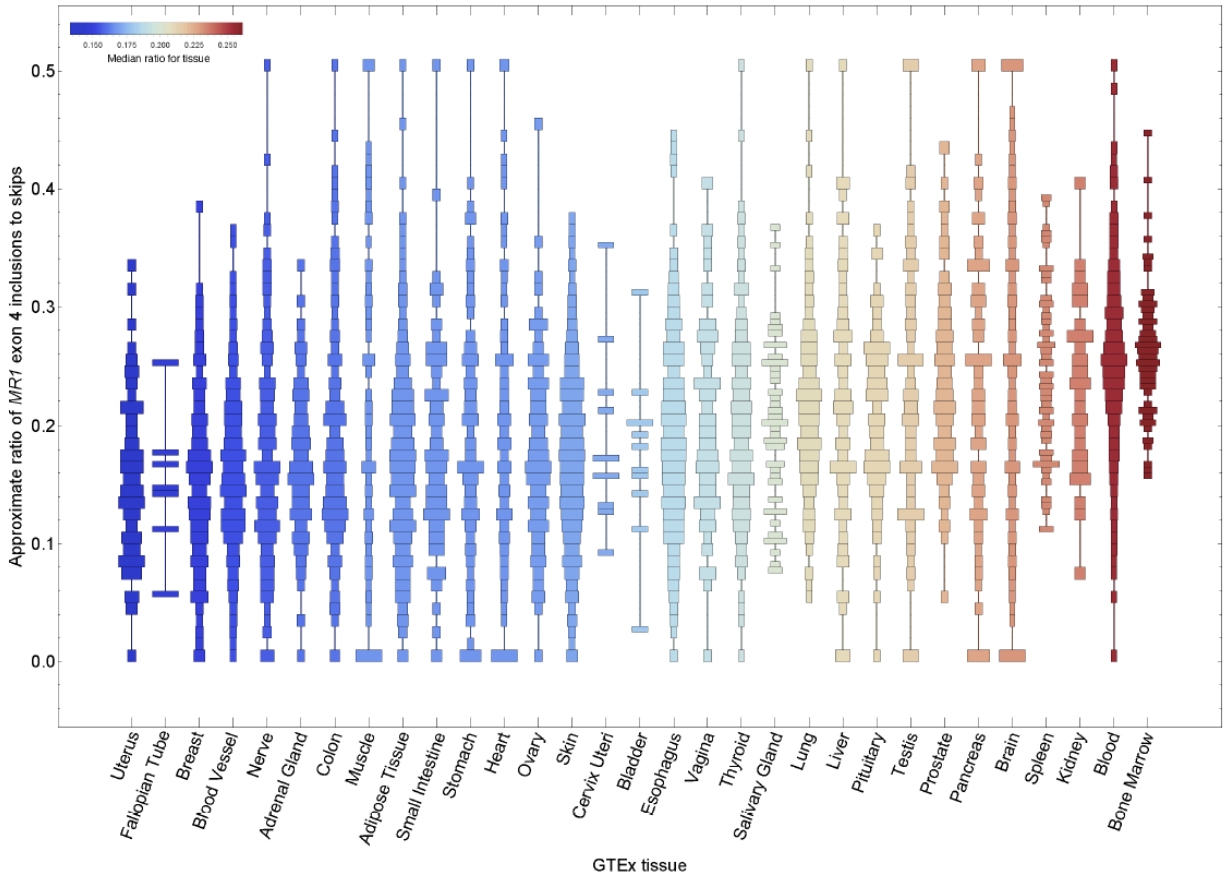

Figure 4.1: Distribution of relative *MR1A/MR1B* transcript across the GTex dataset

The Snaptron tool to quantify splicing events was used to query human transcriptome data from the publicly available GTex dataset of non-malignant human tissues. Relative *MR1A/MR1B* mRNA expression was measured by quantifying junctional inclusion ratios of exon 3 inclusions (*MR1A*) to exon 3 skips (*MR1B*). The x-axis represents each tissue site, arranged from lowest ratio of *MR1A/MR1B* transcript to highest ratio of *MR1A/MR1B* mRNA. The y-axis represents the relative *MR1A/MR1B* ratio. Data are represented as box-and-whisker plots, with the width of each bin determined by the Snaptron algorithm.

Quantitative RT-PCR analysis reveals differing levels of MR1 splice variants in antigen presenting cells

To explore the relative MR1 splice variant expression in antigen presenting cells (APCs), we utilized quantitative real-time PCR (qRT-PCR) following isolation of APCs from human blood and lung tissue. We designed primers to be specific for each isoform (Figure 2A), and generated a standard curve for each splice variant by using plasmids specific to each isoform. In this manner, we were able to quantify absolute and relative amounts of the MR1 splice variants. From PBMC, we generated monocytes which were then differentiated by plastic adhesion and cytokine stimulation to generate dendritic cells or macrophages (Romani et al. 1996). We observed that in both dendritic cells (DCs) and macrophages, both splice variants were expressed at detectable levels, however, *MR1A* and *MR1B* mRNA expression was relatively equal across donors, with slightly more *MR1B* transcript observed for both macrophage and DC donors. (Figure 2B).

To query the expression of MR1 splice variants in human tissue, we generated single cell suspensions from lung parenchyma and small intestine lamina propria and subsequently sorted epithelial cells by EpCam (CD326) staining (Figure 2C). We then performed qRT-PCR on these cells to show that in both lung and lamina propria epithelial cells, expression of *MR1A* was again similar to *MR1B,* with relatively stable expression of both splice variants in these cells. Interestingly, both lung and lamina propria cells expressed nearly 2-log lower levels of *MR1* splice variants than cells generated from

PBMC, however, these cells were not known to be exposed to MR1 ligand, and therefore, exposure to ligand may change the expression of MR1.

We have previously shown that primary large airway epithelial cells (LAEC) are capable of presenting mycobacterial antigen to stimulate MR1T cells (Gold et al. 2010). Therefore, we generated LAEC from human airway tissue, which we validated by surface staining for EpCam by flow cytometry and microscopy (Figure 2D). We then performed qRT-PCR on these cells and show that in the absence of infection, LAEC isolated from 5/6 donors expressed higher levels of *MR1B* mRNA to *MR1A,* concurrent with RNASeq analysis. Overall transcript expression of MR1 splice variants was similar to levels observed in APCs generated from PBMC. *MR1A* mRNA levels were more stable across donors, ranging from 1.0E-06 to 2.1E-06, while *MR1B* transcript ranged from 2E-07 to 4E-06. Of note, one donor, D24, expressed much higher (4-fold) expression of *MR1B* than *MR1A*, however, the other LAEC examined expressed 2-5-fold higher *MR1A* to *MR1B.*

Taken together, these results indicate validity of the GTex approach for querying splice variants, but highlights heterogeneity within tissues of MR1 splice variant expression.

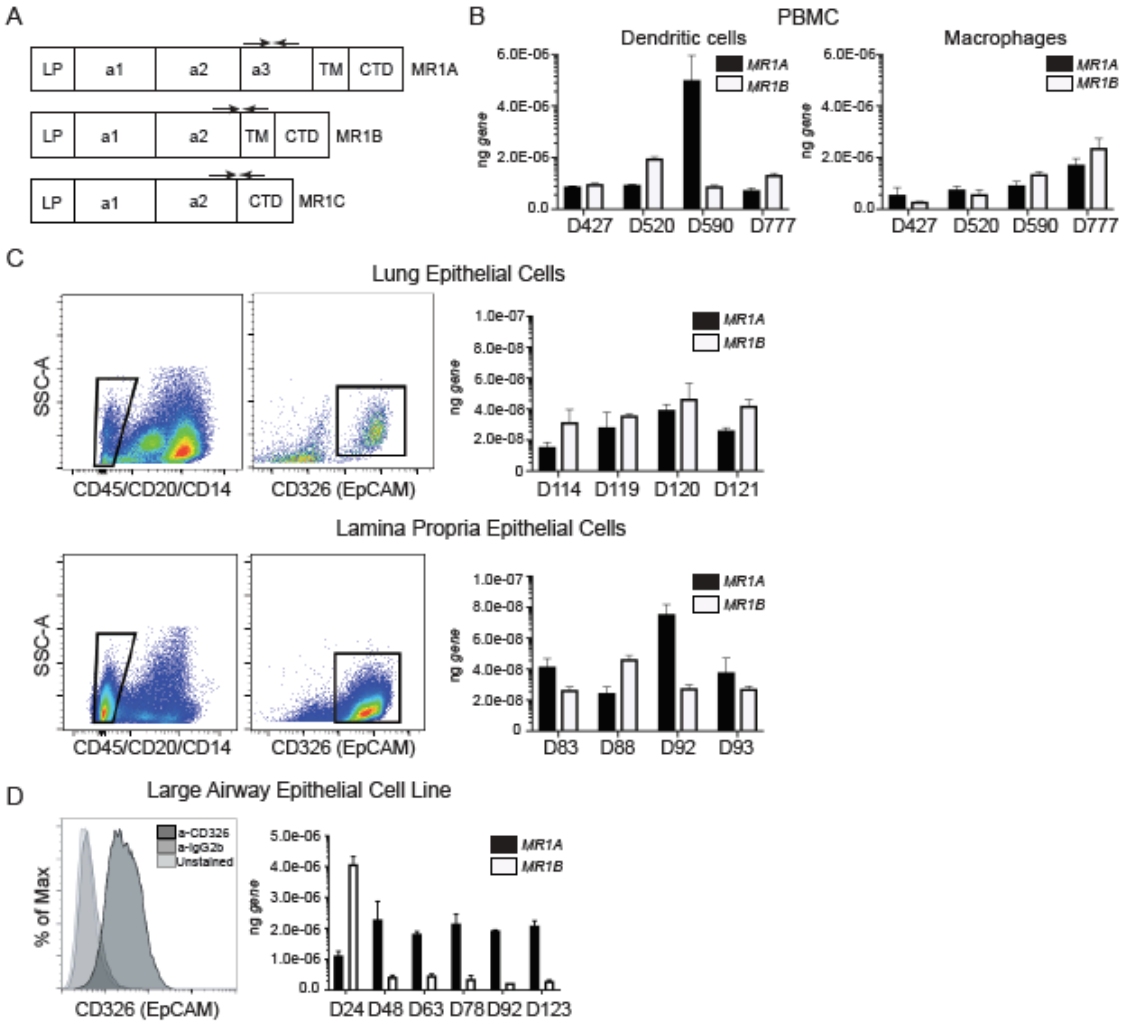

Figure 4.2: MR1A and MR1B mRNA are detectable in antigen presenting cells isolated from human tissues

Quantitative real-time PCR (qRT-PCR) primers were designed to specifically amplify either *MR1A*, *MR1B,* or *MR1C* transcript (A). Adherent monocytes were generated from PBMC of 4 human donors and differentiated for 5 days into macrophages, or for 5-days with the cytokines IL-4 and GM-CSF to generate dendritic cells. mRNA was isolated and qPCR was performed to determine the absolute expression of *MR1A* and *MR1B* mRNA, as quantified by using a standard curve generated by plasmids specific to each splice variant (B). (C) Lung parenchyma and small intestine lamina propria epithelial cells were stained with antibodies against CD45, CD20, CD14 and CD326 (EpCAM). CD45- CD20- CD14- CD326+ cells were sorted for mRNA isolation and cDNA synthesis. qRT-PCR was performed as described in (B) to determine the absolute expression of *MR1A* and *MR1B* mRNA for each donor. (D) Human Large Airway Epithelial Cells (LAEC) were isolated and cultured from the upper airway and stained with an antibody to either isotype (IgG2b) or CD326 (EpCAM) to verify surface expression of EpCAM. mRNA was isolated and qRT-PCR performed as described in (B) to measure absolute expression of *MR1A* and *MR1B* mRNA. Error bars represent mean and standard error from triplicate wells

