THE UNIVERSITY OF CHICAGO

# THE IMPACT OF MR1 LIGAND DIVERSITY ON THE CELLULAR IMMUNE RESPONSE TO CANCER AND INFECTION

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## Abstract

Major histocompatibility complex-related protein 1 (MR1) is a highly conserved non-polymorphic antigen presenting protein that is well known for the presentation of microbially-derived riboflavin metabolites to mucosal-associated invariant T (MAIT) cells. Once thought to be limited to this one modality, recent work has shown that MR1 and the T cells that recognize it are much more diverse than previously appreciated. Most notably, recent reports of MR1-restricted T cell (MR1T) clones that have shown MR1-dependent cytotoxic activity against cancerous tissues has opened the door for the study of potential endogenous ligands for MR1. The work described in this dissertation provides context for these recent developments, positing that the newer "atypical" members of the MR1T family may be more unified in their recognition of MR1 both in homeostasis and in the context of cancer. My characterization of a new subset of endogenously derived MR1 ligands, endogenous pterin (EP) molecules, answers the outstanding questions around the ability of MR1 to present "self" metabolites. Further investigation of these molecules reveals that they can activate cancer-associated MR1Ts, but beyond this cancer paradigm, I also show that these same ligands can activate 6-FP reactive and "atypical" non-cancer-associated MR1Ts. This implies that these unmodified endogenous metabolites may play a much broader role in MR1T biology, perhaps explaining the selection of these cells in the thymus or their maintenance in the periphery. Finally, I show how previously discovered microbial ligands for MR1 can be used to define new populations of MR1T cells, introducing the first instance of antigen specific recognition of MR1 by a γδ T cell. Altogether, this work emphasizes the functional versatility of MR1, and through the discovery of new ligand classes, and the reassessment of others, it provides answers for some of the major outstanding questions surrounding the biology of MR1T cells.

For William Rodden,

I did not get to know you long,

but feel blessed to have known you at all.

For your curiosity, your support, and your kindness.

Thank you.

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## Acknowledgments

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# Chapter 1. Introduction

## 1.1 The cellular response to cancer and infection

### 1.1.1 The cellular branch of the adaptive immune system is comprised of T cells that carry a uniquely rearranged T cell receptor

The immune system has evolved with the purpose of both protecting the host from threats and maintaining tissue homeostasis, primarily accomplishing these tasks through the determination of "self" and "non-self"[1]. While almost all multicellular organisms have developed some way to carry out these functions, jawed vertebrates achieve host defense through the synchronized response of the innate and adaptive immune systems[2]. Cell types belonging to the innate branch of the immune system - such as monocytes, macrophages, and neutrophils - utilize germ-line encoded receptors to quickly recognize and respond to the conserved molecular motifs present in common microbes. These pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs) are generally derived from molecules that pathogens require to survive, or common molecules released when tissue damage occurs[3, 4]. The adaptive branch of the immune system, on the other hand, is dependent on highly variable receptors that have the potential to recognize any sort of "non-self" molecular pattern through the generation of remarkable receptor diversity. While the cells that make up the adaptive arm of the immune system respond with much slower kinetics than innate cells, they hold the potential for trained immunity - the ability to "remember" pathogenic insults through expansion and persistence of cells that respond to the primary insult[5]. The adaptive arm of the immune system can be further subdivided into the humoral branch, comprised of B cells and their variable receptor the B cell receptor (BCR), and the cellular branch, consisting of T cells and their variable receptor the T cell receptor (TCR).

Each T cell expresses one TCR made up of two separate polypeptide chains which combine to form a conserved tertiary structure made from a highly variable amino acid sequence. Of the estimated $10^{11}$ total T cells in the human body there are approximately $10^{10}$ unique TCRs that can be detected among them, each defining a unique T cell clonotype[6]. In order to maximize the potential for receptor diversity, while at the same time avoiding an exceptionally large genome, the adaptive immune system has evolved a way to stochastically recombine gene segments, a process called V(D)J recombination, which allows the immune system to generate this remarkable receptor diversity with only about 200 small gene segments (**Figure 1.1A**)[7, 8]. However, receptor diversity is not solely the product of V(D)J gene recombination, as further diversity in the variable regions of the TCR can be added through the excision of templated nucleobases and the random addition of non-templated nucleobases at the V-(D)-J junction[9]. Taken together these processes allow for the potential total diversity of TCR rearrangements to exceed $10^{15}$ unique TCRs[8]. The antigen binding domain of each TCR is comprised of three amino acid loops that interact with the target called "complementarity-determining regions" (CDRs). Two of these loops are encoded within the V gene selected for the given TCR chain (CDR1 and CDR2), but one loop, termed the CDR3 loop, is the product of gene editing at the V-(D)-J junction and the resulting amino acid sequence of this loop is completely unique to an individual T cell clone (**Figure 1.1B**)[10, 11]. As a consequence of a highly regulated development process, the genes used, and variable amino acid sequences generated at gene junctions during V(D)J recombination largely dictate what targets the T cell can recognize in the periphery.

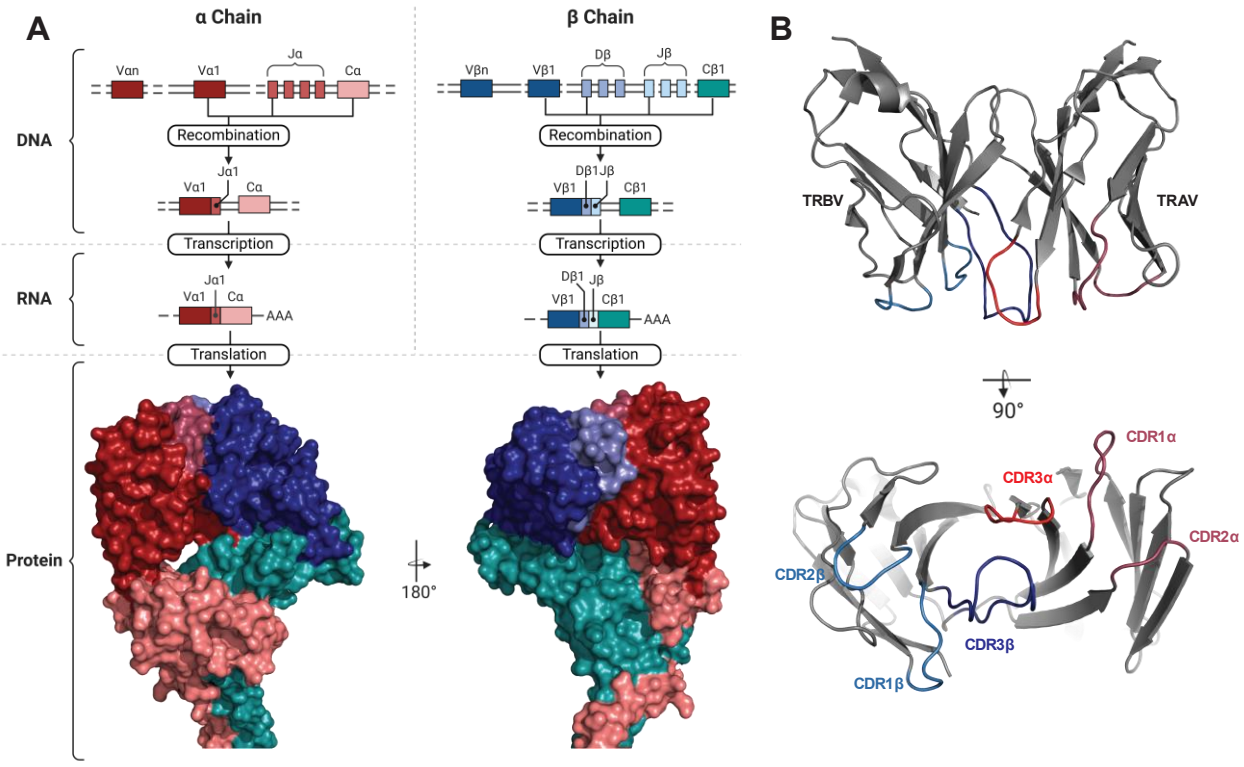



**Figure 1.1** TCR receptor diversity is maintained through VDJ recombination. A) The process of VDJ recombination as it occurs at the DNA level during gene rearrangement through to the generation of an RNA transcript coding for a unique TCR. The fully formed TCR is shown as a protein surface representation with coloring that matches the gene segments from the cartoon above. B) The variable domains of a TCR represented as a cartoon. The non-variable residues are shown in grey with the CDR loops highlighted in red (TRAV) and blue (TRBV). PDB: 6JXR.

### 1.1.2 Conventional αβT cells react to processed peptide antigens presented in the context of polymorphic HLA molecules

T cells can be divided into two groups depending on the genes used to form the TCR during recombination. Alpha beta T cells (αβ T cells) recombine α and β V, D (only for the β chain), and J gene segments while gamma delta T cells (γδ T cells) recombine γ and δ V, D (only for the δ chain), and J gene segments[12]. αβ T cells do not recognize their antigen directly with their TCRs and instead rely on antigen processing and presentation within an antigen presenting molecule prior to recognition[10]. Conventional αβT cells recognize protein antigens that have been processed within the antigen presenting cell (APC) into peptides that are loaded on major

histocompatibility complex (MHC) molecules[13, 14]. While sequestered within the endomembrane system of the APC, MHC molecules bind short peptides in a long groove that is located on the membrane-distal portion of the molecule[15, 16]. When these peptide-loaded MHC molecules make it to the surface of the cell, they are "sampled" by αβ T cells, who probe the collective peptide-MHC complexes present to determine the presence of "self" or "non-self" peptides. At any given time, the surface of an APC is covered by MHC molecules containing various peptides derived from host proteins ("self") or, in the case of infection, pathogens ("non-self"); it is the job of the T cell to delineate between the two. In this way, αβ T cells survey the health of the cells in the body and are capable of "seeing" antigen that may be hidden within them.

There are two major classes of MHC molecules, MHC-I and MHC-II, that can be defined by their molecular characteristics and by the types of T cells that engage them. MHC-I molecules, which engage with CD8+ cytotoxic αβ T cells, have a single membrane bound heavy chain comprised of three domains α1, α2, and α3 that associates non-covalently with a light chain comprised of a single invariant immunoglobulin (Ig) domain called $\beta_2$ microglobulin ($\beta_2$M)[17]. In humans MHC-I molecules can be further subdivided into two sets of human leukocyte antigen (HLA) molecules, three HLA-Ia genes (HLA-A, HLA-B, and HLA-C) and three HLA-Ib genes (HLA-E, HLA-F, and HLA-G)[18]. HLA-Ia genes comprise what are referred to as the "classical" MHC-I molecules. These proteins are expressed in all nucleated cells in the body and are used by conventional CD8+ T cells to survey these cells for signs of intracellular infection[16]. HLA-Ia molecules are the most polymorphic genes in the human genome with well over 4000 different alleles discovered across the human population for each locus[19]. Much like the CDR loops of the TCR, this polymorphism is concentrated in one

specific location - the peptide-binding groove formed by the α1 and α2 domains[20]. This allows for a diversity of peptide antigens to be presented by the HLA-Ia molecules of an individual, and an even larger diversity of peptides can be captured across an entire population due to the major variations in the peptide-binding grooves of HLA-Ia observed between individuals. In this way, T cells monitor the peptidome of all other cells extensively and the level of polymorphism between individuals ensures that no single pathogen affects the population in the same manner[21, 22]. On the other hand, the "non-classical" HLA-Ib molecules are monomorphic or oligomorphic, meaning that there is a limited number of functional alleles for these genes in the human population. These molecules have been shown to have conserved, specialized functions that are carried out in a context or tissue dependent manner. It is likely that the sequence of these molecules has been conserved over the span of evolutionary time due to the conserved functions they serve in each individual[10].

MHC-II molecules (in humans referred to as HLA-DP, HLA-DQ, and HLA-DR) are made up of two membrane bound chains each comprised of two domains each, α1 and α2, where the peptide binding groove of these molecules is formed by the composite arrangement of these two separate chains[23, 24]. Across the human population these molecules also show a high degree of polymorphism which is concentrated in the peptide binding groove, much like HLA-Ia molecules. Unlike classical MHC-I molecules, the expression of MHC-II is limited to "professional" APCs, immune cells that are specialized to help survey, gather, and present antigens to CD4+ T cells[16]. The polymorphism observed in TCRs and in MHC-I and MHC-II is likely no coincidence, as there is strong evidence that these molecules coevolved under selection to be able to capture as much diverse peptide material as possible to help aid T cells in their quest to detect and remove pathogens[10, 25, 26]. While the diversity of TCRs and MHC

molecules is key to T cell mediated immunity, the vast array of potential T cell phenotypes allows for the context dependent action and plasticity necessary to fight all types of threats in the various tissues of the body.

### 1.1.3 T cells have access to an array of potential phenotypes and effector functions used to protect the body from infection and cancer

Conventional αβ T cells can display a wide variety of phenotypes that can be categorized in two main ways, either by the coreceptors they present on the surface that dictate their restriction to either MHC-I or MHC-II, or by the outcome of their response when stimulated - called the effector function[27]. The majority of this programming is achieved in the thymus where naïve T cells undergoing selection prove they are both restricted to the proper MHC molecules found in the body yet are not reactive to the body's own proteins[28]. During this selection, the TCR rearranged by the thymocyte dictates the expression of the major coreceptor for activation, CD4 or CD8, through its ability to recognize either MHC-I or MHC-II[29]. This is the first major decision point as the naïve T cells leaving the thymus continue to roll down a branching epigenetic landscape that will eventually decide their final phenotype and effector function. Broadly speaking, αβ T cells can be grouped by coreceptor expression, where CD8+ T cells, or cytotoxic T cells, are restricted to MHC-I molecules and CD4+ T cells, or helper T cells, are restricted to MHC-II[30, 31]. This restriction is directly mediated by the coreceptor where CD4 and CD8 both interact with the more conserved, membrane proximal portion of their respective MHC during TCR docking[32]. Upon exiting the thymus, naïve CD4 and CD8 T cells have already acquired a basic effector phenotype during selection. CD8 T cells are primed to be cytotoxic to targets presenting a foreign peptide that activates their TCR and CD4 T cells, which

interact mainly with professional APCs presenting MHC-II, are primed to carry out helper functions which can range from releasing stimulating cytokines, activating APCs or B cells, or releasing other effector molecules that can help deal with extracellular threats [29].

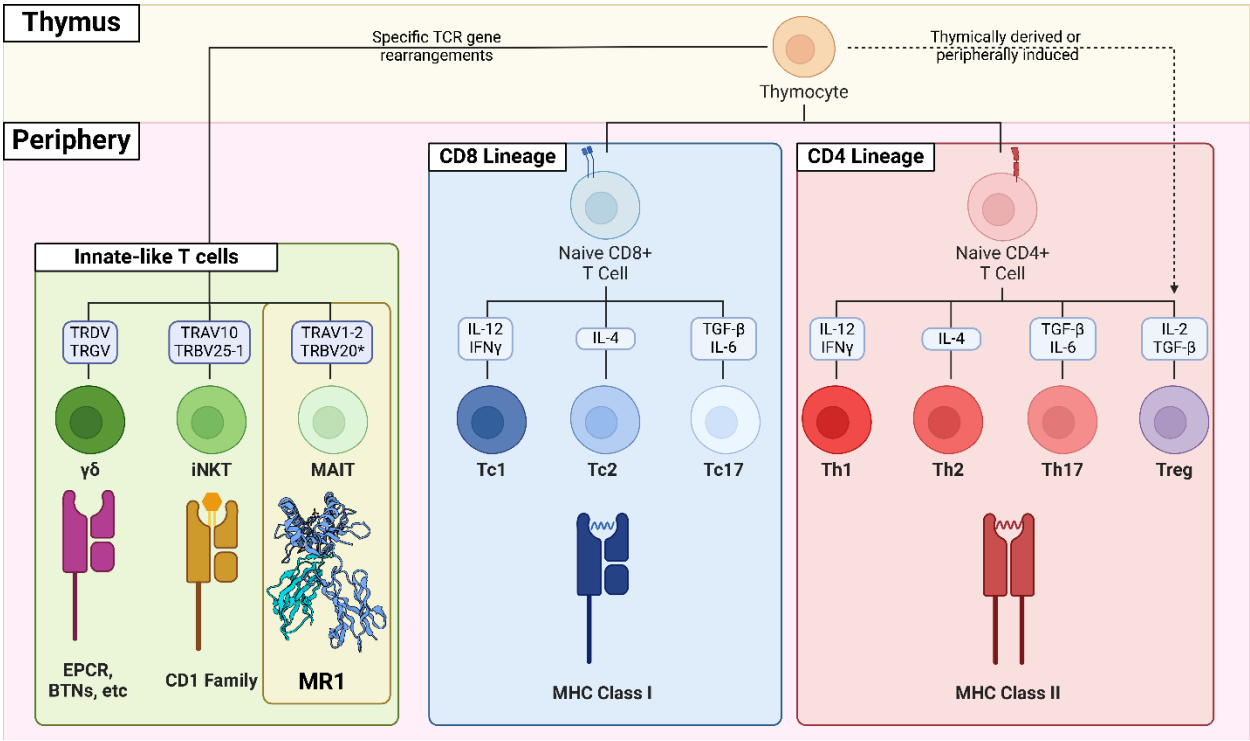



**Figure 1.2** An overview of the major T cell subtypes and innate-like T cells found in humans. For the conventional αβ T cells (CD8 and CD4 lineages) this is a non-exhaustive summary of common effector phenotypes. The cytokines present as a "third signal" during primary activation of a naïve T cell in the periphery to produce each subtype are shown above each clade. The TCR gene combinations most frequently observed for successful selection and development of innate-like T cells (green) are shown above their respective clade. For each subtype, the restriction element (or examples of the most common restriction elements) for each subtype is shown below each clade.