MR1B inhibits T cell activation by MR1A

We wished to assess the function of the MR1 isoforms without the presence of endogenous MR1. To accomplish this, we generated a bronchial epithelial cell line (Beas2B) that lacked the gene for *MR1* (Beas2B:MR1_KO) by utilizing a CRISPR/Cas9 system that had previously been developed to generate an A549 *MR1* knockout cell line (A549:MR1_KO) (Laugel et al. 2016). Following limiting dilution to generate Beas2B clones lacking *MR1*, we screened the candidate Beas2B:MR1_KO cell line with 6-formylpterin (6FP), a folic acid metabolite that stabilizes MR1 on the surface of cells (Eckle et al. 2014). Using this system, followed with antibody staining against MR1, we show, as compared to wild type Beas2B, Beas2B:MR1_KO cells did not express MR1 on the cell surface upon exposure to ligand (Figure 3A). Additionally, we screened Beas2B:MR1_KO cells for their ability to stimulate MR1T cells following infection with *Mycobacterium smegmatis* (*M.smegmatis*) using IFNγ release ELISPOT. Beas2B:MR1_KO cells are incapable of promoting MR1T production of IFNγ upon infection with M. smegmatis (Figure 3B). As antigen presentation to HLA-E restricted or HLA-B45 restricted T cells is not decreased by Beas2B:MR1_KO cells, knockout of MR1 appears to be specific (Figure 3C)

To study the function of the MR1 splice variants in isolation, we generated expression vectors containing the genes for *MR1A*, *MR1B,* or *MR1C* under the control of the CMV promoter. To distinguish MR1A from MR1B or MR1C following transfection, we tagged MR1A with GFP and MR1B and MR1C with RFP (pCI:MR1AGFP, pCI:MR1BRFP, pCI:MR1CRFP). We then transfected these plasmids individually into

Beas2B:MR1_KO and A549:MR1_KO cells and infected the cells with *M.smegmatis* overnight in order to stimulate MR1T cells in an IFNγ release assay. Compared to wild type Beas2B cells, cells transfected with an empty pCI vector were incapable of stimulating MR1T cell production of IFNγ (Figure 3D). Expression of pCI:MR1AGFP reconstituted MR1T cell release of IFNγ in Beas2B:MR1_KO and A549:MR1_KO cells, while expression of pCI:MR1BRFP or pCI:MR1CRFP in MR1 deficient cell lines did not result in MR1T production of IFN γ. As MR1B was detectable inside cells, as measured by quantifying RFP expression by flow cytometry, this indicates that MR1B alone does not present mycobacterial antigen to activate MR1T cells. MR1C was not observed to be detectable inside cells, and we were unable to detect soluble MR1C in the culture supernatant (data not shown), therefore, we believe that MR1C is unable to be expressed as protein.

We addressed the possibility that MR1B or MR1C functions in concert with MR1A to modulate MR1T activation. We coexpressed pCI:MR1AGFP with pCI:MR1BRFP or pCI:MR1CRFP in Beas2B:MR1_KO and A549:MR1_KO cells, and subsequently infected the cells with *M. smegmatis*. IFNγ ELISPOT was performed as described above. Coexpression of MR1A with MR1B resulted in a 40% decrease in MR1T cell activation in both cell lines (Figure 3E). This inhibition was also seen upon infection with M. bovis BCG for Beas2B:MR1_KO cells coexpressing MR1A and MR1B. However, coexpression of MR1C with MR1A did not lead to inhibition of MR1A-mediated presentation of *M.smegmatis*-derived ligand. MR1A expression was measured by flow cytometry detection of GFP when coexpressed with either the pCI empty vector or MR1BRFP, and

did not decrease upon expression of MR1B, indicating that MR1BRFP is not mediating a dominant-negative effect of MR1A (Figure 3F). Additionally, MR1BRFP expression in MR1A-expressing Beas2B:MR1_KO cells did not impact HLAE-restricted or HLAB45-restricted T cell activation, indicating that this effect is specific to MR1T cell activation. Taken together, these results indicate that MR1B may be functioning specifically to inhibit T cell activation by MR1A.

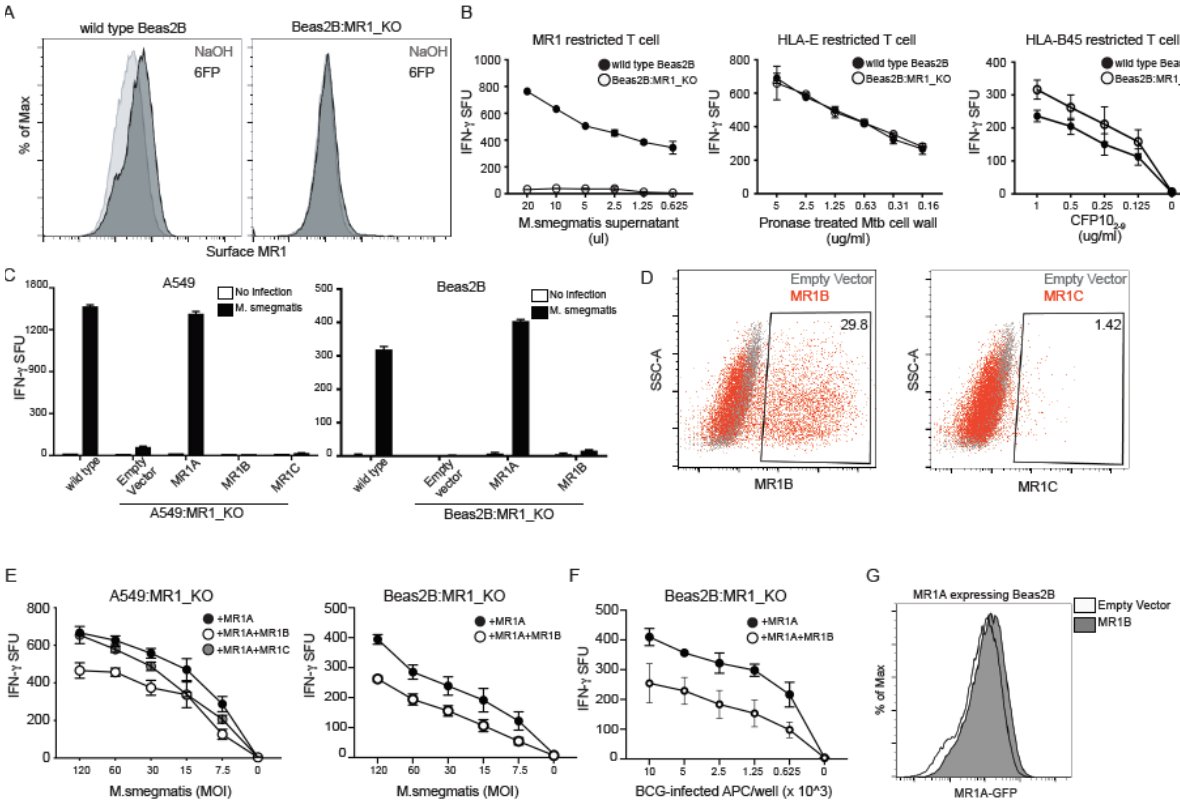

Figure 4.3: MR1B inhibits T cell activation by MR1A

Wild type Beas2B cells were transduced with a lentivirus designed to target the gene for *MR1* using CRISPR/Cas9-mediated gene editing to generate a Beas2B MR1 knockout cell line (Beas2B_MR1KO). Limiting dilution was performed to generate Beas2B_MR1KO clones, which were subsequently screened by flow cytometry and ELISPOT to verify phenotypic and functional knockout of MR1. (A) wild type Beas2B (left) and Beas2B_MR1KO (right) were treated for 16h with 50 uM 6-formylpterin (6FP) or NaOH vehicle to stabilize MR1 expression on the cell surface. Cells were then stained with an antibody to MR1 (α-MR1, clone 26.5) and flow cytometry was performed to measure surface MR1, which was absent in the

Beas2B_MR1KO cell line. (B) wild-type Beas2B and Beas2B_MR1KO cells were treated with either a titrating dose of *M.smegmatis* supernatant (left), pronase treated Mtb cell wall (middle), or $CFP10_{2-9}$ and (right) subsequently utilized as antigen presenting cells to stimulate either a MR1-restricted T cell clone (left), an HLA-E restricted T cell clone (middle), or a HLA-B45 restricted T cell clone (right). IFN-γ production is measured by ELISPOT and reported as IFN-γ spot forming units/5000 T cells (IFN-γ SFU). Error bars represent mean and standard error from duplicate wells. (C) A549_MR1KO and Beas2B_MR1KO cells were transiently transfected with plasmids encoding either MR1AGFP, MR1BRFP or MR1CRFP, or a pCI empty vector. 48h post transfection, cells were infected overnight with *M. smegmatis* at a multiplicity of infection (MOI) of 3 and utilized as antigen presenting cells to stimulate MR1T production of IFNγ in an ELISPOT as described in (B). Untransfected wild type cells were utilized as a positive control for MR1T activation following infection. IFN-γ production is measured by ELISPOT and reported as IFN-γ spot forming units/5000 T cells (IFN-γ SFU). Error bars represent mean and standard error from duplicate wells. (D) Beas2B_MR1KO cells were transfected with plasmids encoding an empty vector, MR1BRFP or MR1CRFP and 48h post transfection, MR1B or MR1C expression was measured by detection of total RFP by flow cytometry. (E) A549_MR1KO and Beas2B_MR1KO cells were cotransfected with plasmids encoding MR1AGFP and the pCI empty vector, MR1AGFP and MR1BRFP, or MR1AGFP and MR1CRFP. Cells were infected for 1h with a titrating MOI of *M. smegmatis* and used as antigen presenting cells to stimulate MR1T cell production of IFN-γ, as measured by ELISPOT as described in (B). Data are pooled from at least 3 independent experiments with duplicate wells and error bars represent mean and standard error. (F) Beas2B_MR1KO cells were cotransfected with plasmids encoding MR1AGFP and a pCI empty vector, or MR1AGFP and MR1BRFP and infected overnight with *M. bovis* BCG at an MOI of 15. Cells were then used as antigen presenting cells to stimulate MR1T production of IFN-γ with the amount of infected Beas2B cells titrated 2 fold from 10000 APC/well to 625 APC/well. IFN-γ production by MR1T cells was measured by ELISPOT as described in (B). Data are pooled from at least 2 independent experiments with duplicate wells, and error bars represent mean and standard error. (G) Beas2B overexpressing MR1AGFP were transiently transfected with either the pCI empty vector or a plasmid expressing MR1BRFP. Flow cytometric analysis was performed to quantify total MR1A expression, as measured by detection of GFP, following transfection

MR1B resides in the same intracellular compartments as MR1A

To elucidate the mechanism by which MR1B is inhibiting antigen presentation to MR1T cells, we examined the subcellular localization of MR1A and MR1B. We individually expressed pCI:MR1AGFP or pCI:MR1BRFP in Beas2B:MR1_KO cells and performed live cell imaging based on GFP or RFP fluorescence as a surrogate for MR1A or MR1B expression (Figure 4A). MR1A was detectable inside both the endoplasmic reticulum as well as intracellular vesicles, as previously described (Harriff et al. 2014, Harriff et al. 2016). MR1B, additionally, resided inside these same compartments, with no MR1BRFP detected on the cell surface at steady state. When Beas2B:MR1_KO cells coexpressed pCI:MR1AGFP and pCI:MR1BRFP, we observed that MR1A and MR1B resided in the same intracellular vesicles (Figure 4B). This suggests that MR1B may be mediating its effects in close proximity to intracellular MR1A.

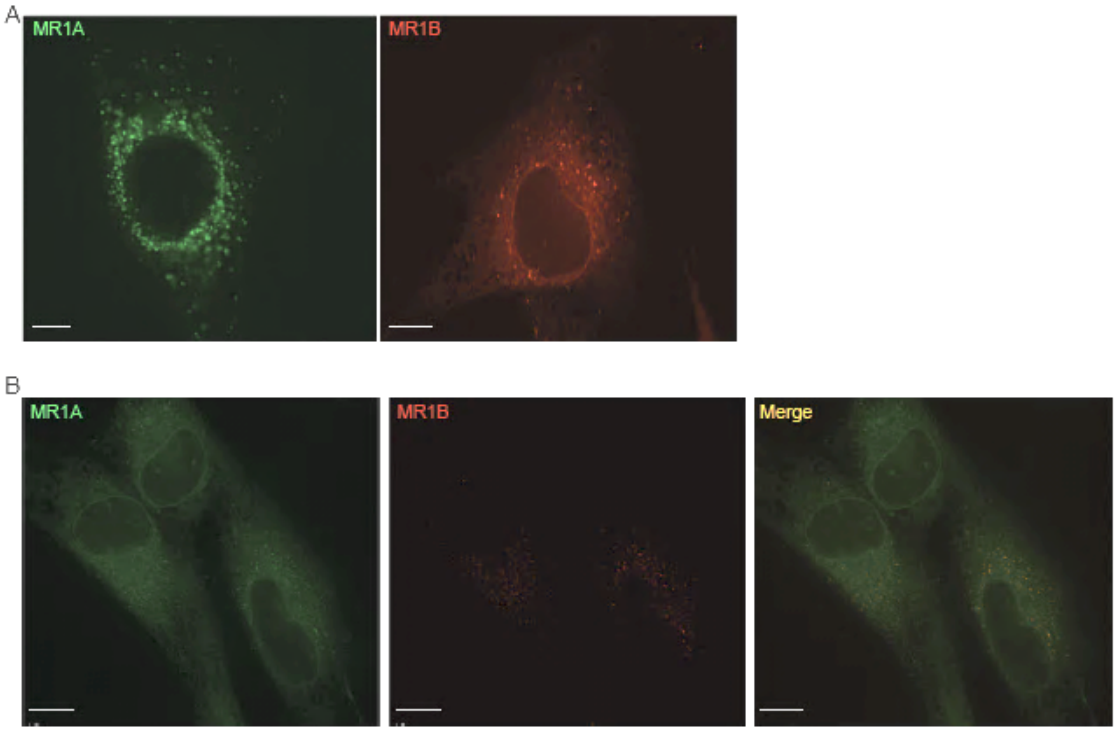

Figure 4.4: MR1A and MR1B reside in the same intracellular compartments

(A) Beas2B cells were transfected with plasmids encoding either MR1AGFP or MR1BRFP. 48 hours post-transfection, live cell imaging was performed using a CoreDV microscope to detect intracellular MR1A (left) or MR1B (right). Scale bars represent 10 μm. (B) MR1A-overexpressing Beas2B cells were transfected with a plasmid encoding MR1BRFP and live cell imaging was performed as described in (A) to determine the subcellular localization of MR1A and MR1B. (left) MR1A alone, (middle) MR1B alone, right (merge). Scale bars represent 10 μm.

MR1B does not traffic to the cell surface upon exposure to ligand

We have established that MR1B functions to inhibit antigen presentation by MR1A and that at steady state, MR1A and MR1B reside in the same intracellular compartments. One possible mechanism is that MR1B can localize to the cell surface, and either inhibits MR1A trafficking to the cell surface or engagement with the MR1T T cell receptor. Beas2B:MR1_KO cells transfected with pCI:MR1BRFP were exposed to 6FP for 16h and live cell imaging was performed to determine whether MR1B can be expressed on the cell surface. As compared to Beas2B:MR1_KO cells expressing pCI:MR1AGFP, which did demonstrate surface MR1A expression following exposure to 6FP, MR1B did not traffic to the cell surface upon exposure to exogenous ligand (Figure 5A). This suggests that MR1B does not directly engage with the MR1T TCR in order to inhibit MR1A function. When 6FP was administered overnight to cells coexpressing MR1A and MR1B, MR1A was still detectable on the cell surface, while MR1B remained in intracellular vesicles (Figure 5B). Intriguingly, flow cytometry demonstrated that coexpression of MR1A and MR1B followed by administration of 6FP overnight did not result in a decreased surface expression of MR1A, as measured by antibody staining for surface MR1A and compared to cells expressing pCI:MR1AGFP alone (Figure 5C). Therefore, these results indicate that MR1B-mediated antagonism is mediated inside the cell, prior to MR1A localization to the cell surface.

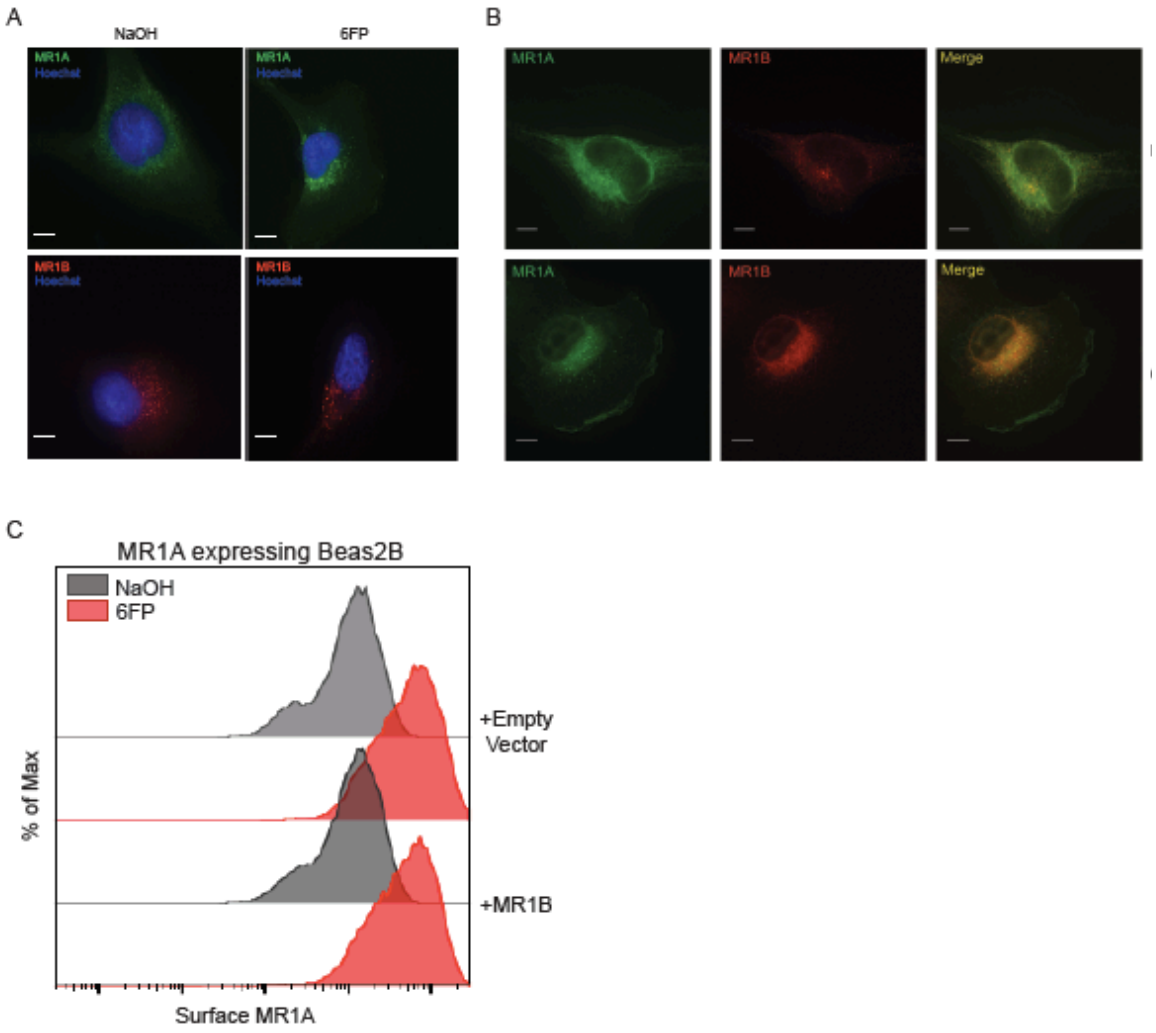

Figure 4.5: MR1B does not traffic to the cell surface

(A) Beas2B cells were transfected with plasmids encoding either MR1AGFP or MR1BRFP, and subsequently treated with 50 uM 6FP or NaOH for 16 hours. Live cell imaging was performed as described in (Fig 4) to determine localization of MR1A or MR1B with or without 6FP. Scale bars represent 10 μM. (B). Beas2B cells were cotransfected with plasmids encoding MR1AGFP and MR1BRFP and treated with 50 uM 6FP or NaOH for 16 hours. Live cell imaging was performed as described in (A). (C) Beas2B cells overexpressing MR1AGFP were transfected with a plasmid encoding MR1BRFP and treated with NaOH or 6FP as described previously. Cells were stained with an antibody to detect surface MR1A (α-MR1, Clone 26.5), which was detected by flow cytometry.

MR1B expression limits abundance of MR1A protein

As MR1B does not appear to block trafficking of MR1A to the cell surface, we explored the possibility that MR1B may be inhibiting MR1A expression. To accomplish this, we stably transduced Beas2B:MR1_KO cells with a lentivirus encoding a doxycycline-inducible MR1AGFP (Beas2B:KO_Tet_MR1AGFP) and sorted the transduced cells based on GFP expression following overnight administration of doxycycline as a surrogate for MR1A (Karamooz et al. 2019)(Figure 6A). We confirmed that these cells were capable of presenting *M. smegmatis*-derived antigen to MR1T cells upon induction of MR1A expression with doxycycline. Using this cell line, we were able to induce MR1A expression by doxycycline either before or after transfecting the cells with pCI:MR1B_RFP (Figure 6C-schematic). Interestingly, transfection of pCI:MR1BRFP into Beas2B:KO_Tet_MR1AGFP cells prior to the induction of MR1AGFP expression by doxycycline resulted in a 20% decrease in MR1AGFP protein, as compared to cells with doxycycline-induced MR1A expression prior to the transfection of pCI:MR1BRFP (Figure 6C). MR1BRFP expression was equal across all conditions. To ensure that MR1AGFP expression was not artificially increased due to prolonged exposure to doxycycline, we also measured MR1AGFP levels pre- and post-transfection of the pCI empty vector and show that MR1AGFP expression remained steady at ~86% GFP expression in both conditions. This indicated that the decreased protein abundance was due to MR1B expression prior to induction of MR1AGFP. We also expressed pCI: MR1CRFP pre or post-induction of MR1A expression, and show that MR1A expression, measured by detection of GFP, was not changed (Figure 6E).