Beyond these two clades of αβ T cells defined by coreceptor expression, each subset goes through further differentiation after gaining experience with antigen in the periphery. The cytokine milieu or other external signals present at the time of activation influence a naïve T cell as a third signal that comes along with TCR and coreceptor signaling (**Figure 1.2**)[33, 34]. The nature of this signal informs the developmental trajectory of the cell as it matures during activation and expansion giving it access to distinct transcriptional phenotypes that equip the T

cell to deal with threats ranging from intracellular pathogens to multicellular parasites. Beyond the classical Th1, Th2, Th17, and regulatory phenotypes that are accessible to conventional αβ T cells as outlined in **Figure 1.2**, there exists other T cell subtypes that can be identified by their biased TCR gene usage and unique transcriptional profiles. These T cell subsets which include invariant natural killer T (iNKT) cells, γδ T cells, and mucosal associated-invariant T (MAIT) cells are considered to be "innate-like" T cells as they exhibit properties of both adaptive and innate immune cells. Namely, while they make use of a T cell receptor and exhibit T cell effector functions, they also respond to less pathogen-specific stimuli than conventional αβ T cells and do so rapidly, with kinetics more akin to an innate response[35-37]. It seems that a major reason these T cells can be programed for such a rapid, innate-like response is due in part to the conserved or non-polymorphic antigen presenting molecules they are restricted to[38]. iNKT cells and some γδ T cells have been shown to be restricted to CD1 family molecules while MAIT cells have been shown to be restricted to the MHC-related protein MR1 (**Figure 1.2**)[39-41]. Unlike the MHC molecules discussed previously, these molecules exhibit very little polymorphism in the human population and the antigens they present are not derived from foreign proteins at all, but conserved features of microbial metabolism such as lipids and metabolites.

## 1.2 MR1 presents a wide array of metabolite antigens to T cells

### 1.2.1 MR1 is a highly conserved, non-polymorphic antigen presenting molecule

As the name "MHC-related protein 1" suggests, MR1 is an antigen presenting protein that is evolutionarily, structurally, and functionally related to MHC-I molecules[42, 43]. MR1 was first identified in the human genome in 1995 through a polymerase chain reaction-based

strategy to amplify and identify genes with sequence similarity to MHC-I[44]. Interestingly, MR1 was shown to be located outside of the MHC region of the genome near other non-classical antigen presenting molecules like the CD1 family. Further investigation into this putative antigen presenting molecule revealed that MR1 exhibits the canonical MHC-I fold (as indicated by the presence of predicted α1, α2, and α3 domains) and the ability to associate with $\beta_2$M (**Figure 1.3**)[42, 45]. Initially, genetic analysis suggested that MR1 was monomorphic in the human population, having one functional allele (MR1*01), but more comprehensive population sequencing and reports of loss-of-function MR1 mutations have revealed the existence of other alleles[46-49]. The alleles identified by sequencing vary in only one to two amino acids from MR1*01 and make up a very minor proportion of the total gene distribution in the population that was surveyed - MR1*01 comprised 71% and MR1*02 comprised 25% with the remaining 4% split between the other 4 alleles identified[49]. Further analysis showed that the MR1 amino acid sequence was also highly conserved in mammals with very little polymorphism observed in the α1 and α2 regions between species[50]. This conservation of the structure of the antigen binding domain of MR1 strongly suggests two things: first, MR1 must serve some critical role in the survival of its mammalian hosts and second, if this molecule does present an antigen, the antigen too would likely be conserved and important for early identification by the host organism[51, 52].

Early clues to the possible identity of this conserved ligand came in the form of basic biochemistry and immunology experimentation. First, it was discovered that the much like the known MHC-I and CD1 family proteins, the heavy chain of MR1 associated with $\beta_2$M and MR1 is likely retained in the endoplasmic reticulum (ER) as the majority of it was found to be endoglycosidase H sensitive when retrieved from cell lysate[42, 45]. Further probing revealed

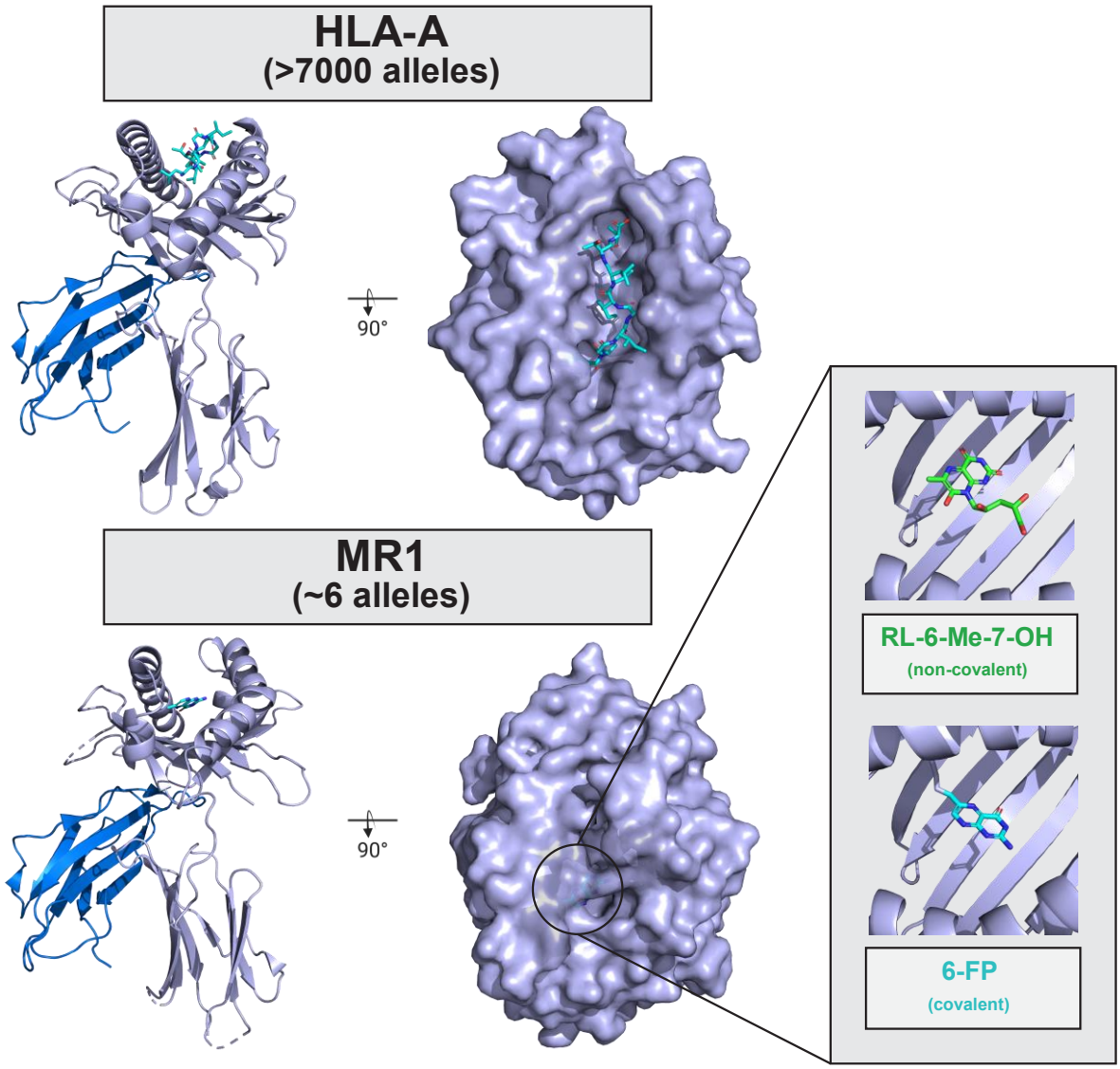



**Figure 1.3** A comparison of HLA-A and MR1. An allele of HLA-A (HLA-A*02) loaded with the MART-1 peptide is shown at the top. Below, MR1 loaded with the pteridine antigen 6-FP is shown in a similar orientation. For each, the heavy chain is shown in light blue while the non-covalently associated light chain (β2M) is shown in dark blue and the antigen is shown in cyan. Examples of non-covalently bound (RL-6-Me-OH) and covalently bound (6-FP) MR1 ligands are shown on the right, with Lys43 depicted as sticks where bound to 6-FP. PDB: 2GTW, 4GUP, and 4LCW.

that, the MR1-$\beta_2$M complex was only stable when the ectodomain was expressed in the presence of yeast extracts or cell culture medium[45]. Combined with the knowledge that MR1 has very little to no surface expression at homeostasis, these data implied that, much like MHC-I-$\beta_2$M complexes, MR1 was unstable in the absence of a ligand, but unlike MHC-I complexes, stabilizing ligands are not always readily available[53]. Discovery of MR1 ligands, however, was impeded for years until the MR1-restriction of MAIT cells was deconvoluted, and it was shown that these cells could only be activated in the presence of bacteria or low molecular weight filtrates of bacterial lysate[36, 40, 54, 55]. The identification of MAIT cells eventually led to the ultimate discovery of a ligand for MR1 and later, much to everyone's surprise, the discovery that this invariable antigen presenting molecule can accommodate an ever-growing array of distinct metabolite antigens.

### 1.2.2 In the context of bacterial or fungal infection MR1 is indispensable in its ability to capture metabolites belonging to B-vitamin biosynthesis

The first structurally validated ligands for MR1 were described in 2012 as molecules related to two separate B vitamins[56]. The first of which, 6-formylpterin (6-FP), is a pteridine molecule generated as a product of the photo-degradation of folic acid (vitamin B9)[57, 58]. This pteridine binds strongly to MR1 through a Schiff-base covalent bond mediated by K43 located in the A' binding pocket of MR1 and multiple hydrogen bonds formed with the surrounding amino acid side chains (**Figure 1.3**). Interestingly, despite this molecule's ability to stabilize MR1 on the cell surface and allow for the production of a stable recombinant form of MR1, it was not shown to activate MAIT cells[56, 59]. It is worth noting that additional pteridine ligands have been described for MR1 that are synthetic derivatives of 6-FP (and are therefore not detected in the human body) that also do not seem to activate MAIT cells (**Figure 1.4 A**)[60, 61]. The fact

that these folate-derived pteridine ligands, which are generally thought to not be physiologically relevant due to their dependence on photo-activation, are capable of binding strongly to the MR1 antigen presenting pocket may suggest that the pteridine core structure is conserved for the binding of other undescribed MR1 ligands. It is quite possible that the strong binding of 6-FP and its derivatives by MR1 may occur due to a conserved interaction MR1 has with other similar but physiologically relevant pteridines that serve an important purpose in MR1T biology[62].

While the strong interaction between 6-FP and MR1 provided great insights into the structure and potential purpose of MR1, the concurrent discovery of MR1 ligands that were capable of activating MAIT cells stole the limelight from these MR1-pteridine interactions[56]. These ligands, termed ribityl lumazine molecules (**Figure 1.4 B**), are derived from intermediates in riboflavin (vitamin B2) biosynthesis – a pathway that is active in many bacteria and yeast cells but not found in mammalian cells. The discovery of activating ribityl lumazine molecules like 6,7-dimethyl-8-(1-D-ribityl)lumazine (DMRL) and the related compounds 6-hydroxymethyl-8-D-ribityllumazine (rRL-6-$CH_2OH$) and 7-hydroxy-6-methyl-8-D-ribityllumazine (RL-6-Me-7-OH) met all of the anticipated factors for what an MR1 ligand was thought to be[43, 56]. First, since these molecules – primary or secondary biosynthetic metabolites – are created, at least in part, through a metabolically essential process, the microbes that make them cannot easily evolve away from their production[63]. Secondly, the conserved features of this class of chemicals could explain why the sequence and structure of the MR1 binding pocket has been conserved across the mammalian class of organisms[50]. Finally, MAIT cells, which are primarily found in the mucosa where these types of pathogens would enter the body, recognize these ligands in the context of MR1 with their conserved semi-invariant TCR[64]. For all these reasons, the discovery of ribityllumazine molecules as ligands for MR1 fit well with the notion that the non-

polymorphic antigen presenting molecule should present conserved microbial antigens to the semi-invariant TCR repertoire of its cognate T cells.

A follow-up study published in 2014 found that the condensation products of 5-A-RU, an intermediate in the riboflavin biosynthesis pathway (RBP), and methylglyoxal or glyoxal led to the formation of 5-(2-oxopropylideneamino)-6-D-ribitylaminouracil (5-OP-RU) and (5-(2-oxoethylideneamino)-6-d-ribitylaminouracil) (5-OE-RU) respectively (**Figure 1.4 C**)[65]. These molecules were shown to form spontaneously when incubated with their respective condensation partner, molecules that are thought to be omni-present in both bacteria and eukaryotic cells as by-products of glycolysis. This study included crystal structures of MAIT TCRs bound to MR1 in complex with these two new pyrimidine ligands 5-OP-RU and 5-OE-RU. Strikingly, the ribitylpyrimidine ligands were able to form a Schiff base with K43 of MR1, much akin to what was shown for 6-FP, while other bicyclic ribityl lumazine molecules like DMRL and RL-6-Me-7-OH were not[43, 65]. While these ligands shared a similar position in the binding pocket and the same ribityl chain protruding out of the pocket, 5-OP-RU and 5-OE-RU were shown to be much more potent activators of MAIT cells[61, 65]. For some time after this discovery, 5-OP-RU was thought to be the only relevant ligand and that MAIT cells were the only relevant cognate T cell population for MR1 – indeed, MR1-5-OP-RU tetramers are still used by some groups to define the entire MR1T cell population in humans and mice[66, 67]. It was not until a few years later that the unbiased assessment of the contents of MR1 made in the presence of various bacteria challenged the idea that 5-OP-RU is the only relevant stimulatory MAIT ligand for MR1[68]. This work began to expand the ligandome of MR1 to include molecules such as photolumazines and even non-RBP derived ligands like the drug metabolites diclofenac (DCF) and 3-formyl salicylic acid (3-FSA) (**Figure 1.4 D and E**)[43, 68-70]. At the same time, other

reports in the literature were challenging the perceived homogeneity of MAIT cell populations and were introducing new non-MAIT T cells that recognized MR1 in the absence of microbial infection altogether.

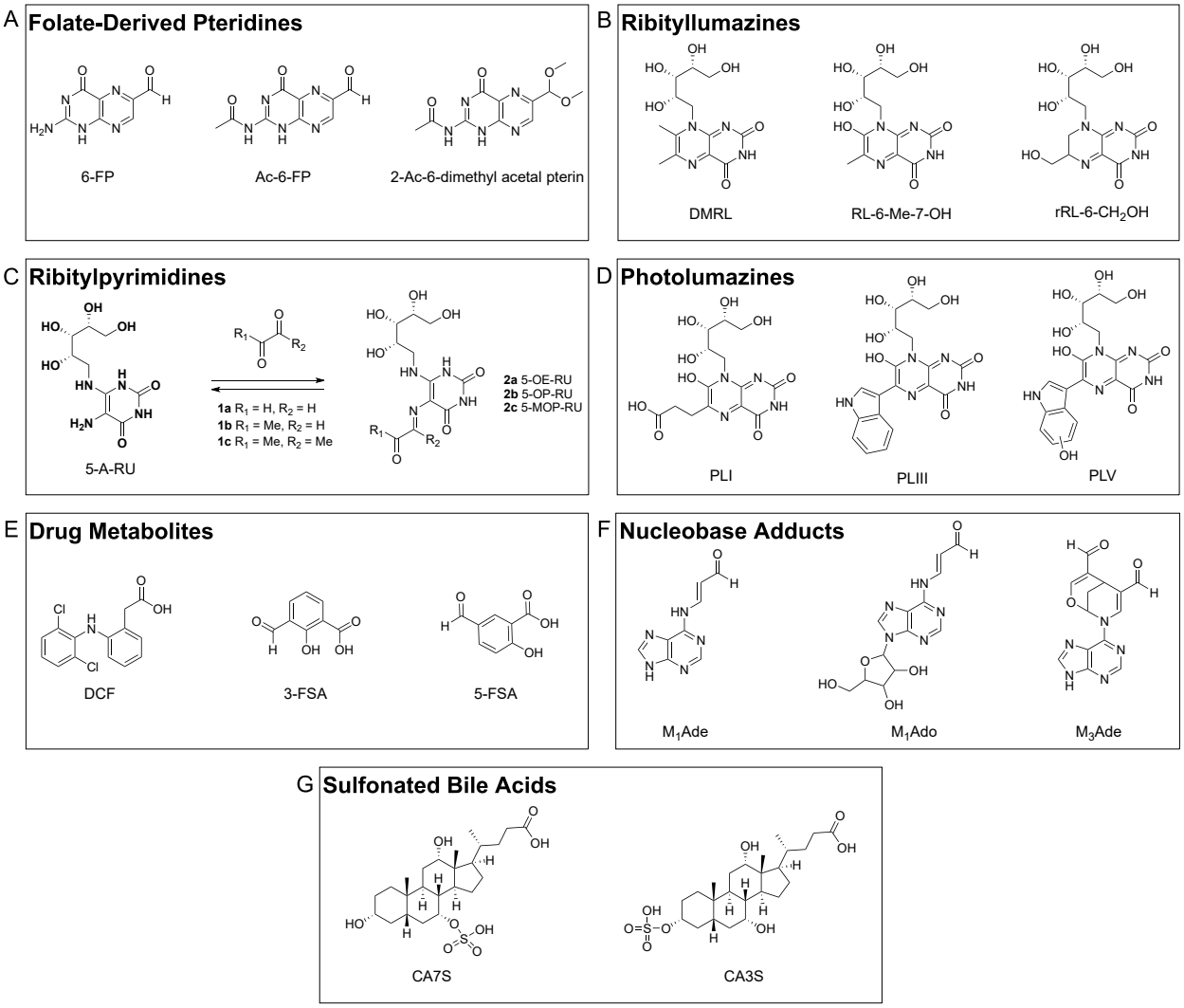



**Figure 1.4** The many classes of known MR1 ligands. Examples from each class of MR1 ligands including folate-derived pterins (A), ribityllumazines (B), ribitylpyrimidines (C), photolumazines (D), drug metabolites (E), nucleobase adducts (F), and sulfonated bile acids (G). This list is non-exhaustive, featuring the most prominent examples from each ligand class.