To assess the functional significance of MR1BRFP expression prior to induction of MR1A expression, we repeated the experiment as described in Figure 6B, and infected the cells with *M. smegmatis.* These cells were then utilized as antigen presenting cells to stimulate IFN-γ production by MR1T cells in an ELISPOT (Figure 6F). We observed that, compared to the no-doxycycline control, Beas2B_MR1KO:Tet_MR1AGFP transfected with an empty vector were capable of stimulating MR1T cell activation upon the administration of doxycycline. Upon transfection of MR1B in MR1A expressing cells, we observed that when MR1A and MR1B were coexpressed, there was a 40% decrease in MR1A-mediated activation of MR1T cells, as seen in prior experiments (Figure 4.3). However, we observed up to a 60% inhibition of maximal MR1T activation as compared when MR1B was expressed prior to induction of MR1A protein, which corresponded to the lower MR1A protein abundance under these conditions. Expression of pCI:MR1CRFP pre or post induction of MR1A expression did not impact levels of MR1A protein or MR1A-mediated antigen presentation, again, suggesting that MR1B is specifically mediating its effects on MR1A. Thus, prior expression of MR1B may inhibit the abundance of MR1A protein, either through competing for chaperones necessary for MR1A folding, or by competing for factors required for protein synthesis of MR1.

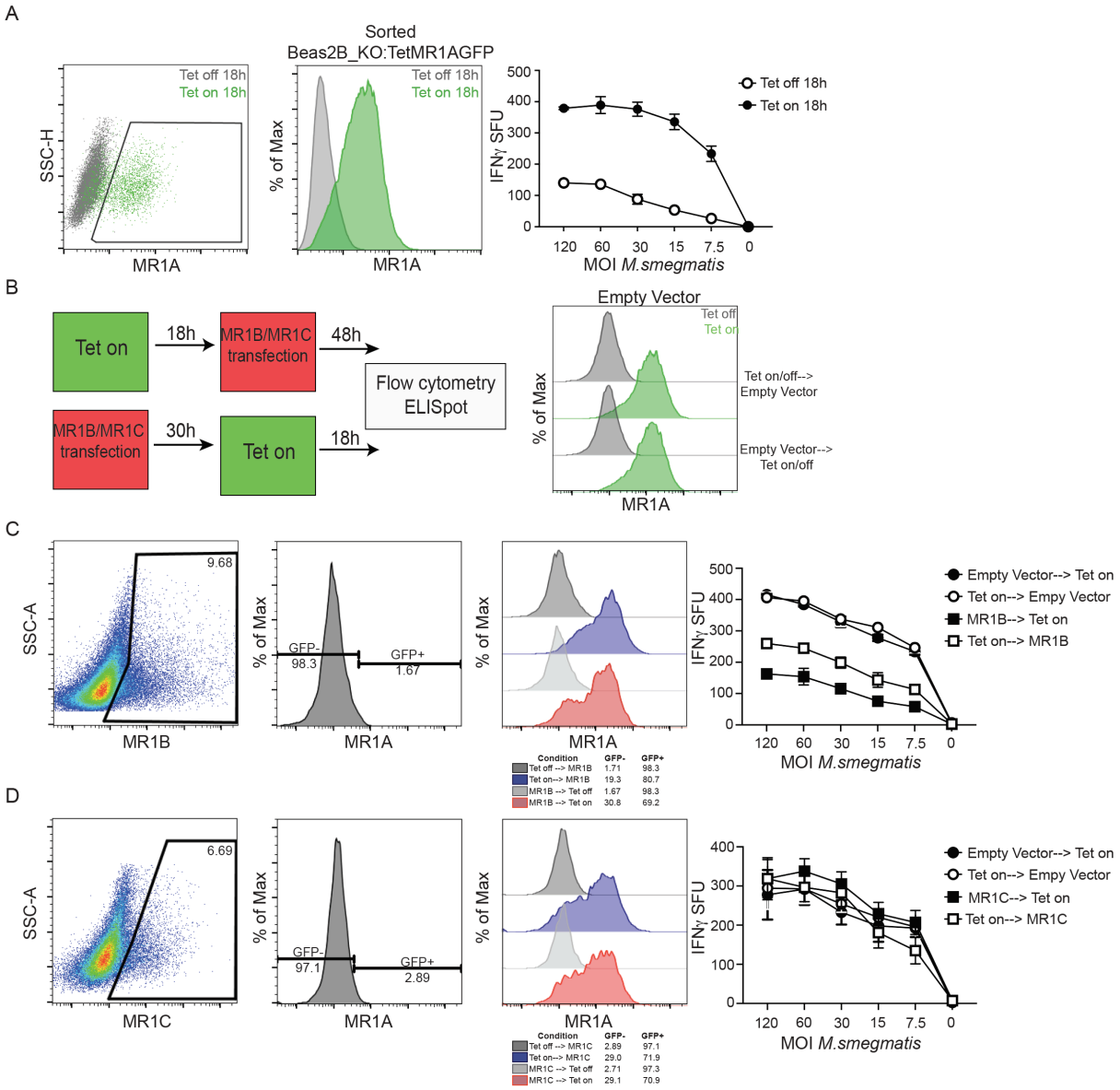

Figure 4.6: Prior MR1B expression decreases the abundance of MR1A protein

Beas2B-MR1KO cells were transduced with a lentivirus encoding a tetracycline inducible MR1AGFP and sorted (A) based on MR1AGFP expression following overnight administration of doxycycline. Sorted cells were verified to be MR1AGFP+ following administration of doxycycline. Beas2B_MR1KO_TetMR1AGFP cells were treated with doxycycline overnight and infected with *M.smegmatis* at a titrating MOI for 1h. Infected cells were used as antigen presenting cells in an ELISPOT assay as described above to stimulate MR1T production of IFN-γ. (B) Schematic of experiments to study the timing of MR1A and MR1B expression. Briefly, doxycycline was added to Beas2B_MR1KO_TetMR1AGFP cells prior to or following the transfection with MR1BRFP, MR1CRFP, or a pCI empty vector. MR1AGFP expression pre and

post transfection with a pCI empty vector was measured by flow cytometry (right) (C)Transfected cells were assessed by flow cytometry, gated on MR1B expression using RFP (left), and then assessed for GFP+ and GFP- expression, which was based on a dox-off control (second). (third) Overlay histograms of MR1B transfection pre and post induction of MR1A expression with doxycycline. Doxycycline off-MR1B transfection conditions are included as a control. gMFI of MR1A+ expression, as measured by GFP+ expression is reported below. (Right) ELISPOT of cells transfected according to (B) and used as antigen presenting cells following infection with *M.smegmatis* at the indicated MOI. Cells were incubated with a MR1T clone and IFN-γ production was measured. (D) Transfected cells were assessed as in (C) by flow cytometry, gated on MR1C expression using RFP

MR1 splice variant expression contributes to antigen presenting capacity in human cell lines

As RNASeq analysis (Figure 1) suggested that uterine endometrial and breast tissues express lower relative *MR1A* mRNA as compared to *MR1B*, we obtained uterine endometrial cell lines, as well as breast epithelial carcinoma cell lines. Using these, along with lung epithelial cells (Beas2B), and dendritic cells generated from PBMC, we sought to measure MR1 expression and function. We first measured MR1 transcript expression by qRT-PCR, as described earlier (Figure 7A). Indeed, we observed that our results corresponded with the transcriptomics analysis-uterine (KLE) and breast cancer (MCF7) cell lines did display lower relative *MR1A/MR1B* mRNA, compared to dendritic cells. These cells were then utilized as antigen presenting cells in an ELISPOT assay to measure MR1T activation upon exposure to *M. smegmatis* supernatant (Figure 7B). We show that as expected, dendritic cells from blood displayed a stronger antigen presentation capacity to MR1T cells than KLE and MCF7 cells. This suggests that while different cell lines are capable of stimulating MR1T cells, their capacity to do so varies, and may be related to MR1 isoform expression.

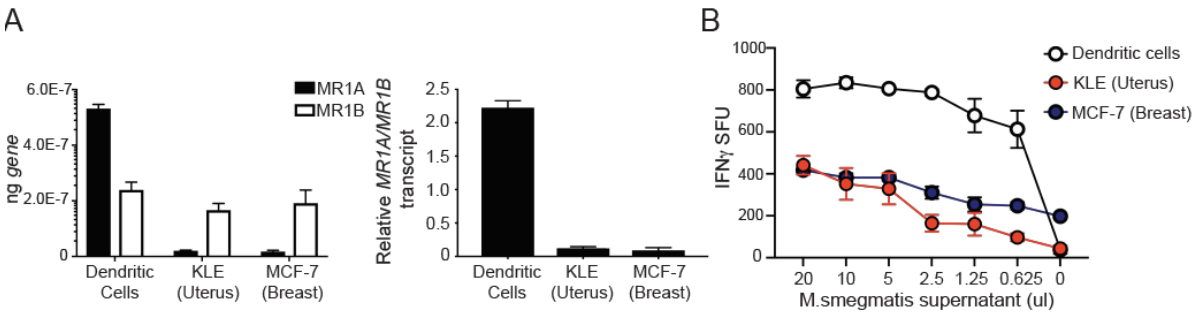

Figure 4.7: Breast and Uterine Epithelial cell lines are less capable of stimulating MR1T cells

Dendritic cells from PBMC, the KLE uterine adenocarcinoma cell line, and the MCF7 breast epithelial carcinoma cell line were assessed for splice variant expression following RNA isolation (A), qRT-PCR was performed to assess the absolute (left) and relative (right) amounts of *MR1A* and *MR1B* transcript. Error bars represent mean and standard error from triplicate wells. (B) Dendritic cells, the KLE cell line, and MCF7 cell line were incubated with *M.smegmatis* supernatant and used as antigen presenting cells in an ELISPOT to stimulate MR1T clone production of IFN-γ. The y-axis represents IFN-γ spot forming units (SFU).

Expression of MR1 splice variants in double positive MR1-expressing thymocytes

In the majority of cells, surface expression of MR1A is not detectable in the absence of infection or ligand (Chua et al. 2011). However, we have previously shown that CD4+ CD8+ double positive (DP) thymocytes express detectable MR1 on the cell surface. The percentage of MR1-expressing DP thymocytes varies from 0.5% to 15%.(Gold et al. 2013) These cells are capable of presenting mycobacterial ligand to stimulate MR1T clone responses, but the role of these cells in the thymus is not elucidated. To further characterize these cells, we isolated MR1-expressing DP thymocytes from human thymus using magnetic selection. We show that these DP thymocytes are capable of acting as APCs to stimulate MR1T production of IFN-γ (Figure 8A).

To assess whether relative expression of the MR1 isoforms is associated with surface expression of MR1A, we sorted MR1 expressing DP thymocytes from nine donors (Figure 8B). We then measured expression of *MR1A* and *MR1B* mRNA by qRT-PCR, which we then compared to surface MR1A expression in these donors (Figure 8C). We observe that there was not a strong association between *MR1A* mRNA and surface expression of MR1 (R^2=0.03354, p=0.637), but there was a moderately significant association between *MR1B* transcript and frequency of MR1+ DP thymocytes (R^2=0.4486, p=0.484). Intriguingly, we observed a strong association (R^2=0.8196, p=0.0008) between relative *MR1A/MR1B* transcript and frequency of MR1-expressing

thymocytes, indicating that mRNA expression of the MR1 splice variants may be associated with the development of these cells.