### 1.2.3 MR1 plays a role in the T cell-mediated cancer surveillance by presenting neo-antigen metabolites to MR1T cells

It was not long after the discovery of 5-OP-RU that initial reports emerged detailing MR1-restricted T cells (MR1Ts) that seemed to react to MR1 on the surface of MR1 overexpressing cell lines in the absence of any microbe. The initial report published in 2017 detailed a way to find these rare T cell clones in the PBMC of healthy volunteers by expanding them on APCs that are overexpressing MR1[71]. This study described a panel of clones that reacted to a series of melanoma and myeloid leukemia cell lines as long as the lines were engineered to over express MR1 on the cell surface. These cells were shown to have diverse TCR chain usage and effector phenotypes – where some were cytotoxic to their targets, others showed helper functions by inducing dendritic cell maturation in an MR1 dependent manner[71]. The major question that arose from this report was: what could MR1 possibly be presenting to these MR1T cells? The ligands seemed to be specific to the cancer cell lines as evidenced by the absence of microbes from the cell culture and through the activation of these MR1Ts using plate bound MR1 loaded with cancer cell lysates. When another report was published in 2020 describing a cytotoxic T cell clone that was derived from a healthy donor (in a similar way as Lepore et al. 2017) and exhibited “pan-cancer reactivity” in an MR1 dependent manner, it became clear that MR1 may be playing roles in the immune system outside of just microbial surveillance[72].

Very recently, multiple studies have been published that outline the discovery of putative cancer-associated MR1 ligands[73-75]. These ligands seem to come in two different groups owing to their formation in cancer cells over that of healthy cells. The first group appear to be nucleoside and nucleobase adducts that have reacted spontaneously in the cytosol of cancer cells

and are bound by MR1 through a covalent Schiff-base bond with K43 in the antigen binding pocket (**Figure 1.4 F**). These ligands were capable of upregulating MR1 on the cell surface of APCs and broadly activated a panel of MR1T clones[73]. A follow up paper from the same group investigated another nucleobase-adduct MR1 ligand that was discovered in the same way called $M_3$Ade[74]. This study included a crystal structure of MR1 bound to this antigen which is the condensation product of malondialdehyde (MDA) and the nucleobase adenine, a reaction that is reported to happen spontaneously within a cancer cell that is experiencing carbonyl stress.

Another recent publication highlighted that microbially derived metabolites can become ligands for MR1 specifically in the context of cancer as they are concentrated within cancer cells for metabolic benefit[75]. This study was able to identify different "vitamers" of vitamin B6 in samples of MR1 derived from squamous cell carcinoma cell line, C1R, and was able to show that MR1 can bind pyridoxal and pyridoxal 5'-phosphate by making a Schiff base bond with K43 in the ligand binding pocket. Interestingly, they were able to show that the TCR from the T cell clone studied in Crowther et al [72] was able to be activated by MR1-pyridoxal complexes, identifying a possible ligand that allows for this T cell to recognize and kill cancer cell lines. While, like all B vitamins, vitamin B6 is an essential metabolite derived from intestinal bacteria, the authors point out that its role in MR1-mediated cancer surveillance may be context (and concentration) dependent, as it has been reported that cancer cells "horde" this vitamin as a way to enhance their ability to synthesize DNA and also to deprive responding lymphocytes of an essential nutrient[76, 77].

All of these studies describing cancer-associated MR1 ligands were published in 2024 which highlights that the scientific community is keenly interested in what ligands MR1 can accommodate during neoplastic disease and legitimizes the role of MR1 in the immune

surveillance of cancer. Some of these ligands can only form via spontaneous reactions in the cytosol of cancer cells [73, 74] while some of them are microbially derived metabolites that seem to be concentrated within cancer cells to benefit their survival[75, 76], but what remains to be seen, is if these ligands fully encompass the cancer-associated ligandome for MR1 or if it is possible that unmodified endogenous metabolites can play a role in this axis as well.

### 1.2.4 Other newly categorized MR1 ligands may be important for the development and maintenance of MR1T/MAIT cells

In the previous parts of this section, I have spoken primarily about microbially sourced ligands that activate MAIT cells and cancer derived oncometabolites that are capable of activating MR1T cells. However, a major question that has remained in the field is: if these ligands are exclusively recognized by these MR1T subsets, then how are these cells educated in the thymus, an organ that is devoid of microbes or cancerous cells, and after selection, how are these cancer-associated MR1Ts supported in healthy individuals? Indeed, all atypical and cancer-associated MR1T clones that have been profiled to date were derived from healthy human PBMC samples, implying the existence of some pool of endogenous ligands that can select and support these cells [71-73]. Beyond this, it has been shown that the ligand repertoire of MAIT cells is more diverse than previously appreciated, as different β chain usage can imbue these cells with the ability to recognize pteridine ligands like 6-FP and acetyl-6-formylpterin (Ac-6-FP) which were categorized previously as non-agonist MR1 ligands for MAIT cells[56, 61]. Notably, this phenomenon has also been observed in some MAIT and MR1T T cell clones that have been labeled MR1-autoreactive, recognizing MR1 on the surface of an APC independent of the ligand in the binding pocket[78].

Some recent work has shed light on possible ligands that are comprised of microbiota-modified-metabolites that may be important for maintenance of MAIT cells in the periphery or perhaps their selection in the thymus. These sulfonated bile acid ligands were discovered via screening of fractionated mouse intestine contents for MAIT activating ligands. Cholic acid 7-sulfate (CA7S), cholic acid 3-sulfate (CA3S), and cholic acid 12-sulfate (CA12S) were shown to stabilize MR1 on the cell surface of APCs when added at high concentrations and CA7S and CA3S were shown to activate MAIT cells in an MR1 dependent manner when added at similarly high concentrations (**Figure 1.4 G**)[79]. The authors also report that a knockout (KO) of Sult2a$^{1-8}$, the enzyme isoforms that are responsible for the creation of CA7S, resulted in fewer thymic MAIT cells in mice. In vitro experiments showed that although no ligand-dependent expansion of MAIT cells was observed with CA7S added to the culture, these cultures had significantly higher levels of MAIT cells after a 6-day incubation in the absence of exogenously added cytokines. While these data suggest a possible role for sulfonated bile acids in the maintenance of MAIT cells, and possibly their selection in the thymus, it remains a mystery how these ligands (or other highly unstable microbially derived ligands that have been suggested to be important for MAIT selection[80]) travel to the thymus and populate it at high enough concentrations to induce MR1 upregulation and subsequent recognition by developing thymocytes. Indeed, in the same study reporting CA3S, CA7S, and CA12S to be important endogenous MR1 ligands for MAIT maintenance it was shown that not only do these sulfonated bile acids rely on the microbiota to be made, but of the three putative ligands, only CA7S could be detected in the intestine where this would occur. This information, combined with the low concentration of CA7S that was detected in thymus (which is not detected at all in germ free mice) and the fact that MAIT cells still develop, albeit at lower numbers, in germ-free[80] and Sult2a$^{1-8}$ KO

mice[79] raises doubts about the "endogenous" nature of these ligands and the role they play in the thymic development of MAIT/MR1T cells under physiological conditions. This outstanding mystery in MAIT/MR1T development underlines the importance of searching for truly endogenous ligands that would have a higher probability of being found at elevated levels in the thymus.

## 1.3 MR1T cells are a large family of T cells comprised of conventional αβ T cells, semi-invariant αβ MAIT cells, and γδ T cells

### 1.3.1 MAIT cells are critical for barrier homeostasis, can recognize an array of MR1 ligands, and have access to context-dependent phenotypes

As described briefly in the previous section, the T cells that use their TCR to recognize MR1 are broadly termed MR1T cells. A subsection of these are MAIT cells which have a conserved phenotype defined by the expression of CD161, CD26, PLZF, T-bet, RORγt, and a select number of chemokine receptors (**Figure 1.5(left)**)[81-84]. The most important defining characteristic of MAIT cells, however, is their semi-invariant TCR which is comprised of the TRAV1-2 gene segment rearranged with TRAJ33/20/12 gene segment[36, 40, 54]. These TRAJ genes encode a conserved tyrosine residue (Y95) that has been shown to make a hydrogen bond with activating microbial ligands which is essential for the activation of canonical MAIT cells[43, 61, 65]. Because of this highly conserved mechanism of activation in this semi-invariant T cell population, the types of ligands that activate MAIT TCRs are also constrained to having certain molecular features. The most important molecular features on MAIT-stimulatory ligands like 5OPRU or DMRL is the ribityl moiety that is allowed to protrude slightly from the MR1 binding pocket[43, 61, 65]. It is the exposed primary hydroxyl group on this chain that

forms the hydrogen bond with Y95 on the MAIT TCR, which is essential for the activation of canonical MAIT cells. The β chain of MAIT TCRs is more variable, but sequencing experiments have revealed a preference for TRBV20-1 and TRBV6 family gene usage [54, 85]. While the TCRα chain is the most conserved and contains the residue necessary for MAIT activation, more recent work has implicated the TCRβ chain in playing a role in MAIT/MR1T ligand promiscuity as outlined in **section 1.2**[61, 78, 86].

From an immunological perspective, MAIT cells are defined as innate-like T cells due to their expression of PLZF and their memory/tissue-resident effector phenotype which allows them quick access to type 1 and type 17 responses that do not require priming or coreceptor stimulation[84, 87]. Due to this independence of coreceptor stimulation during activation, MAIT cells can be found in the body as CD8αβ+CD4-, CD8αα+CD4-, CD8-CD4-, and very rarely CD8-CD4+ or CD8+CD4+. The majority of MAIT cells in humans are CD8+CD4-, expressing a higher proportion of CD8αα homodimer than other αβ T cell populations[66, 67]. Interestingly, more recent work has shown that the CD8 co-receptor is capable of binding to MR1 and enhancing MAIT/MR1T cell TCR sensitivity to ligands that were thought to be non-stimulatory, like the pteridine ligand 6-FP, which may explain why the majority of MAIT cells are CD8αβ+ or CD8αα+[88]. Access to both the T-bet driven type 1 cytotoxic program and the RORγt driven "extracellular threat" type 17 program means that MAIT cells can participate in the immune response by direct killing of infected cells, macrophage/monocyte recruitment, and can secrete cytokines and other factors that are critical for barrier homeostasis and wound healing[89-92]. Remarkably, MAIT cells have been shown to be major players in the anti-viral immune response despite viruses not producing any known MAIT ligands as they lack a metabolism of their own[93]. This response is thought to be purely TCR independent, as MAIT cells can become

activated in vitro just from exposure to type 1 cytokines like IL-12 and IL-18 in the absence of other factors[94], but this does not preclude the possibility that viral infection may stimulate the overproduction of unknown endogenously sourced MR1 ligands in infected cells or activated immune cells. Incredibly, it has been shown that human MAIT cells are able to "decide" between these effector programs based purely the cytokine/metabolic milieu (the "terroir" of the surrounding tissue) and whether they are experiencing TCR stimulation[90]. Taken together it seems that for an "invariant" T cell population MAIT cells show a remarkable ability to process a myriad of simultaneous immune signals and prepare an appropriate measured response.

Finally, it is worth noting that MAIT cell populations have key differences between species which makes the use of mouse models challenging. For example, mice have only a fraction of the circulating MAIT cells that humans do and their MAIT cells leave the thymus with the expression of either T-bet *or* RORγt – not both like in humans[95-98]. The former difference makes studying them in mouse models difficult due to a general lack of cells and the latter implies major functional differences between murine and human MAIT cells that make findings in mice hard to translate to humans. Indeed, it has been shown that murine MAIT cells are epigenetically "locked in" to either a MAIT-1 or MAIT-17 phenotype while human MAIT cells have access to both phenotypes in a context dependent manner[96, 98]. Although recently published research is beginning to challenge the idea that murine MAIT cells are completely rigid in their effector phenotypes, there are still other major differences that have been noted in MAIT development and distribution as well[81, 87, 96].

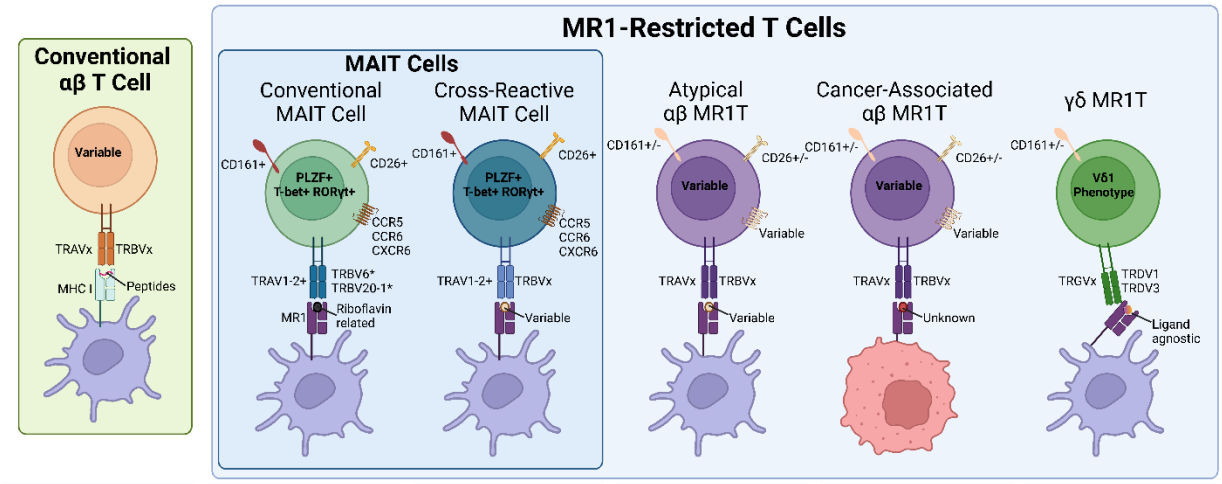
### 1.3.2 αβ MR1T cells are more similar to conventional αβ T cells and interact with MR1 both in the context of infection and cancer

MR1T cells can generally be defined as any T cell that expresses a TCR that is restricted to MR1. However, this designation is generally used to refer to αβ T cells that do not utilize the TRAV1-2 gene segment and are therefore not defined as MAIT cells [99]. While the nomenclature on how to refer to MR1T T cell subtypes varies greatly in the literature, here I will define that the category of αβ MR1T cells can be divided into two main subdivisions: atypical MR1T cells and cancer-associated MR1Ts (**Figure 1.5**). Atypical MR1T cells were first discovered by using MR1-6-FP tetramers as well as MR1-5-OP-RU tetramers to survey the blood of healthy volunteers, and their existence within healthy individuals has been confirmed by other studies that have followed[66, 85, 86, 100, 101]. In these studies, it was observed that a small, but significant population of T cells that were TRAV1-2 negative bound to either the 5-OP-RU, 6-FP, or both tetramers. While some of this TRAV1-2 negative population was shown to have the same resting blood phenotype as canonical MAIT cells, the majority did not, and made use of their diverse TRAV and TRBV gene segments to recognize both MR1 loaded with 5-OP-RU, 6-FP, or both ligands[67, 86]. Interestingly, one study revealed that there were MR1Ts in the body, like the clone MAV21, that did not react to the canonical MAIT ligand 5-OP-RU at all, but instead were activated only by MR1 loaded with the pteridine antigen 6-FP[86]. Other studies have shown that a larger proportion of circulating T cells termed “MR1-autoreactive” (referred to as cross-reactive MAIT cells in this dissertation) that fit the definition of MAIT cells, both phenotypically and through TRAV1-2 gene usage, could bind to MR1 tetramers loaded with various antigens including the pteridines 6-FP and Ac-6-FP and could also react to APCs loaded with 6-FP or without any exogenously added ligands at all[78, 86]. Due to the questionable

physiological relevance of 6-FP and the fact there seemed to be a small, but conserved proportion of MAIT/MR1Ts in healthy people that reacted to MR1 loaded with this ligand, it implies the existence of other similar pteridine ligands that may be made by microbes or sourced completely endogenously.

At the same time that these atypical MR1T cells were being described, the phenomenon of cancer-reactive MR1T cells was an emerging area of study - first with the work outlined in Lepore et al 2017, and then highlighted again by Crowther et al in 2020[71, 72]. These MR1Ts also have access to a full repertoire of TCR gene rearrangements, but unlike most atypical MR1Ts and cross-reactive MAIT cells, they are agnostic to microbially derived MR1 ligands (**Figure 1.5**). The cancer-associated MR1Ts described to date also have access to a wide range of effector phenotypes and in this way, they approximate the appearance of conventional αβ T cells rather than innate-like MAIT cells. As described in **section 1.2.3**, these cells have been derived through the coincubation of PBMCs from healthy donors with irradiated MR1-overexpressing cancer lines – selecting for T cells that show signs of expansion and activation under these conditions. These cells are primarily CD8+, but do not all show a cytotoxic phenotype. Indeed, a few of the clones that were originally validated were able to exhibit helper T cell functions like inducing the maturation of DCs in an MR1 dependent manner[71]. That being said, this subgrouping of MR1Ts has mostly been studied in the context of their cytotoxic reactivity to cancerous cells, where many of them have been shown to be able to lyse a variety of cancer cell lines in an MR1-dependent manner[72]. Some of the MR1 ligands that activate these cells have been described (outlined in **section 1.2.3**) and it has been noted that these cells seem to show promiscuous reactivity to a variety of carbonyl-based ligands that are reportedly only formed in cancer cells[73, 74]. However, if we take anything from the history of ligand diversity for MR1,

it is likely that these are not the only ligands these cells can recognize. This hypothesis becomes even more probable when we recall that these cells were derived from healthy people, meaning that their biological function at homeostasis revolves around ligands that are endogenous to both healthy cells and malignant cells – perhaps acting as sensors for general metabolic distress at steady state and also for the reactive carbonyl species that only arise during neoplastic transformation.




| | | | | | |
|---|---|---|---|---|---|
| **TCR gene usage** | TRAV1-2/TRAJ33/12/20 *TRBV enriched for TRBV6 TRBV20-1 genes | TRAV1-2+ Variable TRBV | Variable TRAV Variable TRBV | Variable TRAV Variable TRBV | Mostly Vδ1 and Vδ3+ Variable TRGV |
| **MR1 Ligands Recognized** | 5-OP-RU and riboflavin intermediates (ribityl-lumazines) | Microbially derived ligands. Folate-derived pteridines Drug metabolites | Microbially derived ligands. Folate-derived pteridines **Unknown endogenous ligands** | Carbonyl oncometabolites **Ligand diversity largely unknown** | Direct MR1 recognition. **Capacity for direct antigen recognition is unknown** |
| **Resting Phenotype** | CD161+, CD26+, Tissue homing effector memory | CD161+, CD26+, Tissue Homing Effector Memory | Variable Some exhibit a conventional MAIT phenotype | Highly Variable | Tissue resident memory phenotype typically associated with Vδ1 γδT cells |
| **Examples in the literature** | G11 AF-7 | M33-64 AM3 BC75B38 | MAV21 MAV36 | MG.7.C5 DGB129 Tc5A87 | G7 G83.C4 |

**Figure 1.5** The family of MR1-Restricted T cells. Each MR1T cell subtype is shown, highlighting differences in TCR gene usage, effector phenotype, and the classes of MR1 ligands recognized by each. For clarity, the names of clones from the literature that are examples for each subtype are listed at the bottom.