MR1+ DP thymocytes are thought to play a role in the selection of MR1T cells (Seach et al. 2013). To explore the relationship between surface MR1 expression and MR1T abundance in the thymus, we stained the above nine thymocytes with antibodies against CD3, as well as with a tetramer consisting of MR1 bound to 5-OP-RU (MR1/5-OP-RU). In all total CD3 thymocytes, we observed low frequencies of MR1T cells, albeit with variation across donors. Intriguingly, the frequency of MR1+ thymocytes appears to associate with the frequency of MR1/5-OP-RU+ thymocytes (R^2= 0.5118, p=0.0302). However, the absolute amounts of *MR1A* and *MR1B* mRNA did not appear to be significantly correlated with frequency of MR1/5-OP-RU+ thymocytes, nor did the ratio of *MR1A/MR1B* transcript. We did observe that thymocytes did also stain with a control tetramer consisting of MR1 loaded with 6FP (MR1/6FP), and that this staining also corresponded with both *MR1A/MR1B* relative expression and surface expression of MR1A (data not shown). While transcript expression of the MR1 isoforms associated with the frequency of MR1+ thymocytes, it is clear that there are other factors contributing to the selection of MR1T cells in the thymus.

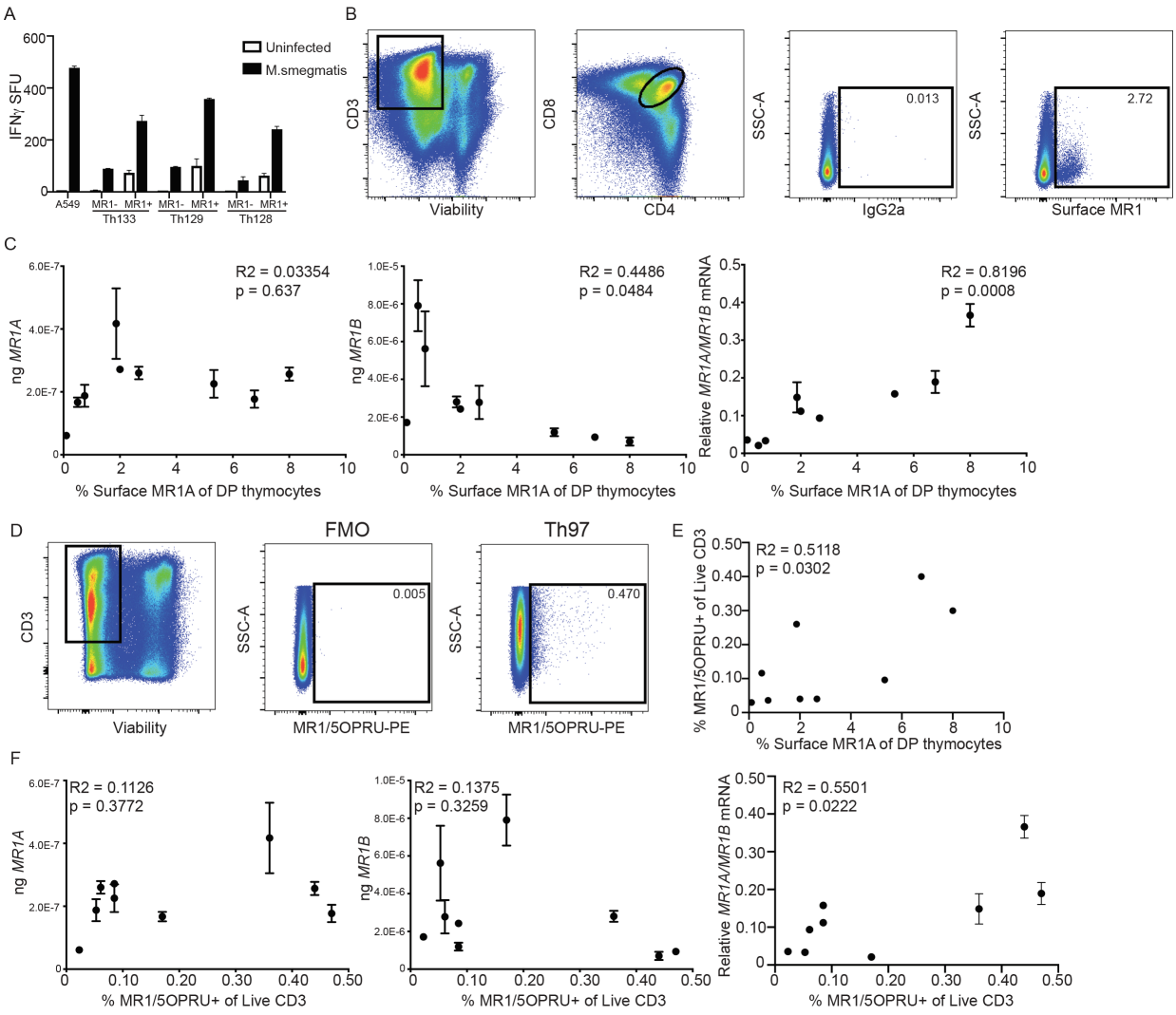

Figure 4.8: Double positive thymocyte expression of surface MR1A is associated with transcript expression and MR1T frequency in the thymus

MR1+ thymocytes were isolated using magnetic sorting (A) and used as antigen presenting cells following infection with *M.smegmatis* for 1h to stimulate MR1T production of IFN-γ, As a control MR1- cells and A549 cells were also included in the ELISPOT. Error bars represent mean and standard error of duplicate wells. T cell production of IFN-γ is represented as spot forming units (SFU). (B) Gating strategy for sorting MR1+ CD4+ CD8+ (DP) thymocytes from human thymus for qRT-PCR of MR1 splice variant expression. Cells were gated on live CD3+ CD4+ CD8+ MR1+ thymocytes based on an isotype control for MR1. (C) Absolute (left, middle), and relative (right) amounts of MR1A and MR1B transcript in DP MR1+ thymocytes versus surface expression of MR1 in 9 thymus donors. Pearson's correlation was calculated and R^2 and statistical significance is reported. (D) Gating strategy to identify MR1T cells in human thymus. Gates were set based on a negative control (FMO). Briefly, cells were stained

with markers for viability, CD3, and the MR1/5-OP-RU tetramer or the MR1/6-FP control tetramer (not shown). (E). Frequency of MR1/5-OP-RU tetramer+ MR1T cells plotted versus surface MR1A expression in DP thymocytes from 9 donors. Pearson's correlation was calculated and R^2 and statistical significance is reported. (F) Same as (C), only expression of MR1A and MR1B transcript in DP thymocytes is plotted versus frequency of MR1/5-OP-RU tetramer staining MR1T cells. Pearson's correlation was calculated and R^2 and statistical significance is reported.

Discussion

Though it has long been known that MR1 undergoes alternative splicing, the function of these splice variants has not been elucidated. We show that MR1A and MR1B, an alternatively spliced form of MR1, are expressed across human tissues, however, there is variation between both cells and donors. Using a cell line generated to lack endogenous MR1, we demonstrate that MR1B inhibits mycobacterial antigen presentation to MR1T cells by the full length variant, MR1A.

Analysis of MR1 splice variant transcript levels showed that relative *MR1A/MR1B* expression varied across tissues. Intriguingly, across tissues, there was overall *MR1B* mRNA detected. It is possible that in the absence of infection, *MR1B* transcript expression remains higher, so as to inhibit inappropriate T cell activation. Further analysis of antigen presenting cells from lung and blood showed variability between both cell type and donors. The mechanism behind this variability raises questions about how MHC gene expression is regulated. It has long been known that alternative splicing and post-transcriptional modifications are often regulated by small nucleotide polymorphisms (SNPs), particularly intronic SNPs. These SNPs have been shown to mediate recruitment and activity of the spliceosome, as well as modulate promoter or enhancer binding to vary gene expression or protein translation (Lalonde et al. 2011, Lee and Rio 2015, Seth et al. 2008). Recently, expression of an intronic SNP in *MR1* was shown to be associated with susceptibility to TB-meningitis (Seshadri et al. 2017). Additionally, there is a growing body of evidence that epigenetic modifications could also impact donor-donor variability in gene expression(Gutierrez-Arcelus et al. 2015). These

questions remain to be addressed in the context of non-classical gene expression and could help define how non-classical MHC molecules, including MR1, are expressed across tissues and individuals.

The mechanism of MR1A translocation to and from intracellular compartments to engage with ligand is not well elucidated, but likely requires chaperone proteins distinct from those needed by classical MHC Class I molecules (Harriff et al. 2014, Harriff et al. 2016). It is possible that MR1B may compete for chaperone proteins necessary for MR1A trafficking to the plasma membrane. This is less likely, as expression of MR1B did not appear to diminish the ability of MR1A to traffic to the cell surface. However, the possibility remains that MR1B is inhibiting protein expression of MR1A, either by binding chaperone proteins necessary for folding and stability, or by competing for the translational machinery needed by the MR1 splice variants. Our results support this hypothesis, as expression of MR1B protein prior to induction of MR1A expression resulted in not only decreased abundance of MR1A protein, but also a stronger inhibition of MR1T cells. These results raise the possibility that regulation of antigen presentation can be controlled at the level of gene expression. Exposure to ligand or microbial infection may be playing a role in changing the expression of antigen presenting molecules, which may be key in understanding the regulation of antigen presentation by MR1.

Our results suggest a potential link between relative *MR1A/MR1B* expression and frequency of MR1 expressing thymocytes, which we have shown can robustly stimulate MR1T cells.. Along with B cells, double positive thymocytes have been suggested to play

a critical role in the selection of MR1T cells in the thymus (Seach et al. 2013). We showed, using an MR1 tetramer, that the frequency of MR1T thymocytes associated with the percentage of MR1A-expressing DP thymocytes. Our data suggest that MR1+ thymocytes may play a role in the selection of MR1T cells, as MR1+ thymocytes can activate MR1T cells, and splice variant expression in these cells is associated not only with surface protein expression, but also with frequency of MR1T cells. How double positive thymocytes function in MR1T development represents an intriguing avenue of research. It is hypothesized that there is a self-ligand presented by MR1 that is required for the development of MR1T cells in the thymus, and how this ligand engages with both MR1A and MR1B would lend insight on MR1T development.

Alternative splicing has been estimated to occur at high frequency in individuals and represents an important means of controlling gene expression (Park et al. 2018). Dysregulation in alternative splicing events has been associated with disease, including diseases of autoimmunity and inflammation. However, the role of alternative splicing in adaptive immunity is poorly defined, with few reports available on the function of splice variants in MHC. Alternative splicing of MICA resulted in splice variants that were both agonists and antagonists for NKG2D, while an α3 domain deletion mutant of HLA-A11 was suggested to promote HIV evasion (Zhang et al. 2017, Gavlovsky et al. 2016). These reports suggest that a putative role for alternative splicing is to control immune cell activation in order to properly target immune responses to microbial infection. Our results suggest that cell lines derived from uterus and breast are less capable of presenting mycobacterial antigen to MR1T cells. Modulation of immune responses in the uterus is

critical during pregnancy, in order to prevent immune killing of the developing fetus. Therefore, MR1B may be playing a key role in the uterus to inhibit MR1T activation in the absence of microbial infection. Our results raise the possibility that alternative splicing of MR1 functions as one means of regulating inappropriate or unwanted T cell activation.

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. De-identified lungs, upper airway, or small intestine were obtained from the Pacific Northwest Transplant Bank (PNTB). Our exclusion criteria included significant tobacco smoking history (>1 pack-year), drowning, crushing chest injuries, lobar pneumonia, and HIV/HBC/HCV infection. Deidentified thymuses were obtained from children undergoing cardiac surgery at Oregon Health and Science University Dorenbecher Children's Hospital. The majority of children were no less than 4 mo. and no more than 4 years old. As the thymuses were obtained as deidentified medical waste under an exempt IRB protocol, no other information is available on the status of the donors.

Reagents and antibodies

Doxycycline (Sigma-Aldrich. St. Louis, Missouri) was resuspended to 2mg ml^{-1} in sterile water and was used at 2 μg ml^{-1}. 6-formylpterin (Schirck's Laboratories, Bauma, Switzerland) was resuspended to 3mg/mL in 0.01M NaOH. Control vehicle used was 0.01M NaOH**.**

Microorganisms and preparation of APCs

M. smegmatis and *M. bovis* BCG were utilized from frozen glycerol stock. For *M.smegmatis* infection, indicated cells were infected for 1h in a 96 well ELISPOT MSHA nitrocellulose plate. For *M.* bovis BCG infection, cells were plated for four hours in a 6 well tissue culture plate and infected overnight at a MOI of 15. Subsequently, *M. bovis* BCG infected cells were harvested, washed, counted, and added to the ELISPOT assay at the indicated amount of antigen presenting cells. To generate *M.smegmatis* supernatant, *M. smegmatis* was cultured with shaking for 24 hours then pelleted. The supernatant was passed through a 0.22 μm filter to remove any bacteria. The supernatant was aliquoted and stored at -80C and utilized at the indicated volume in an ELISPOT assay.

Cell lines

BEAS-2B and A549 cell lines was obtained from ATCC. A549_MR1KO were generated as described previously (Laugel et al. 2016). The KLE uterine epithelial adenocarcinoma cell line was obtained from ATCC. The above four cell lines were cultured in DMEM supplemented with 10% fetal bovine serum and antibiotics. The MCF-7 breast epithelial carcinoma cell line was a generous gift from Shiuh-Wen Luoh (Knight Cancer Institute, Portland, OR) and cultured in RPMI 1640 media supplemented with 10% fetal bovine serum and antibiotics. All cell lines were confirmed to be mycoplasma free.

Generation of a Beas2B MR1 knockout cell line

Wildtype Beas2B cells were seeded in a 6 well plate to 70% confluency and transduced by spinoculation with a lentivirus encoding the CRISPR/Cas9 machinery with guide RNA specifically targeted to MR1 that had been previously used to generate an A549 MR1 knockout cell line (Laugel et al. 2016). Following this, limiting dilution was performed on transduced cells, with 1 cell per well in 96 well plates. Cells were grown in DMEM media containing 10% fetal bovine serum and 1% gentamicin antibiotic. Clones were utilized as antigen presenting cells in an ELISPOT assay following incubation with *M.smegmatis* supernatant and an MR1T clone as described below. Positive clones were expanded in a 12 well plate and tested again for functional knockout of MR1 by ELISPOT. Following this, functionally knocked out clones were incubated with 100 μM 6FP versus an equivalent volume of 0.01M NaOH. Surface staining of MR1 was performed as described below and MR1 surface expression was assessed by flow cytometry on a BD Fortessa. Three clones were validated as being knocked out for MR1 protein expression, as well as functionally unable to stimulate MR1T clones, and cryopreserved in 90% fetal bovine serum/10% DMSO. One clone was selected and utilized for all future experiments (Beas2B_MR1KO).

Generation of stably transduced Beas2B cell lines

The Beas2B_MR1KO cell line was transduced with a lentivirus expressing Tet_MR1AGFP by spinoculation. Transduced cells were exposed to doxycycline overnight to promote expression of MR1AGFP and subsequently sorted on an InFlux cell

sorter based on GFP expression. Cells were subsequently cultured, validated for MR1AGFP expression by flow cytometry following administration of doxycycline, and utilized for further experiments.

Monocyte-derived DCs

PBMCs obtained by apheresis were resuspended in 2% human serum in RPMI and were allowed to adhere to a T-75 flask at 37 °C for 1 h. After gentle washing twice with PBS, nonadherent cells were removed and 10% human serum in RPMI containing 30 ng ml^{-1} of IL-4 (Immunex) and 30 ng ml^{-1} of granulocyte–macrophage colony-stimulating factor (Immunex) was added to the adherent cells. The cells were X-rayed with 3,000 cGray using X-RAD320 (Precision X-Ray Inc.) to prevent cell division. After 5 days, cells were harvested with cell-dissociation medium (Sigma-Aldrich, Gillingham, UK) and used as APCs in assays.

Human tissue sources of antigen presenting cells

PBMCs were isolated from the peripheral blood of healthy donors using Ficoll-Paque gradients.

Lung and small intestine single cell suspensions are prepared from recently deceased donor tissue not suitable for transplant from the Pacific Northwest Transplant bank. Small cubes of lung parenchyma, devoid of airway and lymph nodes, or of duodenal lamina propria, were cut into a cold buffer of HBSS (Gibco) media supplemented with HEPES (Gibco) and PSF antibiotic (Sigma). Tissue was then digested for 30 minutes

at 37C in a DMEM buffer (Gibco) supplemented with PSF antibiotics (Sigma), elastase (15 ug ml^{-1}, Worthington), trypsin I (1.5 ug ml^{-1}, Sigma), DNase I (45 ug ml^{-1}, Roche). The subsequent suspension was further dissociated using a GentleMACS dissociator (Miltenyi). The single cell suspension was then diluted 1:1 with a buffer of HBSS (Gibco) media supplemented with 2% heat-inactivated fetal bovine serum (Gemini Bio Products), HEPES (Gibco) and PSF antibiotic (Sigma) to dilute homogenate and neutralize digest enzymes. This cell suspension is passed through successive filters in this order: metal mesh sieve filter (size 40 then 60, Sigma), and nylon cell strainer (100um then 40 um, BD Falcon). The resulting cell suspension is washed in RPMI supplemented with 10% heat inactivated human serum and used for experiments or cryo-preserved in heat-inactivated fetal bovine serum with 10% DMSO.

Large airway epithelial cells were generated as described previously from the upper airway of human donors (Gold et al. 2010) and cryopreserved in heat-inactivated fetal bovine serum with 10% DMSO.

Thymocytes: Thymus tissue was cut into 3-mm^3 pieces. Each piece was ground in a GentleMACS dissociator with 1 ml of DMEM plus 10% FBS to form a single cell suspension. The suspension was cryopreserved at 2-3x10^8 cells/ml in a 90% FBS/10% DMSO freezing solution with a post-thaw viability of approximately 50%. (Gold et al. 2008)

Expansion of T-cell clones

T-cell clones were cultured in the presence of X-rayed (3,000 cGray using X-RAD320, Precision X-Ray Inc.) allogeneic PBMCs, X-rayed allogeneic LCL (6,000 cGray) and anti-CD3 monoclonal antibody (20 ng ml^{-1}; 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 monoclonal antibodies.

IFN-γ ELISPOT

A MSHA S4510 96 well nitrocellulose-backed plate (Millipore, bought via Fisher Scientific) was coated overnight at 4 °C with 10 μg ml^{-1}solution of anti-IFN-γ monoclonal antibody (Mabtech clone 1-D1K) in a buffer solution of 0.1 M Na_2CO_3, 0.1 M $NaHCO_3$, pH=9.6). Then, the plate was washed three times with sterile PBS and blocked for 1 h at room temperature with RPMI 1640 media containing 10% heat-inactivated HS pool. Then, the APCs and T cells were prepared as described above and co-incubated overnight. Antigen presenting cells and antigen used are noted in the relevant figure. T-cell clones were added at 5×10^3 per well. The plate was incubated overnight at 37 °C and then washed six times with PBS containing 0.05% Tween. The plate was then developed as previously described and analyzed using an AID ELISPOT reader (Harriff et al. 2018).

Construction of MR1B_RFP, MR1C_RFP and transfections.

We utilized our previously described pCI construct (Harriff et al. 2016) for all transient transfections. A gene encoding to MR1B_RFP or MR1C_RFP was ligated into this construct using EcoRI and KpnI, to create pCI_MR1B_RFP or pCI_MR1C_RFP. Confirmatory sequencing was performed. Restriction enzymes and ligation kit were obtained from New England Biolabs (Ipswich, Massachusetts). PCR and gel purification kits were obtained from Qiagen. Transfection of Beas2B or A549 cells using plasmids was done using an Amaxa Nucleofector, Kit T solution (Lonza, Basel, Switzerland), and programs G-016. Each transfection reaction was done with 1e6 cells and 6μg of plasmid. For co transfections, equivalent molar amounts of each plasmid were used for a total of 6 ug of plasmid. A pCI empty vector was used as a control for all transfections. Confirmation of transfection was performed using flow cytometry on a BD Fortessa to detect either GFP (pCI_MR1AGFP) or RFP (pCI_MR1BRFP or pCI_MR1CRFP). All analyses were performed using FlowJo software (TreeStar).

RNA isolation, cDNA synthesis and qPCR analysis

Total RNA was isolated using TRIZOL (LIfeTechnologies) and phenol chloroform extraction. cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). qPCR was performed using SYBRGreen Power Master Mix (ThermoFisher Scientific) on a Step One Plus Real-Time PCR System (Applied Biosystems). Primers were designed to be specific for each gene assay and generated by the DNA services core and IDT Technologies. The absolute quantification method was used using by generating a standard curve with a plasmid specific for each gene assayed.

MR1 surface stabilization

BEAS-2B:MR1_KO cells, either untransfected, or transfected with pCI empty vector, pCI_MR1AGFP, and/or pCI_MR1BRFP, were plated into 6 well tissue culture plates overnight. The next day, the cells were treated with 100 μM 6-FP versus an equivalent volume of 0.01M NaOH. After 16 hours, the cells were harvested on ice and split into two groups for primary staining and isotype control staining. Primary staining was done with an antibody against MR1 (Clone 26.5, gift from Ted Hansen, biotinylated by Biolegend) at 1:100 for 40 min on ice in the presence of 2% human serum, 2% goat serum, and 0.5% FBS. Biotinylated mouse anti IgG2A (Biolegend) served as the isotype control. After washing, streptavidin-Alexa 647 (ThermoFisher Scientific) was added for 40 min on ice. Cells were washed, fixed in 1% PFA and analyzed by flow cytometry on a BD Fortessa, or BD Symphony and data were analyzed on FlowJo (TreeStar).

Fluorescence microscopy

BEAS-2B:MR1_KO cells were transfected with MR1A_GFP or MR1B_RFP and plated into 1.5mm glass bottom chamber slides (Nunc, ThermoFisher Scientific) and incubated at 37C and 5% CO_2. After 48h hours, the cells were stained with NucBlue Live Cell Stain (ThermoFisher Scientific) or analyzed without nuclear staining. Images were acquired on a high-resolution wide field CoreDV system (Applied Precision, Pittsburg,

Pennsylvania) with a Nikon Coolsnap ES2 HQ. Each image was acquired as Z-stacks in a 1024x1024 format with a 60x objective (NA 1.42).