### 1.3.3 γδ MR1T cells are a newly described MR1T cell subset that utilize γδ TCRs to display unique MR1 binding modes

As mentioned briefly in **section 1.1.2**, TCRs can come in two different flavors depending on the V, D, and J genes used to form them[12, 102]. Like αβ T cells, γδ T cells rearrange their TCR through recombination, where the γ chain of the TCR uses only V and J (analogous to the

TCRα chain) and the δ chain of the TCR uses V, D, and J segments (analogous to the TCRβ chain) with junctional diversity added at the CDR3 region for each chain[103]. While the γ and δ loci each have considerably less V and J gene segments than their α and β counterparts, paradoxically, γδ TCRs are capable of maintaining a higher theoretical diversity than αβ TCRs[104]. This expanded potential diversity manifests mainly in the TCRδ chain which is capable of including multiple D segments at the CDR3 junction allowing for extensive nucleotide additions to be made which results in the long CDR3 loops observed in most TCRδ chains[105]. Humans have eight functional TRDV genes, but three (Vδ1, Vδ2, and Vδ3) are the most commonly used. Due to the role that TCRδ and TCRγ chain selection plays in the thymic development of these cells and as innate-like T cells, γδ T cells leave the thymus with a mature effector phenotype, the Vδ genes in the TCR of these cells can be used to define γδ T cell subsets in humans.

Another major difference between γδ T cells and αβ T cells is the diversity of ligands they can recognize and the mode of which they accomplish this. γδ T cells can recognize their antigens directly and recognize a variety of protein antigens that are expressed during cell stress such as MIC-A, EPCR, the CD1 family, and the butyrophilin family of molecules[106]. While some of these antigens resemble MHC molecules, many do not, and some groups have even suggested that γδ TCRs are capable of binding soluble protein antigens and haptens (although the physiological relevance of these interactions are questionable)[107, 108]. This established paradigm of direct recognition of cell surface proteins and other antigens has led to the γδ TCR being labeled as “antibody-like” in its recognition. While this is certainly true in many contexts, γδ T cells have also displayed the capability to act like αβ TCRs by displaying incredibly antigen specific recognition within the context of an antigen presenting molecule. A prime example of

this behavior is displayed by the DP10.7 Vδ1 TCR that only recognizes CD1d when loaded with a specific lipid[106, 109]. As one could imagine with this large variety of binding modes at their disposal, the ligands that can be recognized by γδ T cells is an ever-growing family.

Recently, two related studies have highlighted examples of direct recognition of MR1 by γδ TCRs, yielding the first reports of γδ MR1Ts (**Figure 1.5**). In the first study, a panel of γδ T cells were discovered in the blood of healthy volunteers that bind to MR1 5-OP-RU tetramers[110]. The γδ T cells were primarily Vδ1 with ~25% bearing Vδ3 TCRs. Upon closer inspection of these TCRs, it was shown that the majority of them could be activated by MR1 displayed on APCs and that soluble forms these TCRs could bind to MR1 regardless of the ligand present. Astonishingly, a crystal structure of one of these TCRs (G7) in complex with MR1 showed that the TCR bound underneath the antigen binding domain, primarily targeting the membrane-proximal α3 domain of MR1. This, combined with the activation and binding data, led the authors to speculate that the γδ T cells that recognize MR1 do so in an "autoreactive" manner, as they do not sense the bound antigen directly simply that the MR1 complex is present. The follow up report focused on the same panel of γδ MR1T cells but provided a close look at the direct recognition of MR1 by one of the Vδ3 TCRs that was isolated[111]. Crystal structures of this TCR with 5-OP-RU loaded MR1 and 6-FP loaded MR1 revealed that this TCR bound in a more traditional way (targeting the α1 and α2 domains) although its binding footprint was shifted far past the antigen binding pocket of MR1 binding to the side of the molecule. While this binding mode was closer to the antigen binding domain than that of the G7 TCR, no contact with either 5-OP-RU or 6-FP was made by the TCR. This finding seemed to confirm the author's previous assertion that γδ T cells can only recognize MR1 in an "autoreactive" manner. To an extent, this claim makes sense with what we know about γδ TCR recognition. Afterall, γδ TCRs

have been shown to bind molecules that are only present on the surface of cells when something is wrong (MIC-A, EPCR, etc) and MR1 is generally not found on the surface of cells unless an antigen, microbially derived or otherwise, is present. However, as was the case with the DP10.7 TCR, it is certainly possible for γδ TCRs to react to the antigen as well as the antigen presenting molecule they are recognizing. The question remains: if this type of recognition is possible for the γδ T cells that are restricted to MR1 and if they can recognize MR1 antigens would there be a preference for one type over another?

## 1.4 The importance of identifying novel MR1 ligands and categorizing their effects on cognate T cell populations

### 1.4.1 The potential for immunotherapies that target MR1 and MR1-restricted T cells

In the past three decades both the scientific community and the population at large have become acutely aware of how transformative medicines that can harness or redirect the immune system can be. Monoclonal antibodies (mAbs), adoptive cell therapy, and gene transfer therapies, like TCR transfer and chimeric antigen receptors (CARs), have each foundationally changed the way we think about treating diseases like cancer and autoimmunity[112]. As stated at the beginning of this dissertation, the immune system plays a critical role in keeping the host organism at homeostasis, whether this is regulating the health of other cells or defending the host from “non-self” invaders[1]. It follows logically that in the instances when the immune system falls short of this goal, such as autoimmunity and cancer, therapies that can subtly correct this behavior can be especially transformative. However, a major challenge for designing immunotherapies that can effectively treat a disease and also still be effective for every individual in a population, is that each individual possesses a highly variable immune system

and, in diseases like cancer, a highly individualized disease[113]. For this reason, immunotherapies that target the classical TCR and MHC can be effective, but only for the narrow sliver of the population that share the targeted MHC allele[114, 115]. MR1 presents an attractive target for immunotherapy for several reasons, the chief among them is its near-monomorphic gene distribution in the human population (see **section 1.2.1**)[46, 49]. With the majority of the population sharing the same allele and only <1% bearing an allele that has actually been shown to impact the function and antigen repertoire of the molecule (MR1*04), any therapy that is designed to target MR1 would be broadly applicable to the entire human population (**Figure 1.6**)[49, 75, 116]. Due to the newly appreciated function of MR1 to present cancer-associated antigens to MR1T cells, the potential for a TCR transfer therapy using a TCR or a CAR construct that can distinguish between these antigens in the context of MR1 seems to be a straightforward and potentially transformative therapy to explore. The advantage of this type of approach is not limited to the broad applicability in the population, but due to the fact that MR1 is only stable on the cell surface when an antigen is present; the likelihood of the off-target effects that have plagued TCR-based and CAR therapies would be highly limited if not eliminated entirely depending on the degree of specificity for the cancer antigen and in the receptor used[117].

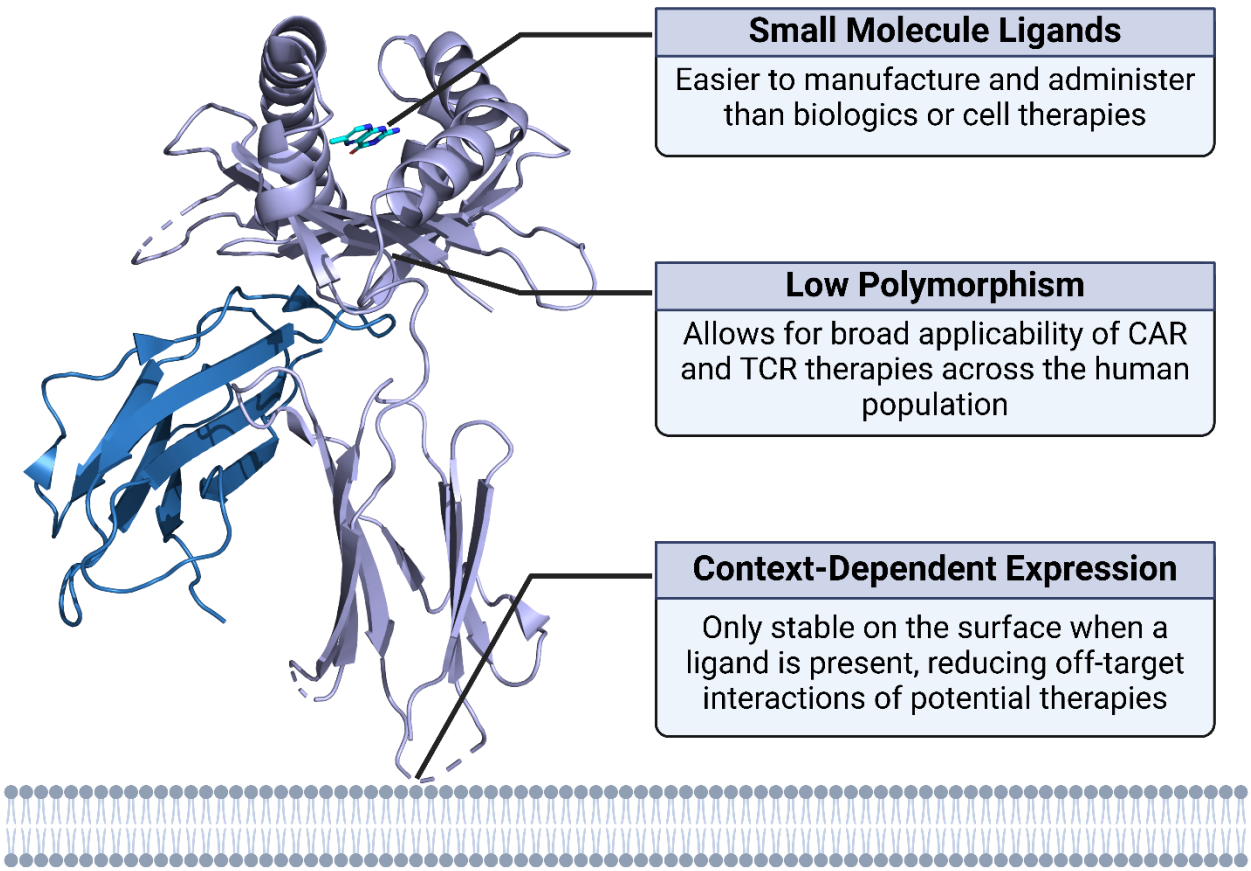



**Figure 1.6** Why MR1 is an attractive therapeutic target. Due to the low polymorphism, context-dependent expression, and its penchant for small molecule ligands, MR1 is a great potential target for immunotherapies.

Beyond using MR1 as a TCR or CAR target, the simple fact that MR1 presents small molecule antigens makes it an attractive target for more traditional therapies as well (**Figure 1.6**). Long considered the gold standard for medicines, small molecules are easier to produce in large quantities, easier to ship and store, and easier to administer than protein based and cell-based therapies[118]. One could imagine if an agonist MR1 ligand or cocktail of ligands were directed to sites of cancer or infection, MR1T cell responses could be enhanced allowing for the clearance of the malignancy. This approach could be particularly effective to upregulate MR1 on the surface of cancer cells that may have escaped traditional αβ T cell surveillance by the down regulation of MHC-I[119]. Certainly, one could imagine a combination therapy of an MR1 targeting TCR therapy administered with ligand-mediated MR1 upregulation within the tumor

being a particularly powerful way to stimulate an anti-cancer response. While maintaining many of the qualities as its related MHC-I cousin, the unique qualities of MR1 make it an exciting target for a wide range of diseases, providing more, and potentially safer, options for the patients that need them.

### 1.4.2 The discovery of novel MR1 ligands can be leveraged to further our basic understanding of the immune system

The immune system is the most ubiquitous, versatile, and intricate organ system in the body as it is becoming appreciated how many roles outside of pathogen defense the immune system plays. Immune cells have now been implicated in regulating many essential functions in the body such as wound healing, pain signaling, neuronal control, fat metabolism, cardiovascular health, and even the fetal development of organ structures[120]. Due to the critical importance of this system for our persistence as a species, it is equally critical to study and understand it. While MR1, the ligands it presents, and the cells that respond to it represent a small portion of the total immune system, the discovery of novel ligands, cells, and forms of recognition help move us forward in our understanding of fundamental biological processes.

As the existence of endogenous MR1 ligands has long been held as a possibility to answer major outstanding questions in MR1 and MR1T cell biology, the identification of MR1 ligands fitting this description goes beyond implications for cancer and cancer therapies[52, 121]. The discovery of truly endogenously sourced MR1 ligands could help answer foundational questions about the thymic development of MAIT/MR1T cells, their maintenance in the periphery, and their potential roles in tissue homeostasis outside of cytotoxic responses to infected or neoplastic cells. As discussed in **section 1.2** and **section 1.3**, MAIT cells persist in the

thymus of germ-free mice and, while there is some evidence that the selection of these cells involves microbial ligands exported to the thymus, it is insufficient to explain the selection of all MAIT cells and their presence in fetal tissues[80, 122]. Unconventional MR1T cells on the other hand have a completely unknown developmental trajectory. MR1Ts have diverse phenotypes and TCR gene usage that is reminiscent of conventional αβ T cells yet react only to MR1 [71, 72]. Does this mean that they develop the same way as conventional T cells, educated on MHC-I in the thymus and then "cross-react" with MR1 in the periphery? Or do they follow the same murky development trajectory as human MAIT cells? Beyond their development, all known MR1T cells that are cancer-reactive were derived from the blood of healthy individuals. As it is unlikely that these cells exist solely to deal with the eventual, and in some cases, hypothetical, neoplastic transformation of healthy cells in the body, there must be some role they play in the normal functioning of their hosts that they are maintained for. Since it has been shown extensively that these cells are agnostic to microbially derived ligands presented in MR1[71, 72], it would follow that their maintenance and function is centered around endogenous ligands that would be present during health and disease. The discovery of these ligands would then allow for further experimentation to answer the questions surrounding the origin, development, and purpose of MR1T cells. A better understanding of the basic biology of these cells can only allow for better therapies that leverage their use.

It is also crucial to develop our understanding of the existing ligands that MR1 presents and probe them for roles not yet appreciated. As discussed in **section 1.2.2**, MR1 presents a vast and ever-growing repertoire of microbially derived ligands, the majority of which interact directly with MAIT cells[99]. However, despite their semi-invariant receptors, it has been shown that MAIT cells can distinguish between these ligands, as different MAIT clones can show

preference for one ligand over another[68, 123]. While this selectivity has been explored from the perspective of the TCR, beta chain usage being the prime suspect for this behavior, it has yet to be fully appreciated how much the ligand within MR1 "selects" the cells it interacts with or if each ligand is capable of eliciting a different response from different cells[66, 85]. As was appreciated in **section 1.3.1**, MAIT cells are capable of a wide breadth of potential effector functions – is it possible that different ligands can be interpreted by the TCR to influence the appropriate response to a given situation? Or beyond this, what would the implication be if it were shown that a different class of T cell altogether were to recognize only very specific microbially-derived MR1 antigens? Only broad, unbiased approaches would be able to identify these types of "unconventional" interactions and only ligand-focused immunological and structural studies would be able to reveal what this selectivity or promiscuity means to the immune system as a whole. As our understanding of the world around us has always hinged on minute incremental progress made with great effort, the answers to these seemingly narrow questions could lead to major breakthroughs that benefit human health for years to come.

### 1.4.3 How the work presented here makes progress toward answering these translational and foundational questions

I hope at this point I have impressed upon the reader that there is a need for us to better understand MR1 and its ability to capture and present ligands that are sourced from the native metabolome. The identification of these ligands can lead us to a better understanding of not only MR1 biology, but also of cancer and T cell biology in general. In **Chapter 2** I will discuss how I was able to leverage a novel mass spectrometry (MS) based pipeline to describe new ligands for MR1 in the context of neoplastic disease, namely multiple myeloma. Identified among the many

other potential MR1 ligands were endogenous pteridine (EP) molecules. These molecules were validated to be true ligands for MR1 in a series of different assays, were found to originate naturally from multiple myeloma cell lines, and were shown to stabilize MR1 on their surface at steady state. The discovery of EP MR1 ligands puts us one step closer to a more holistic understanding of MR1 as an antigen presenting molecule, but also answers one of the oldest questions about MR1 in its capacity to bind and present molecules that are derived from a purely native source.

As outlined in **section 1.3**, the major impetus to search for and identify these endogenous ligands was the initial discovery of T cells that react to tumor cells in an MR1 dependent manner. Therefore, it was critical that I survey the breadth of the MR1T family to identify TCRs that bind and are activated by these ligands. In **Chapter 3**, I utilize MR1T TCR expression in the T cell lymphoma line JRT-T3.5 and human MR1 (hMR1) tetramers loaded with known and novel ligands to address which MR1T cells recognize EP ligands. Through these experiments, I show that cancer associated MR1Ts and the "atypical" MAIT/MR1Ts that have been shown to react to 6-FP (a non-endogenous pteridine) both recognize and are activated by MR1 loaded with EP ligands. These findings have major implications for the function of these cells both in cancer and at steady state within healthy individuals. It remains to be seen if these ligands play a role in the selection of these cells or their maintenance in the periphery, but these results are promising first steps for answering those questions as well. Additionally, through the help of Dr. Jen Cooperrider at the University of Chicago Medical Center, I present detailed information on the presence and phenotype of EP-MR1 reactive cells in the circulation of healthy individuals and myeloma patients.