Dynamics of MR1A and MR1B expression

To assess the temporal dynamics of MR1A and MR1B expression, pCI_MR1BRFP or pCI_MR1CRFP was transfected into Beas2B_MR1KO cells overexpressing a Tet_MR1AGFP lentivirus either 24h prior to or 16 hours post induction of MR1A expression. Flow cytometry was performed as described earlier to measure MR1AGFP expression or MR1BRFP expression using a BD Symphony and data were analyzed using FlowJo software. For functional analyses, cells were harvested on ice, infected 1h with a titrating dose of *M. smegmatis*, and utilized as antigen presenting cells ($5x10^3$/well) in an ELISPOT assay following incubation with an MR1T clone ($5x10^3$/well). The ELISPOT was set up and developed as described above.

Flow cytometry staining and FACS sorting

Lung and small intestine cell suspensions generated as described earlier were thawed, washed with PBS twice and blocked in FACS buffer. 2e06 cells were stained with antibodies against surface CD45, CD326 (EPCam), CD14, and CD20, for 30 minutes on ice in the dark, as well as Live/Dead Fix Aqua for viability. Cells were harvested on ice, washed in PBS, resuspended in FACS buffer, and sorted on a BD InFlux sorter for Live, CD45- CD14-, and CD20- CD326+ cells in order to isolate CD326+ epithelial cells. Cells were sorted into TRIZOL for immediate RNA isolation and subsequent quantitative real time PCR analysis.

Sorting of MR1+ DP thymocytes: Thymocytes were thawed and blocked with FACS buffer, and $2x10^6$ cells were stained with antibodies for the surface markers CD3, CD4, CD8, and MR1-biotin (clone 26.5), or the isotype control (IgG2a-biotin) for 30 minutes on ice in the dark. Cells were washed twice with cold PBS and stained with streptavidin-PE (1:2000 dilution) for 15 minutes in the dark. Cells were washed twice and resuspended in FACS buffer and sorted on a BD InFlux Sorter for Live, CD3+ CD4+CD8+ MR1 expressing thymocytes, which was confirmed based on an isotype control for MR1. Cells were sorted into TRIZOL for immediate RNA isolation and subsequent quantitative real time PCR analysis.

For quantifying the frequency of MR1T thymocytes, thymocytes were thawed, blocked with FACS buffer, and $2x10^6$ cells were incubated with the MR1/5-OP-RU tetramer or the MR1/6FP tetramer (1:500 dilution, NIH Tetramer Core facility), for 45 minutes at RT in the dark. Following this, antibodies to the surface markers CD3, CD4, and CD8 and Live/Dead Fix Aqua viability stain were added at manufacturer recommended concentrations. Cells were incubated at RT in the dark for 15 minutes, washed twice with PBS, and fixed with 1% paraformaldehyde. Acquisition was performed on a BD Symphony flow cytometer and data were analyzed using FlowJo software (TreeStar).

Data analysis

Data were analyzed and plotted using Prism 7 GraphPad Software (La Jolla, California). Statistical significance was determined using unpaired Student's two-tailed *t*-test, unless otherwise indicated. For comparison of DP MR1+ thymocyte gene expression with frequency of DP thymocytes or of MR1T thymocytes, Pearson's correlation calculation was performed. For comparisons of functionality between transfected antigen presenting cells, linear regression analysis was performed and significant differences between slopes and y-intercepts were measured and reported. Error bars in the figures indicate the standard deviation, standard error of the mean, or the data set range as indicated in each figure legend. P values ≤ 0.05 were considered significant (*$P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$).

Chapter 5: Discussion

It has been increasingly clear that the paradigm of innate and adaptive immunity as separate and non-overlapping arms of the immune system is no longer modeling the full picture of the detection between self and non-self. Unconventional T cells are one class of cells that are capable of bridging the gap between innate and adaptive immunity, in order to enable the immune system to more broadly survey the host for infection by pathogen. These cells, though similar in nature to CD8+ T cells in their ability to promote cytokine production and cell killing following recognition of antigen bound to MHC through their TCRs, are distinct in their ability to emerge from the thymus with immediate effector function and their relatively high abundance in tissues. In this manner, while the canonical CD8+ T cell response takes days to mount and requires clonal expansion and trafficking to infected tissues following antigenic exposure, the unconventional T cell response can respond almost immediately and are thought to play an important role in the surveillance of early infection at mucosal sites. Moreover, unconventional T cells, including CD1-restricted T cells, HLA-E-restricted T cells, and MR1-restricted T cells can recognize non-peptide antigen generated by microbes on the surfaces of their cognate antigen presentation molecule. This broadens the scope by which CD8+ T cells can sense microbial antigen and promote host defense.

MR1-restricted T cells (MR1T) are an unconventional CD8+ T cell subset that recognize a broad array of small molecules through the antigen presentation molecule MR1. Though the MR1T TCR was thought to be invariant and bind only a limited antigen

repertoire, which are derived from intermediates the vitamin B synthesis pathway, recent reports have indicated that the opposite is in fact true. The MR1T TCR is highly diverse, particularly in the CDR3α and CDR3β regions that directly engage with ligand (Gold et al. 2014). Additionally, the range of ligands that can bind MR1 and stimulate MR1T activation on the cell surface is much broader than previously thought, and not only includes bacterially-generated small molecules that are not found in the riboflavin synthesis pathway, but also common pharmacologic agents (Harriff et al. 2018, Meermeier et al. 2016, Keller et al. 2017). In Chapter 3 of this dissertation, we show that Mtb-reactive MR1T clones from blood and BAL display a high degree of variation in CDR3α and CDR3β usage, with sequences never before described. It is known that canonical CD8+ TCRs require both the TCRα and TCRβ to mediate contacts with the peptide/MHC Class I complex, while the nonclassical iNKT TCR only appears to require the TCRα chain in the recognition of glycolipid antigen bound to CD1d. The role of the MR1T TCRα chain in recognizing MR1-bound antigen is well established, but there is debate over the importance of the TCRβ chain, whether it directly confers ligand specificity, or primarily functions to fine tune antigen recognition. We show, using paired MR1T clones that share TCRα chain sequences with differing TCRβ sequences, that in some, but not all cases, differences in the TCRβ chain can confer reactivity not only to microbially infected antigen presenting cells, but also exogenous MR1 tetramer bound antigen (5-OP-RU). These results suggest that MR1T cells can require both the TCRα and TCRβ for antigen recognition, however, this is not universal, and in some cases, only the TCRα chain is required to distinguish between both microbial antigen. Recent

reports have suggested that the MR1T TCR repertoire is shaped by exposure to antigen, and our results support the hypothesis that diversity in the MR1T TCR repertoire enables MR1T cells to act as specific sensors of an increasingly diverse microbial metabolome.

MR1T cells have an increasingly diverse TCR repertoire, are highly enriched in mucosal sites, and can bind a wide range of ligands generated by microbes, including commensals present in mucosal sites. Additionally, MR1T cells have been implicated in a wide array of autoimmune conditions, which I have summarized in my introduction, likely through aberrant recognition of either an as yet unknown endogenous ligand or of antigen generated from commensal bacteria. Therefore, the regulation of MR1-mediated antigen presentation to MR1T cells is of importance in understanding the balance between appropriate response to infection and autoimmunity. For the second portion of my dissertation, I focused on further exploring the mechanism by which MR1T responses to infection can be regulated. MR1, the antigen presenting molecule for MR1T cells, is non-polymorphic and highly conserved. Though it adopts a standard MHC Class I fold, MR1 undergoes alternative splicing to produce multiple isoforms, the significance of which is not well known. Prior studies suggested that an alternatively spliced form of MR1, termed MR1B, is expressed on the cell surface and can stimulate MR1T in response to infection with *E. coli*, similar to the full length isoform, MR1A, which encodes all encoded exons and is known to promote MR1T activation. However, these studies are limited in the presence of endogenous MR1 already inside the cell, thereby confounding the ability to examine MR1B independently.

In this study, we utilized CRISPR/Cas9 to generate a MR1 knockout cell line, combined with overexpression vectors to show that while the MR1 splice variants do not individually stimulate MR1T responses following infection with mycobacteria, MR1B acts to inhibit MR1T activation when coexpressed with MR1A. We show that this inhibition appears to occur inside the cell, potentially inside vesicular compartments. Though coexpression of MR1B simultaneously or post MR1A expression does not impact the abundance of MR1A protein, expression of MR1B prior to that of MR1A seems to reduce detectable MR1A, indicating that MR1B may be mediating its effects through competition for factors necessary for MR1A to assemble and fold. There are multiple mechanisms by which this is occurring. 1. MR1B is directly competing for ligand and therefore, preventing MR1A from binding ligand and folding. 2. MR1B is directly competing for components of the protein expression machinery or for chaperone proteins necessary for MR1A folding and localization. The first hypothesis is supported by the fact that MR1A and MR1B both share a ligand binding domain. However, as the nature of the putative self-ligand that enables MR1 folding in the absence of microbial infection or exogenous ligand is currently unknown, it is difficult to study whether MR1B a direct competitor of ligand from MR1A. Mutagenesis of the ligand binding domain in MR1B may help confirm the ligand-binding hypothesis. If a mutant MR1B does not inhibit MR1T activation, this lends credence to the idea that ligand binding is a mechanism by which MR1B is mediating its effects. However, the crystal structure of MR1B is not known, and with the lack of the α3 domain in MR1B, there is a possibility that mutagenesis of ligand binding residues in MR1B may completely ablate MR1B protein expression. MR1A is known to

require unique chaperone molecules that are distinct from MHC Class I in trafficking to the cell surface (Harriff et al. 2016, Karamooz et al. 2019). Though we show that MR1B is not expressed on the cell surface, even in the presence of 6-formylpterin, a ligand that upregulates MR1A on the plasma membrane, the possibility remains that MR1B could be binding proteins that are required for correct MR1A folding and trafficking to intracellular compartments, including intracellular vesicles. In the absence of robust antibody reagents to measure endogenous MR1 or distinguish between MR1A and MR1B, immunoprecipitation studies combined with mass spectrometry using overexpression models could help validate this hypothesis.

Our laboratory has recently shown that the ligand repertoire from two microbes that can stimulate MR1T cells is not only very broad, but also distinct (Harriff et al. 2018). We show in Chapter 3 that MR1T responses to Mtb and M.marinum were highly correlated, as were those to M. smegmatis and E.coli, suggesting that the two pairs of microbes share similar ligand repertoires. MR1B does not traffic to the cell surface upon exposure to 6FP, however, it is possible that MR1B does not bind 6FP, but instead engages with other ligands, perhaps those distinct from the riboflavin or folic acid synthesis pathway. To definitely answer whether MR1B engages ligand, refolding assays and crystallography are essential to defining the structure of MR1B and enumerating the ligand binding pocket in the absence of 6FP. It is also possible that in the context of certain microbial ligands, MR1B plays an inhibitory function, but in the context of other ligands, MR1B may have no impact on T cell activation, or may potentiate T cell responses. Indeed, MR1B was suggested to stimulate T cell responses

in response to fibrosarcoma cells infected with *E. coli* (Lion et al. 2013). The T cell response to pathogen is context specific, and different cell types and environments may impact protein function in distinct ways. Further studies of MR1B in differing contexts would be necessary to gain a broader understanding of its physiological role.