Finally, **Chapter 4** dives into new functions of previously discovered microbial ligands for MR1. As discussed in **section 1.3.3**, a newly discovered member of the MR1T family, γδ MR1T cells, have been described as MR1 "autoreactive" due to their strange antigen agnostic binding modes. Here I describe the discovery of a novel panel of γδ MR1T cells and the surprising discovery that a member of this panel, termed GDM95, exhibits marked antigen selectivity in the context of MR1. PLIII, a ligand described previously by our lab, is found primarily in MR1 samples made in the presence of mycobacteria[68]. Interestingly, the GDM95 T cell clone was recovered from a single cell RNA sequencing (scRNA-seq) data set that was generated with barcoded CD1 and MR1 tetramers used to stain T cells recovered from human lung tissue. This is significant as all of the other γδ MR1T cells that have been studied in depth were from the blood of healthy volunteers, a compartment where Vδ1 T cells are not thought to carry out their main effector functions[102]. The discovery that the GDM95 TCR only binds to hMR1-PL3 tetramers and is activated by APCs loaded with PLIII is also the first instance of a γδ MR1T cell that recognizes MR1 in a "T cell-like" antigen-dependent manner. These findings are significant not only for the novelty of what is being described, but because it presents the possibility that γδ MR1T cells may be playing a larger role in host barrier defense against microbes rather than just recognizing MR1 as another stress related ligand.

With all of the work that is presented here, I hope to impress the reader with one key concept after reading this dissertation: MR1 is an incredibly versatile and absolutely fascinating molecule. The incremental steps forward in our understanding of MR1 that I present in this thesis signify how vast of an impact a single protein can have with wide reaching implications for human health, disease, and development.

# Chapter 2. Metabolomic Analysis of Myeloma-Sourced MR1 Reveals a Novel Class of Endogenous Pteridine MR1 Ligands

## 2.1 Introduction

As discussed in **Chapter 1**, MR1 is a ubiquitously expressed antigen presenting molecule that has very little polymorphism in the human population and across mammals[49, 50]. Initially studied in the context of host-pathogen interactions, MR1 has been shown to capture and present a myriad of small molecule metabolites the majority of which are derived from riboflavin biosynthesis, a process which occurs in various microbes[63]. When bound and presented by MR1, these biosynthetic intermediates are strong activators of the innate-like MAIT cells that patrol the mucosa[50, 56, 64]. Recently, there have been reports of other MR1 restricted T cell clones, MR1Ts, that use a polyclonal TCR repertoire and are phenotypically heterogenous (see **section 1.3.2**)[71, 72, 86, 101]. Interestingly, a subset of these MR1Ts have been shown to exhibit MR1-dependent cytotoxic activity against cancerous tissues in the complete absence of any microbial infection or microbial ligands[71, 72]. This discovery sparked broad interest in defining what ligands could be captured and presented by MR1 in the context of neoplastic disease.

When first considering what type of MR1 ligand would allow for MR1T cells to discern between cancerous and non-cancerous tissue, I considered two main hypotheses (**Figure 2.1**). First, it is possible that the metabolic environment of cancer cells is altered from homeostasis to the point where reactive intermediates, such as reactive oxygen species (ROS), accumulate, leading to spontaneous reactions between metabolites and the generation of “oncometabolites” that bind MR1[124]. Alternatively, it is possible that the metabolic dysregulation in cancer cells and the inflammatory tumor microenvironment drives the aggregation of certain unmodified

endogenous metabolites that, at a heightened concentration, are able to bind MR1[125]. The first of these hypotheses has been addressed in a series of recent publications that outline how carbonyl stress in cancer cells can lead to the formation of nucleobase adducts which bind MR1 and activate cancer-associated MR1T cells[73, 74]. However, these two hypotheses are not mutually exclusive. The discovery of nucleobase adduct ligands for MR1 does not preclude the existence of other, unmodified metabolite antigens; further, these putative endogenously sourced ligands may contribute to interactions with the same, and possibly additional, MR1T cells. Taking this into account, I set out to address if unmodified endogenous metabolites play a role in the MR1T-cancer axis and if these same MR1 ligand metabolites have broader roles in MR1T biology outside the context of neoplastic disease.

To achieve this, I used multiple myeloma cell lines as a model system to search for novel endogenous MR1 antigens. Multiple myeloma cell lines have been shown in the past to have high mRNA and protein surface expression of MR1, which may indicate the enhanced presence of endogenous MR1-stabilizing ligands[126]. I therefore used several orthogonal approaches to generate recombinant MR1 loaded with myeloma ligands and utilized untargeted metabolomics to survey known human metabolites that may be binding to MR1 in the context of this disease. Analysis of these potential ligands for molecular features known to be present in MR1 ligands revealed the presence of multiple ions annotated as isomers of pteridine metabolites. The majority of these endogenous pterin (EP) metabolites are known intermediates in tetrahydrobiopterin (BH4) biosynthesis, an essential metabolic pathway that has been documented to be dysregulated in various cancers including multiple myeloma[127-134].

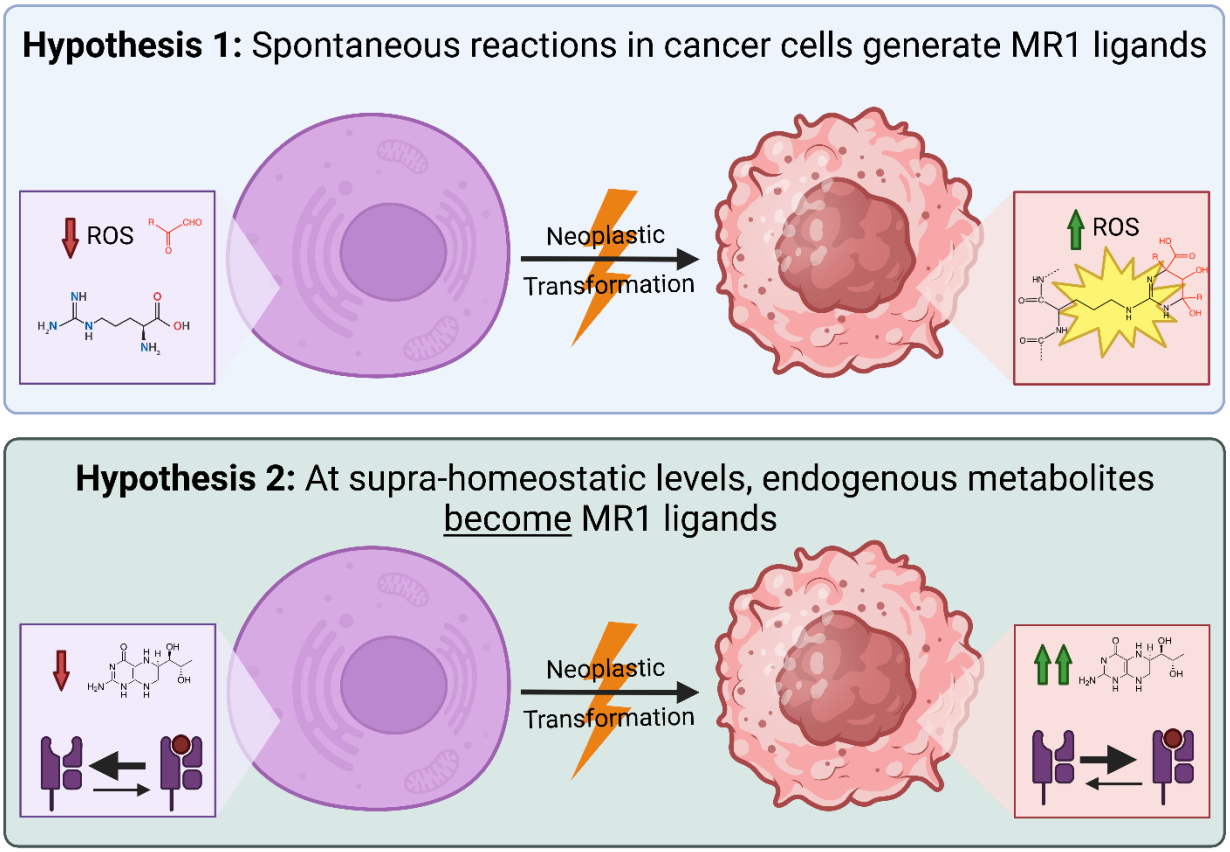



**Figure 2.1** Possible sources of a cancer-specific MR1 ligand. Hypothesis 1 (top) proposes that a cancer specific MR1 ligand may originate via spontaneous reactions that can only occur in the cytosol of a cancerous cell. Hypothesis 2 (bottom) proposes that the buildup of normally well controlled endogenous metabolites in cancer cells (a well-documented phenomenon) could lead to a concentration dependent interaction with MR1 - effectively making a weak binding molecule at steady state into a strong binder when found at heightened concentrations within a dysregulated cell.

A panel of these molecules and other related EPs was assembled, and I was able to show that five of the nine EPs tested had the ability to bind to MR1 in cell surface stability assays. These same MR1 ligands blocked the loading of 5-OP-RU and the subsequent activation of MAIT TCR Jurkats, indicating that they can compete with microbial ligands for the same MR1 binding pocket. Additionally, I was able to show that augmentation of the BH4 biosynthesis pathway, the source of these EP ligands, had a direct and specific impact on the MR1 surface expression of various myeloma and EBV-transformed lymphoblastic B cell lines (BLCLs). Taken together, these data show that EPs are found to be enriched in MR1 samples generated

with myeloma cell lines, are validated to bind to MR1, and comprise an endogenous pool of MR1 ligands at steady state in multiple myeloma cell lines.

## 2.2 The development of novel MR1-based tools for surveying the ligandome of myeloma cell lines

When starting my investigation into the possible myeloma-associated antigens that MR1 could be capturing and presenting on the surface of myeloma cell lines, I spent a considerable amount of time identifying the best ways to produce MR1 that would be preferentially loaded with these types of antigens. Recent advances in our understanding of intracellular MR1 trafficking have described its path through the ER, Golgi, cell surface, and endosome, and have revealed that the majority of MR1 loading likely occurs in ER[135-140]. It is well known that partially folded MR1 is retained in the ER and that ligand loading, which results in the covalent or non-covalent neutralization of the charge on K43, is crucial for it to continue to the surface[136, 141]. However, some evidence has shown that a small percentage of MR1 can make it to the surface without a ligand and can be recycled to the endosome[142]. This has led to the claim that ligand loading, or exchange, could happen in these areas as well (in a fashion analogous to MHC-II)[135, 137]. Although, it is worth mentioning that this assertion has been challenged by more recent studies[141, 143]. When considering the proposed endogenous origin of these antigens, it seemed most likely that loading was occurring within the cell where these ligands originate and where MR1 was being retained in the ER. Therefore, the first approach to generating MR1 loaded with myeloma antigens was to engineer a system where a stable, soluble source of MR1 (human platform MR1 or hpMR1 (**Figure 2.2 A**)[68]) could be produced within the myeloma cells themselves and then purified from the supernatant.

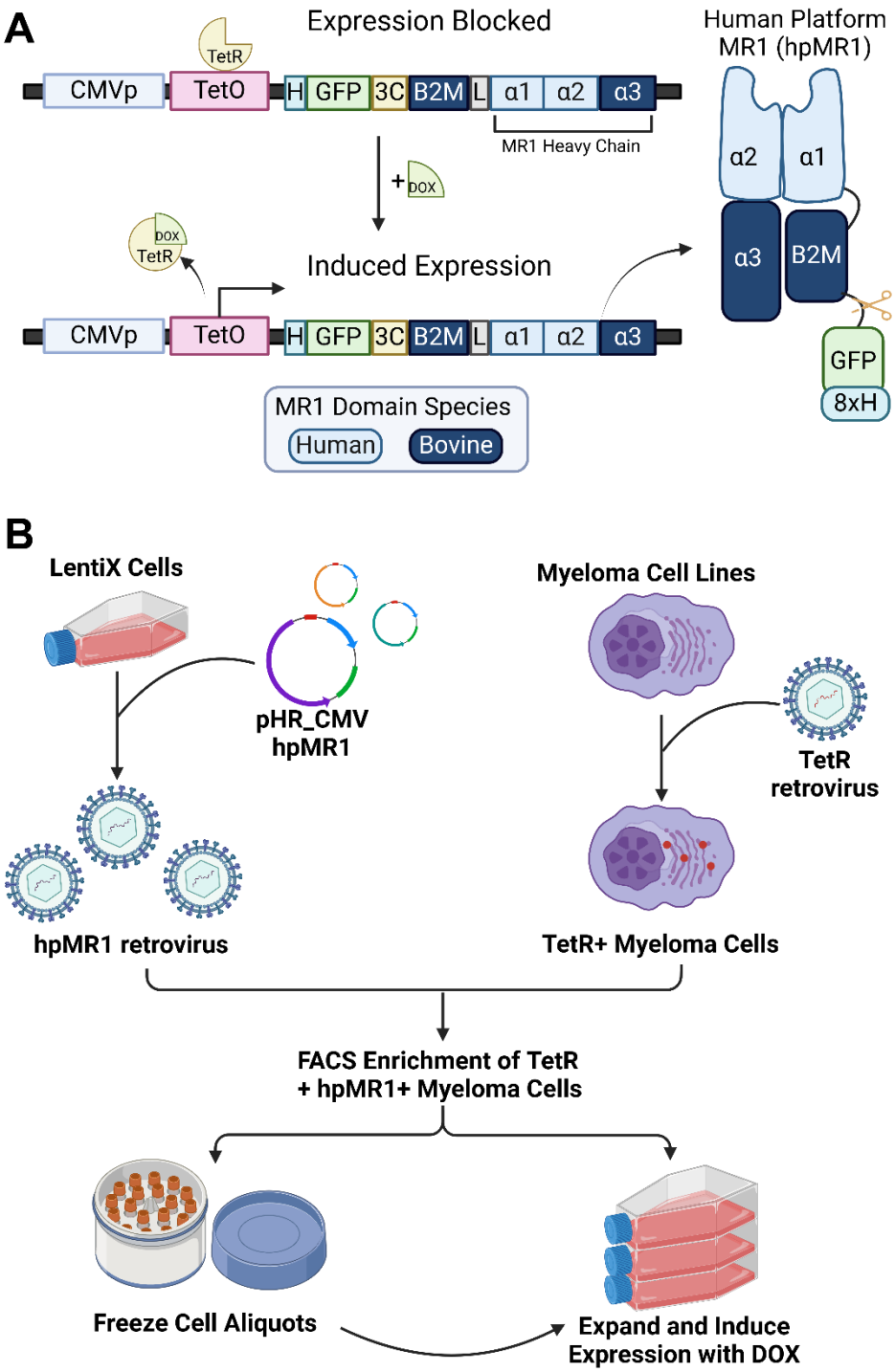



**Figure 2.2** The design of an inducible MR1 expression system for multiple myeloma cell lines. A) An overview of the TetR system and its regulation at the DNA level. The TetR protein is co-expressed within the cell and blocks efficient transcription of the hpMR1 construct until the addition of doxycycline (DOX). The hpMR1 construct is made up of a single polypeptide chain encoding an 8x histidine tag (8xH), GFP, a 3C cleavable linker, bovine β2M, a flexible GS linker, and an MR1 heavy chain comprised of bovine α3 and a human antigen binding domain (α1 and α2). B) The stable integration of both the expression plasmid and the TetR expressing plasmid allows for the streamlined production, storage, and expansion of hpMR1 secreting myeloma cell lines.

As has been discussed in the literature and has been experienced firsthand through my own testing of various systems, myeloma cell lines are particularly hard to transfect through chemical and non-lenti viral means. This led me to investigate the application of an inducible retroviral system to the production of hpMR1 in these myeloma cell lines[144]. This system relies on a lentiviral vector to generate cell lines that stably express the tetracycline repressor (TetR) as well as the given protein expression construct. The expression construct is engineered to have a TetR operator sequence (tetO) between the promoter and protein coding sequence (**Figure 2.2 A**). This approach yielded two major benefits for this project. First, the use of lentivirus allowed for high efficiency transfection of the myeloma cell lines that had previously been very difficult to transfect. Second, engineering stable cells in this way allows for easy expansion of the line without incurring epigenetic silencing of the overexpressed construct operon. The cells could be expanded under normal growth conditions and then protein expression can be induced all at once through the addition of doxycycline to the growth medium, giving higher yields and easily reproducible expressions (**Figure 2.2 B**). Additionally, the hpMR1 protein produced in this method would traverse the majority of the MR1 trafficking pathway and would be exposed to any potential endogenous antigens at the concentrations that are native to these myeloma cell lines.

To control for the possibility of antigen loading outside of the endocytic pathway or any effect that may be caused by using an engineered and overexpressed hpMR1 construct, I devised an orthogonal approach for generating fully human MR1 (hMR1) loaded with myeloma antigens. This approach, termed refold capture, relies on the hMR1 polypeptide to capture stabilizing ligands that are present in the refold mixture during refolding while in the presence of refolded human $\beta_2$M. In theory, ligands that can bind the MR1 pocket will stabilize the formation of

properly folded hMR1, increasing the yield of this species as the unfolded aggregated protein is removed during purification. Indeed, this approach has a precedent in the literature as it was how the first ligand for MR1, the pteridine 6-FP, was discovered to bind MR1 as it was refolded in a background of RPMI media[56]. By performing refolds of hMR1 in the presence of metabolite-extracts from myeloma cell lines, I was able to generate fully human MR1 protein that had the opportunity to capture any potential endogenous ligands present in the sample. This approach has the additional benefit of allowing us to survey not only the ligands captured during refold, but for the enrichment of any molecules captured from the background lysate, as the lysate itself was also submitted as a matched sample for analysis.

### 2.3 Analysis of a wide range of MR1-myeloma samples reveals the enrichment of endogenous pteridine molecules

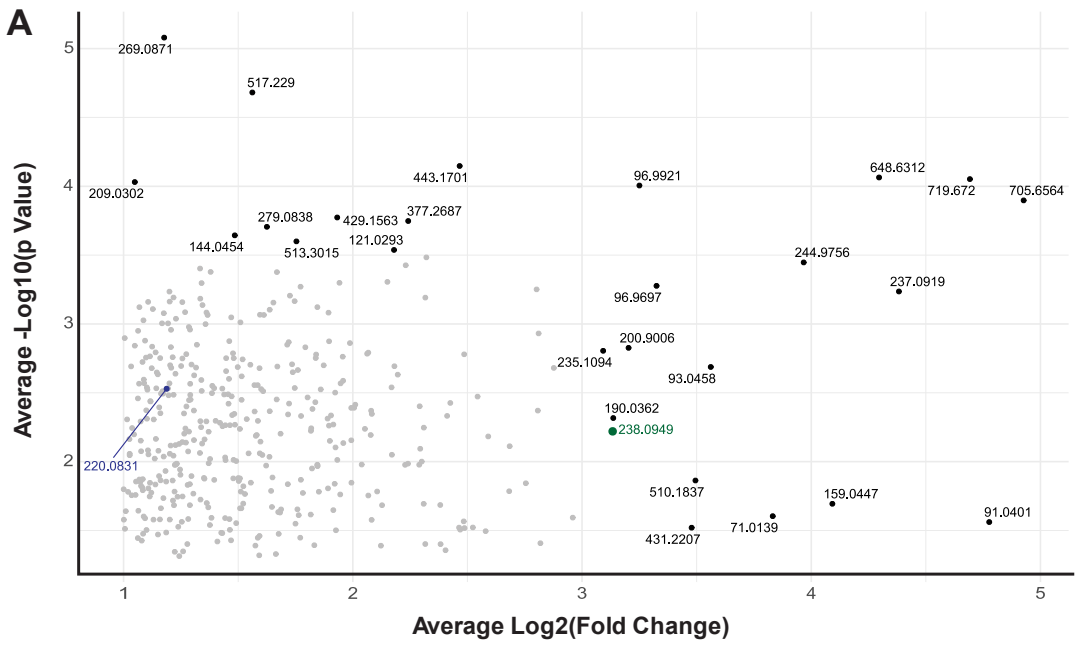
Using the protein production techniques described above, an experiment to survey these MR1 species for myeloma associated ligands was developed (**Supplemental Fig. S2.1 A**). The experiment was designed to have three groups based on the source of the samples and the type of downstream data analysis. First, the recombinant expression (RE) group included hpMR1 made in the myeloma cell lines L363 and U266, which was compared directly against hpMR1 and T22 generated in the BLCL cell line JY. Second, the refold capture (RFc) group included hMR1 refolded in the presence of myeloma or mock metabolite extractions and was compared directly against hMR1-JY and mock lysate refold (UnL) samples. Each metabolite extraction sample was resuspended to a concentration of 4mg/mL in HPLC grade water before being added to its corresponding refold. Finally, the refold enrichment (RFe) group was designed to find ligands enriched in the refolded protein samples as compared to their raw lysate backgrounds

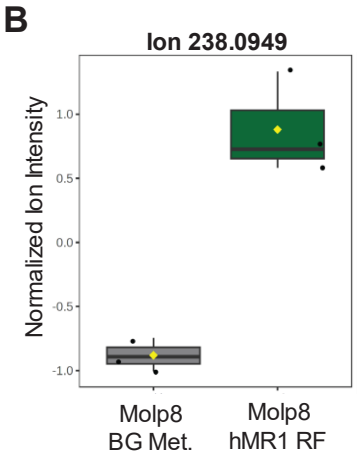
(**Supplemental Fig. S2.1 B**) through the normalized comparison of ion intensity between these samples. 10uL of the same metabolite extract used in the RFc group was taken, diluted, and analyzed as the corresponding metabolite background sample for each RFe comparison. For both the RE and RFc groups, the MR1 protein concentrations were normalized to be 0.5mg/mL prior to ligand extraction with 70% EtOH. Samples were prepared in triplicate (separated by batches) and immediately frozen after extraction. All samples were stored at -80°C until all samples were prepared for submission (time from experiment start to final extraction was 35 days). All samples were submitted for ligand identification via an untargeted MS metabolomics approach which analyzed the samples as well as internal standards in negative mode via direct injection[145]. 5uL of each sample were injected twice, consecutively, within 1 minute to serve as technical replicates and mass spectra between 50 and 1,000 m/z were recorded. The resulting observed ions were matched against HMDB, KEGG and CHEBI metabolite databases, resulting in 1,199 annotated ions with chemical formulae within a 1 mDa annotation threshold of their top match.

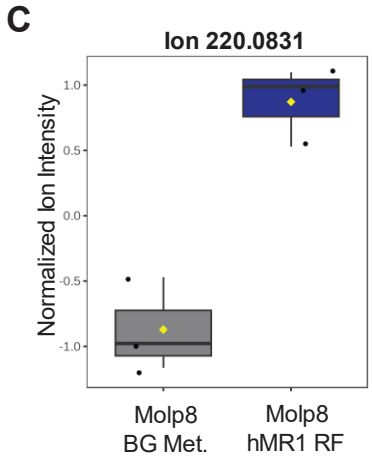
Pairwise analysis of the ions found in the myeloma samples to their corresponding control conditions afforded 19 volcano plots showing 418 ions that were upregulated in at least one comparison (**Supplemental Fig. S2.2 A**). 12 of these comparisons were from the RFc and RE data groups, where the normalized contents of the myeloma samples were compared directly to either the RF-JY/UnL or the RE-JY/T22 samples respectively. The remaining 7 RFe sample comparisons were carried out by first standardizing each ion observed in the refold and background metabolite samples as a percent of the total ion intensity. Next, the significantly enriched ions found between either the mock extraction or JY RFe comparisons were removed from the myeloma samples. Finally, the ions that were enriched in the refold as compared to the

corresponding background ions were found via pairwise comparisons. All comparisons were performed using the metaboanalystR package, where samples in the same group were first normalized to a gaussian distribution prior to an FDR corrected unpaired t-test and fold change analysis of each shared ion.

Of the 418 significant myeloma ions, those with either an average $\log_{10}$(p-value) of <-3.5 or a $\log_2$(FC) > 3 were checked by hand for features that resemble known MR1 ligands (**Figure 2.3 A**). Ions 238.0949 was found to be enriched in both the Molp8 and U266 RFe samples, while ion 220.0831 was found to be significantly enriched in only the Molp8 RFe samples (**Supplemental Figure 2.2 B-D**). Both ions were highly enriched in the refold over the matched metabolite extraction background samples, indicating that the presence of these ligands in the lysate may have stabilized hMR1 during refolding (**Figure 2.3 B and C**). These ions were annotated to be isomers of various EP molecules, which was of great interest to us as one of the first ligands discovered for MR1, 6-FP, is a pteridine molecule with similar molecular features to the isomers attributed to these ions (**Figure 2.3 D**)[56]. This finding prompted us to prioritize the validation of these molecules and to ascertain if they play a known role in the pathogenesis of cancer. Further research into the origin of these compounds revealed that they are all intermediates in the BH4 biosynthetic pathway, an essential metabolic pathway for both healthy and cancerous cells.




D

| Ion m/z | Potential Annotations |
|---|---|
| **220.0831** | 2'-deoxysepiapterin |
| **238.0949** | 7,8-dihydrobiopterin |
| **238.0949** | 1-hydroxy-2-oxopropyl tetrahydropterin |
| **238.0949** | 6-lactoyl-5,6,7,8-tetrahydropterin |
| **238.0949** | quinoid dihydrobiopterin |
| **238.0949** | O2"-4a-cyclic-tetrahydrobiopterin |

**Figure 2.3** The identification of endogenous pteridine ion annotations. A) Plotting the average significance and fold change of each ion that was found to be enriched in at least one set of myeloma over control samples allowed for the discovery of ion 238.0949 and 220.0831. Ion 238.0949 (B) and 220.0831 (C) were found to be significantly enriched in the Molp8 RFe samples. D) The suggested annotations for these ions were endogenous pteridine molecules.

## 2.4 Endogenous pteridine molecules are intermediates of the tetrahydrobiopterin biosynthesis pathway and are implicated in cancer progression

BH4 is an essential cofactor for monooxygenase enzymes, playing an important role in phenylalanine and tryptophan metabolism, as well as acting as a cofactor for nitric oxide synthase (NOS) in the production of nitric oxide (NO)[146]. Along with its role as an obligate cofactor for monooxygenase enzymes, cofactor-independent roles such as reactive oxygen species (ROS) scavenging, mitochondrial respiration, and cellular protection from ferroptosis exist for BH4[132, 147]. Accordingly, the increased production of the pteridine intermediates made during BH4 biosynthesis has been observed during immune activation and, interestingly, these molecules are also found at heightened concentrations in the serum and urine of cancer patients, including those with multiple myeloma[131, 148-151]. The production of BH4 is governed by three major pathways: the *de novo* pathway, which requires guanosine triphosphate (GTP) as a primary substrate; the salvage pathway, which converts the product of the spontaneous degradation of BH4 precursors back into BH4; and the recycling pathway, which recycles spent BH4 in the form of pterin-4α-carbinolamine back into BH4 through the intermediate quinonoid dihydrobiopterin (q-BH2)(**Figure 2.4**)[152, 153]. While the salvage and recycling pathways are vital for maintaining cellular BH4 levels, the vast majority of BH4 made in a cell comes from the *de novo* pathway[146]. The first step in this pathway - which is also the rate limiting step - is governed by the enzyme GTP cyclohydrolase 1 (abbreviated GCH1 or GTPCH1 interchangeably in the literature)[147].

Interestingly, the expression of GCH1 is partially controlled by nuclear factor kappa-B (NF-κB) and Stat1/Stat3 signaling, meaning that inflammatory signals like IFN-γ, TNF-α, and IL-1β - which are present during the immune response to infection or cancer - increase the rate of

GCH1 transcription and subsequently the rate of BH4 production[154, 155]. In fact, recent work has revealed that BH4 biosynthesis plays a critical role in the T cell-mediated immune response to cancer, perhaps providing an explanation for the connection between this pathway and inflammatory mediators[156]. However, the role of BH4 and its intermediate metabolites in cancer progression and control is not clear cut, as many pro-tumoral effects of BH4 and intermediate EP molecules have been described in the literature as well. It is well understood that the direct role of BH4 in NO production leads to enhanced angiogenesis at tumor sites, but more recently, the ability of BH4 to protect against ferroptosis has been shown to aid in cancer cell evasion of programed cell death and, in a mouse model of multiple myeloma, BH4 was also shown to promote proliferation and tumor growth *in vivo*[127, 132, 157-160]. Beyond these examples, it has also been shown that various cancers can gain an evolutionary survival advantage by enhancing BH4 metabolism. Activated KRAS in lung carcinomas can drive abnormal BH4 production, which provides a GCH1-dependent survival advantage; mutations in the BH4 recycling pathway have been directly linked to enhanced progression of PDAC tumors; and, the dysregulation of GCH1 can contribute to enhanced kynurenine metabolism - subsequently limiting the amount of available tryptophan for T cells within the TME[128, 130, 161, 162].

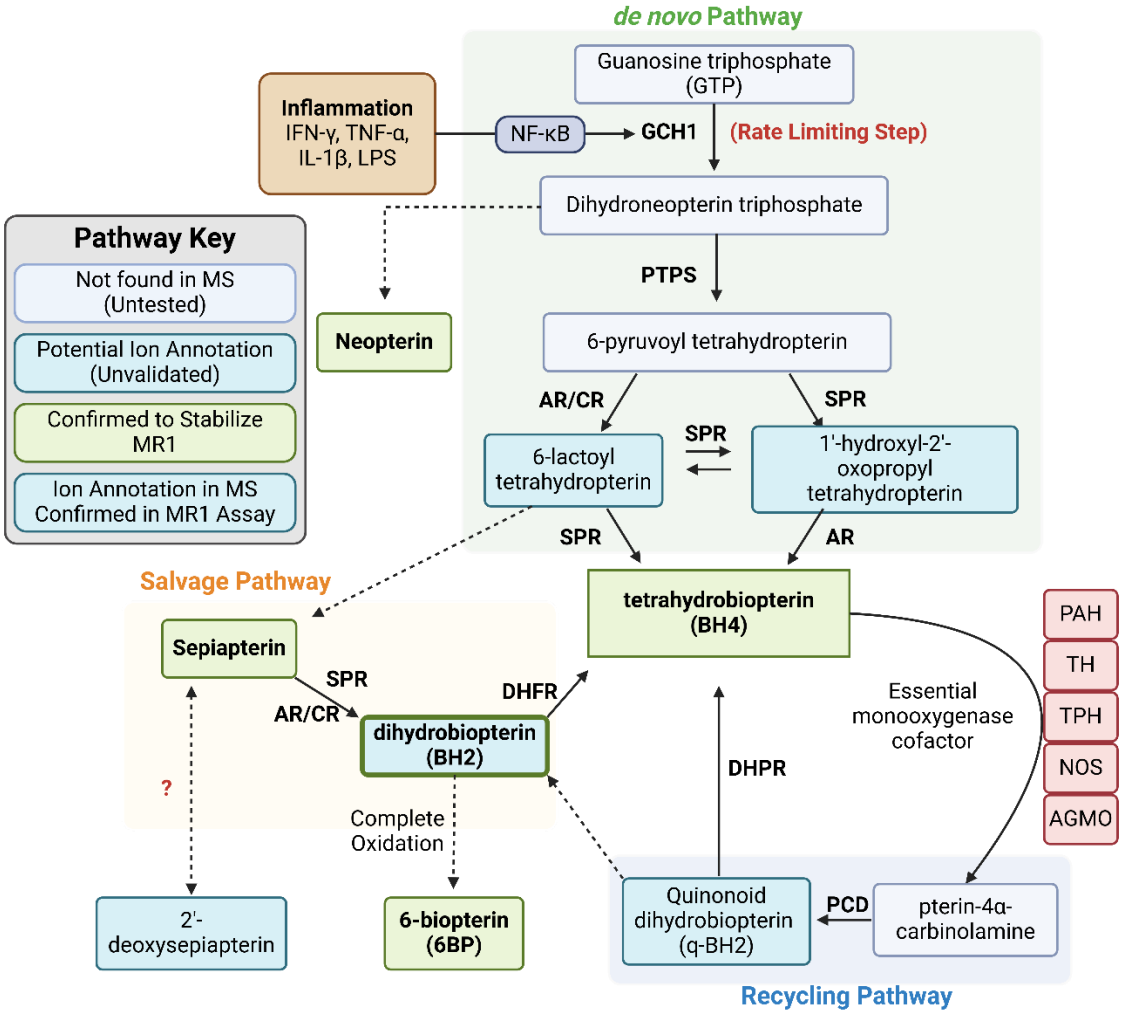



**Figure 2.4** The tetrahydrobiopterin biosynthesis pathway. BH4 is generated through three distinct pathways the *de novo* pathway, the salvage pathway, and the recycling pathway. The pteridine intermediates generated during this pathway are colored by their representation in our MS dataset, our EP panel, and their validation status.

While integrating the findings outlined in these studies paints a complicated picture for the role of BH4 metabolism in the progression and control of cancer[163], for our purposes, it was clear the EP molecules identified in our MS experiment belong to a pathway that is often upregulated or associated with cancer progression and can be leveraged for cancer survival and immune evasion (**Supplemental Figure S2.3**). This, combined with the long-established observation that these molecules build up within tumor cells, the TME, and systemically as a whole, lent credence to our hypothesis that endogenous metabolites upregulated to supra-

homeostatic levels during neoplastic disease can become MR1 ligands[130, 131, 151]. Therefore, it became a high priority to thoroughly investigate EP molecules as cancer-associated endogenous MR1 ligands through multiple avenues of validation.

### 2.5 Neopterin, 6-biopterin, dihydrobiopterin, tetrahydrobiopterin, and sepiapterin are ligands for MR1

The similarity of the EP molecules identified in our MS analysis to known MR1 ligands and the reports of heightened abundance of these molecules in cancer patients, motivated us to procure a panel of pteridine compounds that are either direct intermediates of the BH4 biosynthesis pathway or related to known intermediates (**Supplemental Figure S2.4 A**). This list of EP molecules included 7,8-dihydrobiopterin (BH2), which was one of the possible annotations of ion 238.949. These compounds were first screened at a wide range of concentrations (1uM – 1mM) for any ability to stabilize MR1 on the surface of MR1 overexpressing JY cells (JY.MR1). From this screen, it was observed that five of the nine compounds – BH2, BH4, sepiapterin, neopterin, and 6-biopterin (6BP) - were able to increase the amount of MR1 on the cell surface as shown by the increased fold change in the MFI of an MR1-APC antibody (**Supplemental Figure S2.4 B**). Follow-up investigation on these compounds revealed that the effect these molecules had on MR1 surface stabilization was reproducible and dose-dependent when performed at a narrower range of concentrations (**Figure 2.5 A**). It should be noted that while the concentrations needed for response in this assay are relatively high as compared to covalently bound MR1 ligands like 6-FP, they are in agreement with the concentrations used in similar assays for other recently identified non-covalently bound MR1 ligands[79]. The results of this screen were surprising to us, highlighting that many EP molecules related to BH4 biosynthesis

seemed to stabilize MR1 on the cell surface. It was important to validate these findings further to ensure that this effect was specific to a direct interaction between these molecules and MR1.