We show that relative expression of MR1A and MR1B transcript in human double positive thymocytes is associated with expression of surface MR1A in these cells. Additionally, we were surprised to note that this relative MR1A/MR1B expression in DP thymocytes was also associated with the frequency of MR1T thymocytes. This is intriguing, as DP MR1+ thymocytes are thought to select MR1T cells (Seach et al. 2013). This raises a second potential role for the regulation of alternative splicing in promoting surface expression of MR1 on DP thymocytes and contributing to MR1T selection and development in the thymus. The presence of surface MR1 on DP thymocytes in the absence of known infection raises the question of what the self-ligand is that selects for MR1T cells, or if there are multiple self ligands that help select MR1T cells. How MR1B directly engages with self-ligands or impacts MR1A interaction with selecting ligands to regulate MR1T development represents an intriguing area of research.

The physiological importance of MR1B-medaited inhibition of MR1A in peripheral cells is not yet known. We utilized computational analysis of human transcriptome data, combined with molecular biology techniques in antigen presenting cells isolated from human tissues to show that MR1A and MR1B transcript are expressed across tissues, with variation both between tissue sites and within donors. Analysis of transcriptomics data suggested that a higher MR1A/MR1B transcript ratio was associated with tissues

such as blood, spleen, lung, and liver. However the opposite was observed in uterus and colon. Using cell lines from a variety of tissue sites, we show that uterine and breast cancer cell lines, which expressed low relative MR1A/MR1B, were less capable of stimulating MR1T activation following exposure to mycobacterial antigen than lung epithelial and blood dendritic cells. These data support the hypothesis that MR1B acts as a negative regulator of MR1A-mediated antigen presentation in tissues that require broad immunosuppression. The uterus, in particular, requires a high level of immunosuppression during pregnancy, and many non-classical MHC molecules, including HLA-G and HLA-F appear to play an important role in mediating protection of the developing fetus (Davies et al. 2006, King et al. 2000). Cytotoxic MR1T cells have been shown to accumulate in the placenta, but potentially to act in an antimicrobial response to pathogens that could harm a developing fetus (Solders et al. 2017). However, we postulate that alternative splicing of MR1 functions in the uterus to regulate MR1T responses and prevent tissue damage or harm the developing fetus.

Though our results suggest an important role for alternative splicing in the control of MR1T activation, it is not yet understood how MR1 expression, both at the transcript and protein level, is regulated following exposure to ligand or microbial infection. It is easy to imagine a scenario where, in the absence of microbial infection, MR1B expression is higher than MR1A, but following exposure to pathogen, MR1A is rapidly expressed and antigen presentation can occur. Additionally, the role of TLR signaling in modulating MR1 expression represents an intriguing area of research and could help determine under what precise conditions MR1A expression and protein translation is

stimulated. CHIP-Seq and epigenetic analyses, furthermore, could also be utilized in gaining a broad understanding of MR1 gene and protein expression.

Though alternative splicing has not been reported for many antigen-presenting molecules, there are many reports of splice variants implicated in diseases of host immunity or autoimmunity (Evsyukova et al. 2010). A splice variant of IL-7, a cytokine important for lymphocyte growth and development, was shown recently to be associated with increased risk for multiple sclerosis (Galarza-Munoz et al. 2017). Intriguingly, expression of this IL-7 splice variant also appeared to be dependent on single nucleotide polymorphisms. As intronic SNPs have been generally studied in the regulation of alternative splicing, there remains a possibility that SNPs in MR1 may be of importance in the regulation of MR1 splice variant expression, which may, in turn, influence susceptibility to microbial infection. An intronic SNP in MR1 was recently shown to be associated with susceptibility to tuberculosis, although the significance of this SNP is unknown (Seshadri et al. 2017). Further studies on how SNPs in MR1 impact MR1 expression and splicing, as well as associations with antigen presentation and MR1T function would be of interest.

Intriguingly, a recently described splice variant of HLA-A11 was shown to potentially impact HIV-mediated immune evasion (Zhang et al. 2017). This variant lacked the α3 domain, and modulated NK-mediated cytotoxicity in response to HIV infection. This, combined with a report that the MHC-like molecules, MICA and MICB, expressed splice variants that were both stimulatory and inhibitory to NK cells, places our results in

the context of a growing body of work supporting alternative splicing as a means of regulating host immune responses to infection (Gavlovsky et al. 2016).

In addition to playing a vital role in the early control of microbial infection, MR1T cells have been implicated in the pathogenesis of autoimmunity (Hinks 2016). The role of MR1 in presenting endogenous or commensal antigen to MR1T cells in autoimmune pathology is not established, but remains a possibility. It would be of interest to query tissues from individuals with autoimmune diseases to measure splice variant expression and antigen presentation function. I hypothesize that inflamed tissues isolated from individuals with autoimmune exacerbations will express higher relative levels of MR1A and these tissues will be more efficient at stimulating MR1T responses, as compared to normal tissue, or tissue from healthy patients. Moreover, understanding how autoimmune pathology changes MR1 gene and protein expression, or vice versa, would lend further insight into the balance between immune tolerance and appropriate host response to pathogen.

Taken together, our results show that MR1T cells can efficiently discriminate between microbial antigen through usage of their TCRα and TCRβ, and that these responses can be regulated through alternative splicing of MR1. We propose that alternative splicing of MR1 represents a novel means of regulating T cell activation in the context of infection.

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Appendices

Appendix Table 1: Sequencing of MR1T clones (Chapter 3)

Name	TRAV	CDR3a	TRAJ	MAIT Match Score	TRBV	CDR3b	TRBJ
D0033A1	1-2	CAALDSNYQLI	33	0.9583	4-2	CASSQDMVSITDTQY	2-3
D0033A6	1-2	CAVVDSNYQLI	33	0.9587	4-2	CASSHSSGTGGNEQF	2-1
D0033A10	1-2	CAVTDSNYQLI	33	0.9583	3-1	CASSSGLEVTGELF	2-2
D0033A8	1-2	CVTMDSNYQLI	33	0.9392	6-1	CASSEAGGGYNEQF	2-2
D0033A3	1-2	CVTMDSNYQLI	33	0.9392	6-1	CASSEAGGGYNEQF	2-1
D0033A2	1-2	CAVTDSNYQLI	33	0.9583	3-1	CASSQAETELNTGELF	2-2
D0033A11	1-2	CAVIDSNYQLI	33	0.9582	19	CASSIQGPHESYNEQF	2-2
470E3-2	1-2	CAVRDNDYKLS	20	0.9638	6-4	CASSVGFEHNEQF	2-1
571E3-2	1-2	CAVRDSNYQLI	33	0.9567	6-4	CASSEASGGTDTQY	2-3
571H7-2	1-2	CAVQDSNYQLI	33	0.9576	6-5	CASSDGDSGANVLT	2-6
435A8	1-2	CAVADSNYQLI	33	0.9404	6-4	CASSDSADGQDTQY	2-3
435H6	1-2	CAVLDSNYQLI	33	0.9581	3-1	CASSQEVPFSSGGSSVTQY	2-3
470F8	1-2	CAVINSNYQLI	33	0.9274	20-1	CSARGSRRDYEQY	2-7
432BE6	1-2	CAVRGPSGGSYIPT	6	0.864	4-1	CASSQGGPSNTEAF	1-1
427F10-2	1-2	CAVSDSNYQLIW	33	1	20-1	CSARGTADYQPQH	1-5
403A9	1-2	CAVVDSNYQLI	33	0.9587	20-1	CSARDGGEAYNEQF	2-1
403B3-2	1-2	CAVLDSNYQLI	33	0.9581	3-1	CASSQEVPFSSGGSSVTQY	2-5
403C6	1-2	CAVRSGDYKLS	20	0.9266	6-4	CASSEGLAGGNEQF	2-1
450B9	1-2	CAVMDSNYQLI	33	0.9573	6-1	CASTPSGEFSEAF	1-1

571F3-2	1-2	CAVSDSNYQLI	33	0.959	20-1	CSAREVEGTYEQY	2-7
571B9	1-2	CAVRDSNYQLI	33	0.9567	6-4	CASSEASGGTDTQY	2-3
571G4	1-2	CAVRDSNYQLI	33	0.9567	6-4	CASSEASGGTDTQY	2-3
403D5	1-2	CAGMDSNYQLI	33	0.9557	7-8	CASSPRGRGDGANVLT	2-6
462D5	1-2	CASMDSSYKLI	12	0.9633	4-2	CASSQDGSSGANVLT	2-6
470A1-2	1-2	CAVLDSNYQLI	33	0.9581	6-1	CASSGGQEGNEQF	2-1
450B2-1	1-2	CAAMDSNYQLI	33	0.9576	6-1	CASSGEISYNEQF	2-1
427F5	1-2	CVVMGLIGFGNVLH	35	0.8412	7-8	CASSPRGRGDGANVLT	2-6
427D8-2	1-2	CAVMDSNYQLI	33	0.9573	5-1	CASSLLRQGTEKLF	1-4
450A9	1-2	CAVRDSNYQLI	33	0.9567	20-1	CSAREVEGTYEQY	2-7
470A8-2	1-2	CAGMDSNYQLI	33	0.9557	7-8	CASSPRGRGDGANVLT	2-6
462D7	1-2	CASMDSSYKLI	12	0.9613	4-2	CASSQDGSSGANVLT	2-6

Appendix Table 2: Antibodies used for flow cytometry staining in Chapters 3 and 4

Antibody Name	Fluorophore	Manufacturer	Clone
CD3	FITC	BioLegend	UCHT1
CD8	APC Cy7	BioLegend	SK1
TRAV1-2	APC	BioLegend	3C10
CD4	PE Cy7	BioLegend	RPA-T4
CD326	APC	BioLegend	9C4
CD14	FITC	Biolegend	HCD14
CD20	FITC	BioLegend	2H7
CD45	FITC	Biolegend	30-F11
Live-Dead	Aqua	ThermoFisher	
MR1/5-OP-RU tetramer	PE	NIH Tetramer Core (Emory)	
MR1/6FP tetramer	PE	NIH Tetramer Core (Emory)	

Appendix Table 3: Table of adult lung, airway, and small intestine donors (Chapter 4)

Donor	Gender	Cause of Death	Mechanism of Injury	Age	Smoking history?
OD23	Male	Head Trauma	Blunt Injury	22 months	No
OD48	Male	Cerebrovascular/Stroke	ICH/Stroke	44	Yes
OD63	Male	Cerebrovascular/Stroke	ICH/Stroke	57	No
OD78	Female	Cerebrovascular/Stroke	ICH/Stroke	69	No
OD83	Male	Cerebrovascular/Stroke	ICH/Stroke	65	No
OD88	Female	Anoxia	Asphyxiation	29	No
OD92	Female	Cerebrovascular/Stroke	ICH/Stroke	48	Yes
OD93	Female	Head Trauma	Blunt Injury	52	Not indicated
OD111	Female	Anoxia	Not Indicated	22	No
OD114	Male	Cerebrovascular/Stroke	ICH/Stroke	56	Not indicated
OD119	Female	Cerebrovascular/Stroke	ICH/Stroke	58	No
OD120	Female	Anoxia	Drug Intoxication	39	No
OD121	Female	Anoxia	Not Indicated	6	No
OD123	Male	Cerebrovascular/Stroke	ICH/Stroke	39	No

*Determination of smoking history made based on >20 pack/year history