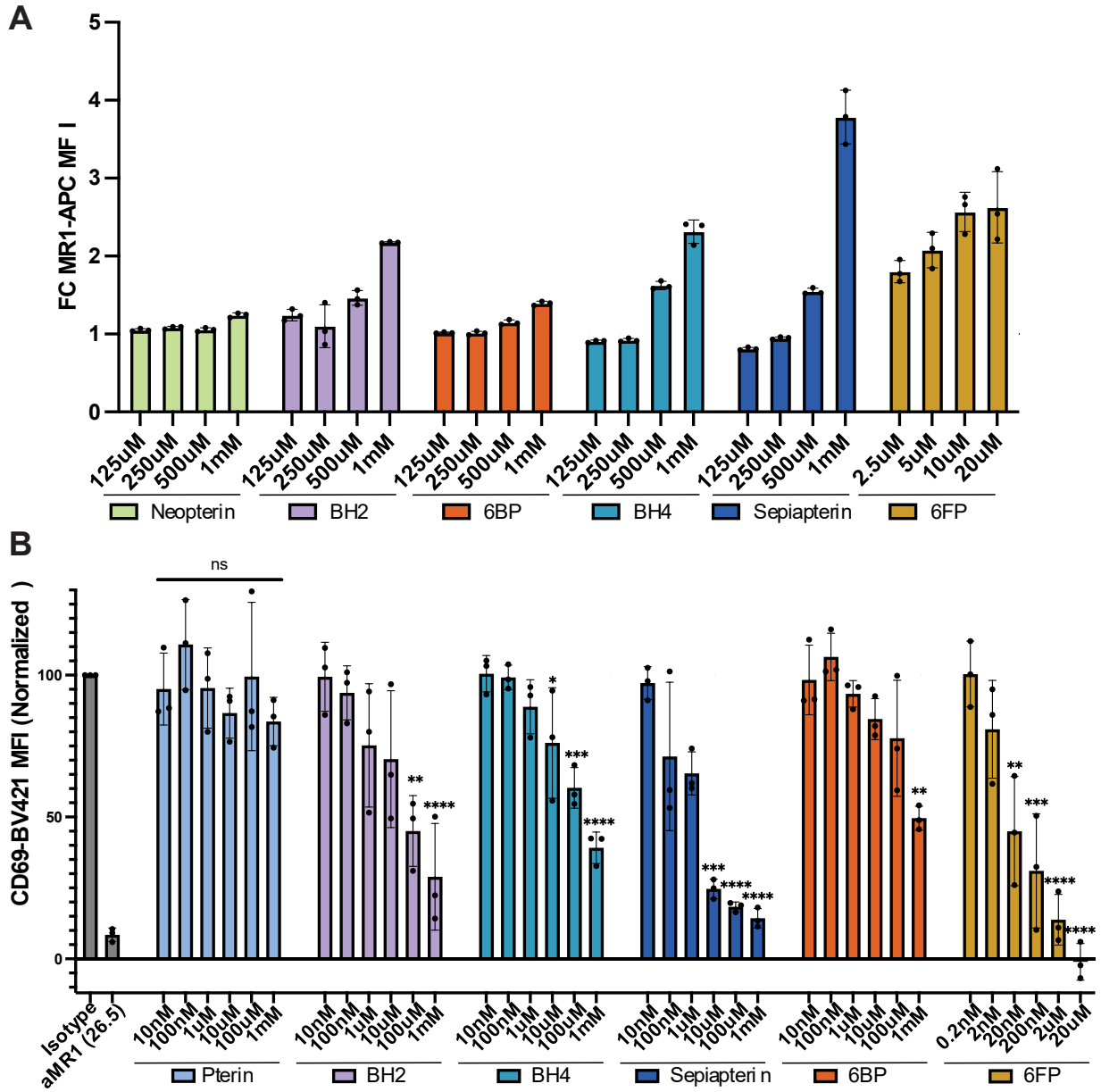



**Figure 2.5** Endogenous pteridine molecules are ligands for MR1. A) The coincubation of JY.MR1 cells with varying concentrations of Neopterin, BH2, BH4, 6BP, and sepiapterin for 16 hours results in the cell surface stabilization of MR1. B) The 5-OP-RU-mediated activation of the canonical MAIT TCR G11 can be blocked in a dose dependent manner as JY.MR1 APCs are preincubated with EP MR1 ligands. Data in A and B represent data from three independent experiments with mean ± SD. For B, the significance of these findings was ascertained by one-way ANOVA with Dunnett's multiple comparisons. P values are reported as *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P≤ 0.0001.

### 2.6 Endogenous pterin MR1 ligands block the activation of canonical MAIT cells

In order to further validate these ligands, an activation blocking assay using JY.MR1 cells as APCs and a $\beta_2$M KO Jurkat JRT3-T3.4 (JrtB) bearing the canonical 5-OP-RU reactive MAIT TCR, D426-G11 (referred to hereafter as G11) was performed[123]. It was observed that the preincubation of JY.MR1 cells with BH2, BH4, 6BP, and sepiapterin decreased the activation of the JrtB.G11 cells in a concentration dependent manner while pterin, which was not able to stabilize MR1 on the surface of JY.MR1 cells in our previous screen (**Supplemental Figure 2.4 B**), had no effect on G11 activation (**Figure 2.5 B**). Interestingly, this blocking effect can be observed at much lower substrate concentrations as compared to our surface stabilization assays, indicating that these compounds can interact with MR1 in the high nM to mid μM range, much closer to the physiological concentrations of EPs that have been reported during inflammation or cancer-induced dysregulation[146, 164]. It is worth noting that the potential for endogenously sourced ligands to bind MR1 and effect the MR1-MAIT axis has been hypothesized since the discovery of MR1[52, 135, 165]. It is quite possible that this in vitro assay highlights a potential purpose for these ligands in the resolution of the MAIT cell response to microbial ligands. Effectively, tuning the reaction of canonical MAIT cells by blocking the loading of stimulatory ligands as the inflammatory conditions continue to increase the concentration of EPs within the local environment. More experimentation with a larger panel of MAIT clones will be necessary to see how broadly applicable this phenomenon is, but these initial results certainly point to this being a potential function of EP MR1 ligands. So far, I have shown that endogenous pteridines can be identified at a significantly higher frequency in extractions from MR1 refolded with myeloma cell line lysate and these ligands are capable of binding to MR1 in *in vitro* assays. Next, it was critical to show that these EP ligands can interact

with MR1 at the natural concentrations found within myeloma cells, perhaps answering the questions around their abnormal basal surface expression of MR1.

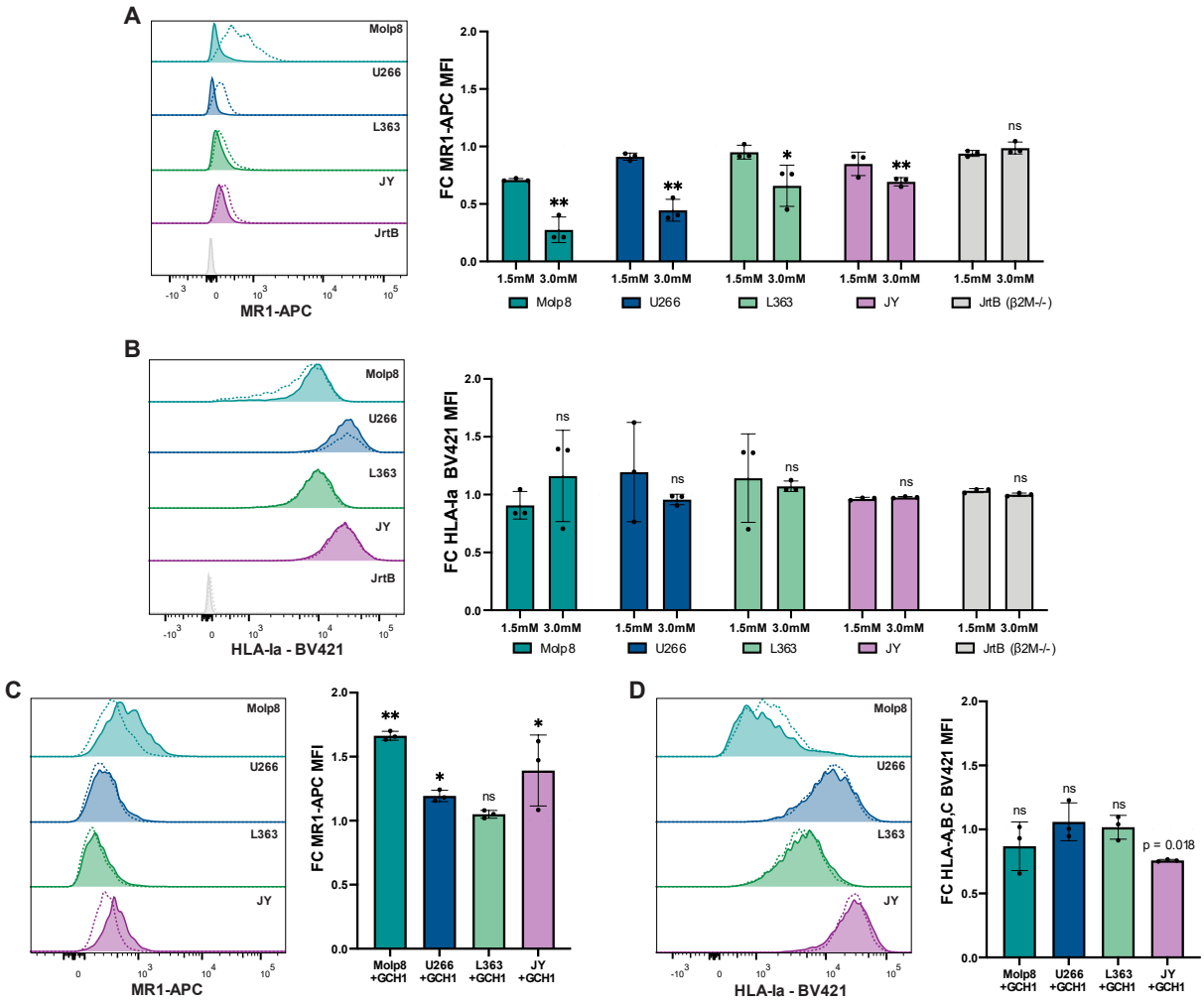



**Figure 2.6** Modulation of GCH1 has a direct impact on the surface expression of MR1. A) The introduction of the GCH1 inhibitor, DAHP, reveals a significant decrease in the surface expression of MR1 between treated (solid lines) and vehicle treated (DMSO, dashed lines) cells. B) This effect is specific to MR1, as the surface expression of the related HLA-Ia family of molecules is not significantly impacted by DAHP treatment. C) The over expression of GCH1 (solid lines) results in a significant increase in the surface expression of MR1 when compared to parental WT cell lines (dashed lines). D) This effect is specific to MR1, as HLA-Ia surface expression does not increase with GCH1 overexpression. Bar graphs represent data from three independent experiments with mean ± SD. Histograms are derived from one experiment that is representative of the triplicate. Significance ascertained by an unpaired, two-tailed Student's t-test between the experimental group and control group. P values are indicated for each significant comparison or reported as *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P≤ 0.0001.

## 2.7 Intermediates of the tetrahydrobiopterin biosynthesis pathway constitute an active pool of endogenous ligands for MR1

Satisfied that these EP molecules can bind to MR1 when added exogenously to the cell culture, we next investigated if these molecules represent a natural pool of MR1 ligands in myeloma cells at steady state. As has been described previously, multiple myeloma and BLCL cell lines exhibit a high level of basal MR1 surface expression without exogenous antigens added to the culture[126]. It is well known that MR1 is unstable without a ligand in the pocket and is primarily retained in the ER until a ligand is present[136, 141]. Therefore, it was hypothesized that EPs constitute a potential pool of MR1 stabilizing ligands in these cell lines under basal conditions. The BH4 biosynthetic pathway is tightly regulated in healthy cells as BH4 is an essential cofactor for many functions within the cell[152]. The target of this regulation revolves around the rate limiting enzyme GCH1, which is responsible for the first step of the *de novo* pathway - converting GTP into dihydroneopterin triphosphate [153](**Figure 2.4**). 2,4-diamino-6-hydroxypyrimidine (DAHP) is a specific inhibitor of GCH1 function and has been shown to disrupt the *de novo* BH4 biosynthetic pathway, thereby decreasing the amount of BH4 and its related intermediates within the cell[166]. If BH4 and its intermediates are binding to MR1 within myeloma cells and stabilizing it on the cell surface, it was anticipated that the addition of DAHP to the cell culture medium would reduce the concentration of available ligands within the cell and subsequently, the amount of stabilized MR1 on the cell surface. In line with this hypothesis, when DAHP was added to myeloma cell culture, the inhibition of GCH1 resulted in a direct decrease in MR1 surface expression in these cells (**Figure 2.6 A**). Due to the central role of BH4 in many metabolic pathways, it was important to show that this effect is specific to MR1 and not a result of a global knock down in protein production or surface expression. By assaying

for their surface expression within the same experiment, it was shown that the highly related HLA-Ia family of MHC class I proteins (HLA-A, B, and C) are not affected by DAHP treatment of these cell lines, indicating that GCH1 inhibition has a specific effect on the surface expression of MR1 (**Figure 2.6 B**).

Previous work has shown that the overexpression of the rate limiting enzyme in BH4 biosynthesis, GCH1, increases the cellular concentration of BH4 and its intermediates[156]. We therefore hypothesized that overexpression of GCH1 would also increase the surface expression of MR1 as EPs increase in concentration within the cell. Indeed, we observed an increase in MR1 surface expression when GCH1 was overexpressed in the JY BLCL cell line and two out of three multiple myeloma cell lines tested (**Figure 2.6 C**). While unexpected, the lack of response in the L363 cell line may be due to the state of GCH1 expression in these cells already being augmented through mutation or overexpression. In line with this explanation, it can be shown on the Cancer Cell Line Encyclopedia hosted by the Broad Institute that L363 has a higher basal mRNA expression of GCH1 in comparison to Molp8 and U266[167]. It is also important to note that the ions annotated as EP ligands were identified to be significantly enriched in Molp8 and U266 RFe samples, but not the L363 samples, so it is quite possible that this EP-MR1 axis is less prominent in L363 cells. Further investigation is warranted to fully explain this interesting observation. Importantly, as with the GCH1 inhibition data, it was ensured that GCH1 overexpression specifically increased the surface expression of MR1, as there was no corresponding increase in the surface expression of the related MHC protein family HLA-Ia (**Figure 2.6 D**). It should be noted, however, that there was a small but significant decrease in HLA-Ia surface levels in the JY cell line when GCH1 was overexpressed. While puzzling, this decrease in HLA-Ia expression does not contradict that heightened BH4 metabolism seems to

cause a specific *up*regulation of MR1 on the surface of these cells. Taken together, these results show that the endogenous pteridine molecules produced by BH4 biosynthesis represent an active pool of potential MR1 ligands in myeloma cell lines which could allow for recognition by cognate MR1T cells.

### 2.8 Additional discoveries from the myeloma-MR1 pipeline

While the major focus of this dissertation is the discovery and validation of the pteridine ligands found to be enriched in the Molp8 and U266 RFe samples, as the scatter plot in **Figure 2.3** shows, there are many other highly significant ions that could have been investigated. This section will focus on a handful of these significantly enriched ions that were picked based off interesting molecular characteristics but were ultimately not pursued further. Many of these additional compounds did not seem to impact MR1 surface stability in our screening assays, which is not an unexpected result. It was known from the start that the MS1 approach we used provides *potential* identities (annotations) for the m/z values identified and we were aware that the crux of this approach would always lie in the validation of the possible MS1 findings. This also means that if a given compound did not perform well in our validation screening assay due to solubility issues, lack of effect at the given time-point of analysis, or an inability to permeate the JY.MR1 cells, it was also discarded from our analysis. This is a major caveat to our approach as these ligands that were shown to have no effect in our screening assay may still be MR1 ligands but were not able to stabilize MR1 under the given screening conditions. It should be noted, however, that ligands that did not stabilize MR1 in these screens were most likely not the right identification for the given m/z value. Taking all these caveats into consideration, there were a few ligands outside of the EPs that I found to have a middling effect on MR1 surface

stability in our assays. These results are very interesting, and these ligands are certainly worth pursuing further but, for reasons I will elaborate on in the rest of this section, may require some ingenuity to fully validate.

When first presented with this data set and after processing it to focus on the 418 ions that appeared to be significant in at least one comparison of a myeloma sample over a control, it was overwhelming to decide which compounds to pursue for validation. This was deepened by the realization that many of the most significant or top reoccurring ions were annotated as either compounds that likely could not be present in these samples, or as compounds that were not easily procurable. Indeed, highly represented ions like 251.0712, 429.1931, and 437.1979 were labeled as natural products known to be derived from plants, and ions found to be highly significant or found at large average fold change ratios like 719.672, 705.6564, and 269.0871 were similarly labeled as metabolites not endogenous to humans. These are likely examples of the caveats mentioned above: if something was a novel compound (not found in any metabolic database), it would not be labeled or labeled inaccurately as a compound from a database that shared the same chemical formula. This made it clear that focusing on this list from the "top down" was not the best approach. I therefore implemented some ground rules to help aid in focusing on compounds that would have a higher likelihood of validation success. Ignoring the significance and recurrence of the ions in my list I instead focused my search on compounds that had some tangible connection to cancer, as this would allow me to focus on human derived endogenous metabolites and ones that may be more likely to be present in a cancer-associated sample. From these, I paid special attention to compounds that resembled the known MR1 ligands described in the literature.

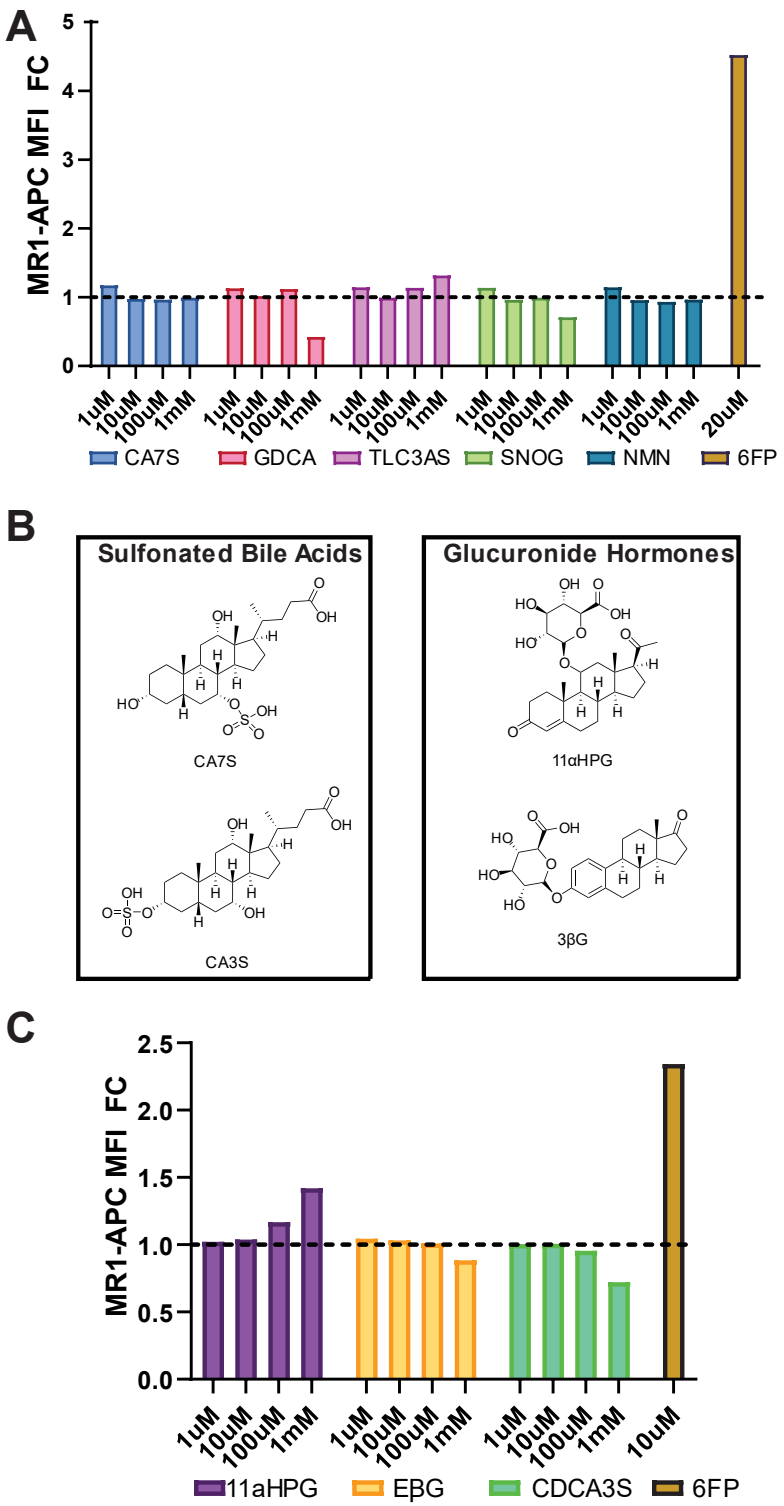



**Figure 2.7** Screening results with cancer-associated metabolites and glucuronide hormones. A) MR1 surface stabilization of cancer associated metabolites SNOG and NMN, as well as cholic acid derivatives CA7S, GDCA, and TLC3AS. B) The chemical structures of known MR1 ligands CA7S and CA3S in comparison to the chemical structures of 11αHPG and 3βG. C) MR1 surface stabilization of glucuronide hormones 11αHPG and 3βG, as well as one additional cholic acid derivative chenodeoxycholic acid 3-sulfate (CDCA3S). Data are representative of one screening experiment each.

The first ions I tried to validate fell more into the first category than the second. Both S-nitrosoglutathione (SNOG, m/z = 335.0663) and nicotinic acid mononucleotide (NMN, m/z = 336.0498) appeared in the recombinant hpMR1 generated from the U266 cell line in comparison to JY hpMR1 and JY T22. It is a well-documented phenomenon that glutathione acts as a scavenger for reactive compounds in the cytosol and that cancer cells use this to their benefit to remove ROS and other reactive metabolites[168], so while bearing no real chemical similarity to any known MR1 ligand, SNOG seemed to be a good candidate to test due to its cancer-relevance. Similarly, NMN is a precursor to NAD which is important to a number of pathways cancer cells leverage for growth and, unlike SNOG, the nicotinic acid moiety found on NMN vaguely resembled the core of smaller drug-metabolite MR1 ligands[70, 169].

At the same time that I had begun to analyze this dataset, the paper that had labeled sulfonated bile acids as MR1 ligands was published[79]. This led me to interrogate my dataset for compounds with similar structures. I was excited to see that one of the compounds identified in this publication, taurolithocholic acid 3-sulfate (TLCA3S, m/z = 562.2518), appeared as a significant ion annotation from two of the RFe samples (Molp8 and JY). Closer inspection of the data set found that related compounds like glycodeoxycholic acid (GDCA) and the focus of their study, CA7S, were also present in the myeloma samples although just missing the significance cut off in a few comparisons. This prompted me to procure a subset of these compounds along with SNOG and NMN and test them at a wide range of concentrations in an overnight MR1 stabilization assay with JY.MR1 cells. SNOG and NMN did not have any measurable effect on MR1 MFI at the concentrations and time point tested (**Figure 2.7 A**). While TLCA3S did not seem to impact MR1 stabilization at the concentrations tested by the Yamasaki group (up to 10uM), I found that at a high concentration (1mM) there was a very slight increase in MR1 MFI

(**Figure 2.7 A**). What was more surprising, however, was that CA7S had no measurable effect on MR1 MFI at any concentration tested (**Figure 2.7 A**). The concentrations I used were equivalent to what was tested in the previously published paper and, while the majority of their studies were done at 6 hours instead of 16, CA7S was one of the few ligands they tested in a similar assay in a time course from 0-16 hours[79]. They reported that while the effect was diminished at 16 hours, there was still a measurable increase in MR1 MFI with CA7S, a result I was not able to replicate. It should be noted that the Yamasaki group used a different cell line for their studies and while in theory this should not impact the assay drastically, it may help explain this variability between our results.

With the potential exception of TLCA3S, these results were disappointing. However, at the same time, I noticed that there was a significant number of larger significant ions (>400m/z) present in my list with chemical structures that resembled these sulfonated bile acids. This class of ions, termed glucuronide hormones, was of particular interest as they shared the cholesterol-like backbone of the sulfonated bile acids, but were compounds known to be associated with the growth of certain cancers (**Figure 2.7 B**)[170]. Ions 505.2437, 463.2329, and 423.1413/425.1461 belonged to hormones or other important growth signaling molecules that had undergone glucuronidation. Not only is the hormonal growth signaling potential of these compounds significant in cancer, but the modification through the addition of a glucuronide group shows processing by UGT enzymes, enzymes commonly upregulated by cancers, including myeloma, to aid in the efflux of chemotherapies and other toxic substances[127, 171]. Furthermore, the addition of this glucuronide group was reminiscent of the bacterial sulfonation of the MAIT activating cholic acid molecules that were described by the Yamasaki group, and, it is also worth noting, that the glucuronide group itself is reminiscent of the ribityl moiety found on traditional

activating MAIT ligands (**Figure 2.7 B**). These coincidental aspects led me to procure a few glucuronide-hormones, including 11-α-hydroxyprogesterone glucuronide (11αHPG, m/z = 505.2437, found in Molp8, JY, U266, and L363 RFe samples) and esterone-3-β-D-glucuronide (EβG, m/z = 463.2329, found in all RFe samples) for testing. I was able to show that at high concentrations (100uM-1mM), 11αHPG was able to increase the MFI of MR1 by almost 50% while EβG had no significant effect in this screen (**Figure 2.7 C**).

TLC3AS and 11αHPG represent interesting compounds not only due to their significance in the MR1 literature or for known roles in cancer metabolism, but also because their presence in our MS dataset has been confirmed through these preliminary screens – putting them at the top of the list of potential MR1 ligands that have yet to be investigated in depth. The major issue with pursuing them further is not academic, but rather technical issues with their solubility and the cost of these molecules is more or less prohibitive for further study. Hormones in general are hard to synthesize and harvesting them from living tissues is time consuming and provides low yields. This is further compounded by the fact we are looking for these molecules only when they are modified in certain ways, which makes procuring them naturally nearly impossible without the proper chemistry knowledge and equipment. Also due to these challenges, purchasing more than 1mg (much more is needed for a refold or to perform multiple cellular assays) is exceedingly expensive. That being said, there are other significant ions in this dataset that are annotated as products of glucuronidation which have yet to be validated that may prove to be better candidates for further study.

## 2.9 Discussion

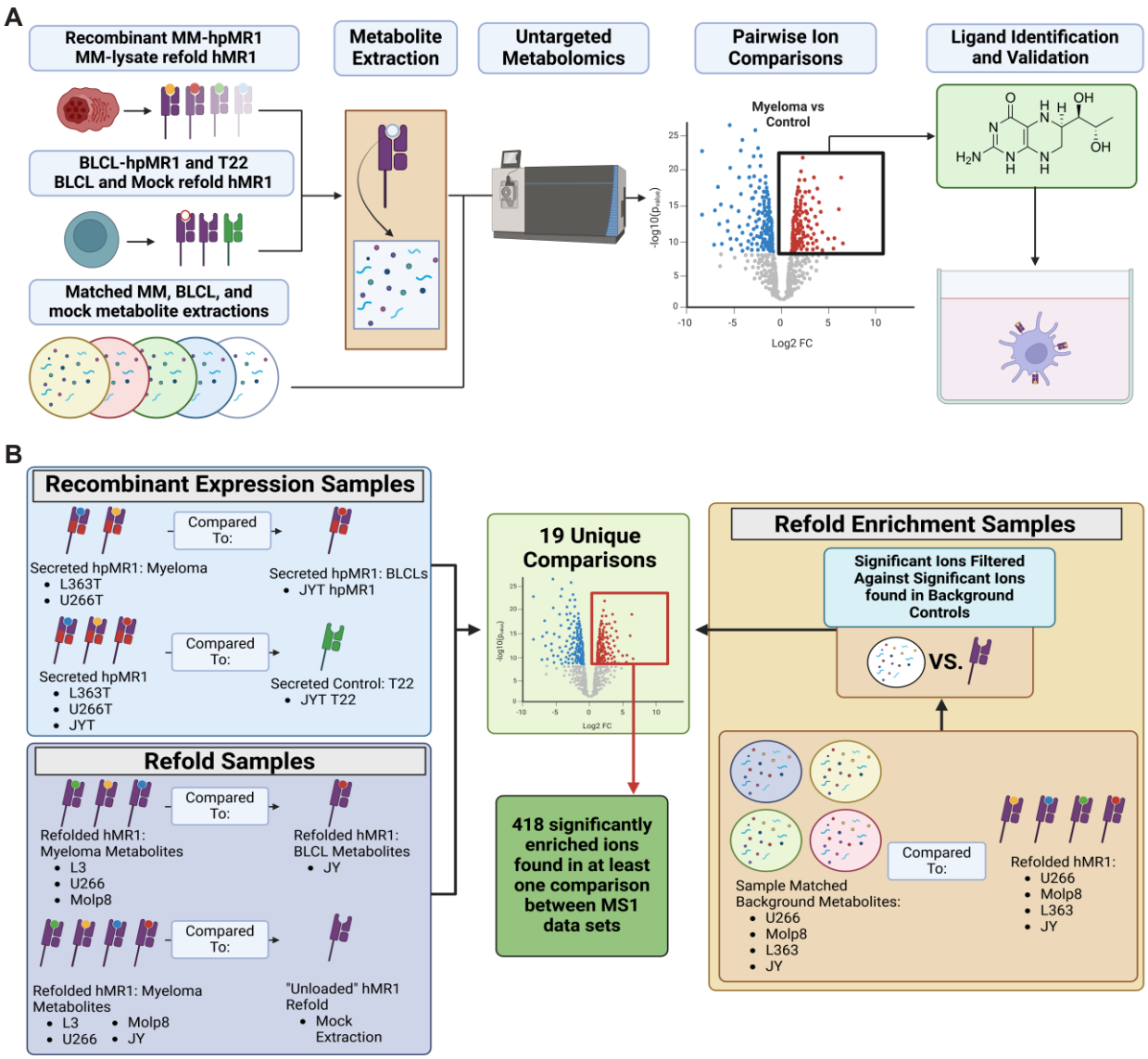
The ligand repertoire of MR1 is a growing body of molecules, some with conserved

characteristics, and some that are wildly different in size or chemical features. Since the early days of the discovery of MR1 it has been postulated that MR1 may be capable of capturing endogenous metabolites and presenting them to MAIT cells[52], but to date no unmodified endogenous antigens have been described to bind MR1. Here, I show that unmodified endogenous metabolites are capable of binding to MR1 and compete for the same ligand binding pocket as 5-OP-RU, effectively blocking canonical MAIT activation. The EP ligands that exhibit these qualities all originate from the same biosynthetic pathway, BH4 biosynthesis, which is controlled in part by the inflammatory signals present during infection and the immune response to cancer. While discovered in the context of multiple myeloma, these results paint an interesting picture for the potential uses the immune system may have for these MR1 ligands in tuning the MAIT cell response to infection. As innate-like T cells, MAIT cells react quickly and potently when stimulated through their TCR, but due to their localization to the mucosa, it is likely that they are exposed to microbially-derived antigens with some regularity[90, 172]. It has been suggested in the literature that a potential pathway for the regulation of MAIT cell responses is through the competition between activating and inhibitory MR1 ligands[172]. However, many of the MAIT antagonist ligands described for MR1 have questionable physiological relevance and are likely not found in the mucosa or the body at any meaningful concentration[57, 58]. It is therefore tempting to speculate that outside of neoplastic disease, the upregulation of pteridines in the inflamed mucosal tissue allows for dampening of the microbial antigen driven activation of MAIT cells through direct competition for the MR1 binding pocket. A natural negative feedback loop would occur as more MAIT-mediated inflammation would cause more pteridine buildup and thus more effective inhibition. More work with additional MAIT clones is necessary

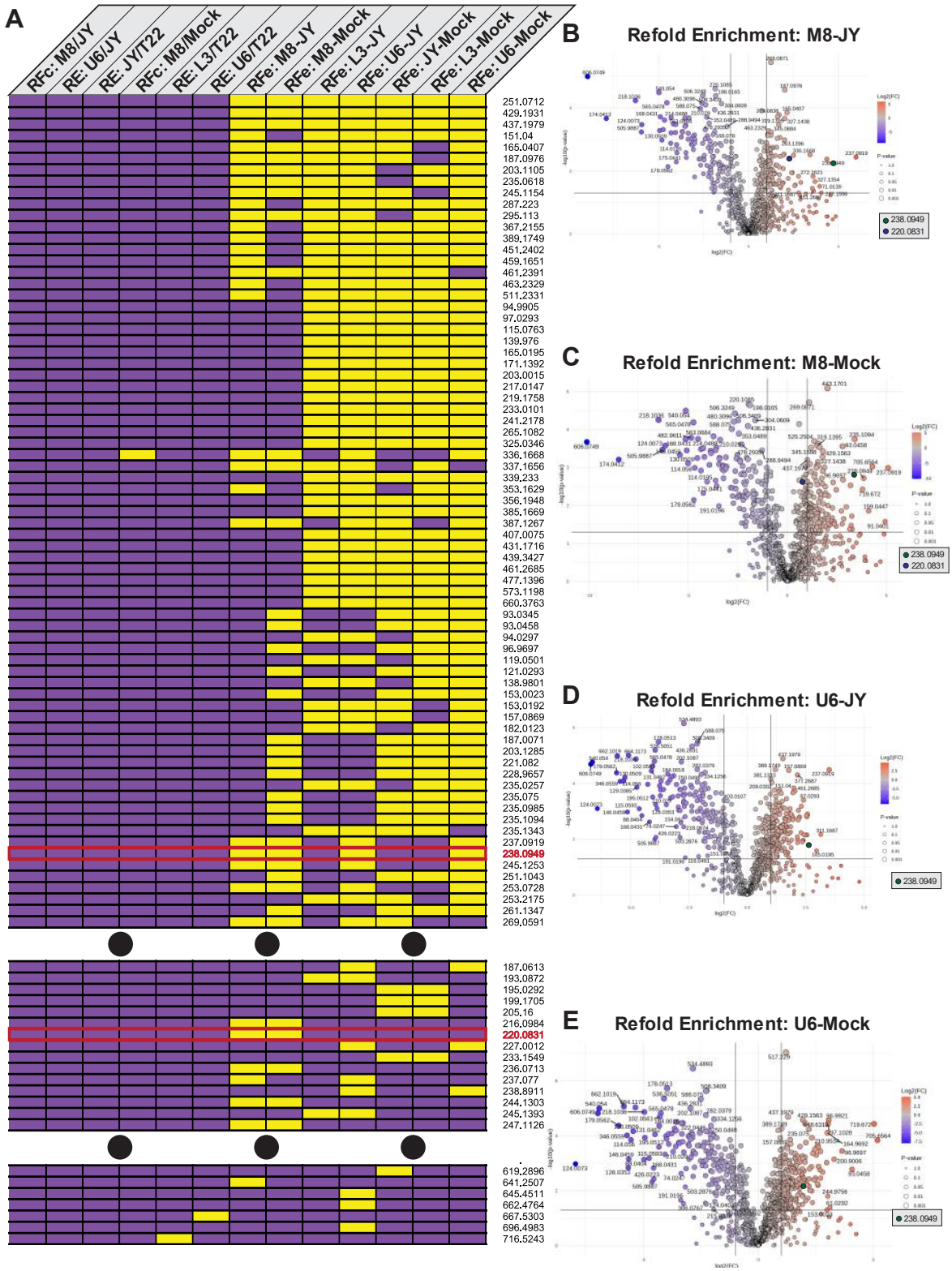
to fully validate this hypothesis, but it remains a plausible function for these novel EP antigens for MR1 in contexts beyond cancer.

While so far, I have shown that these EP ligands for MR1 do not stimulate MAIT cells, there is a more diverse family of MR1T cells present in the human body (See **section 1.3**). These members of the family do not make up the vast cell numbers that MAIT cells do, but are consistently found to be a supported population of cells in healthy people[66, 67, 71, 86, 88]. This portion of the MR1T family ranges from cross reactive MAIT cells that are activated by ligands thought to be MAIT antagonists to cancer-associated MR1T cells that have garnered a large amount of attention due to their broad cancer reactivities[71, 72, 78, 86]. While some activating ligands have been described for these cell types[73, 74], their full ligand repertoire and their functions in health and disease have not been fully described, and many questions remain about their basic biology. In the case of cross-reactive MAIT cells and atypical MR1T cells, the folate-derived pteridines they react to are not thought to be pervasive in the human body[57]. Is it possible that they exist to survey for other forms of endogenously sourced pteridines as monitors of tissue stress or wounding? Furthermore, the cancer-associated MR1T cells that seem to react to the nucleobase-MDA adducts that are only formed in cancer cells have all been derived from healthy people[71, 72]. If their ligands only arise during a disease that these individuals have yet to encounter (or may never encounter), then how are these cells maintained in the periphery and what ligands are they selected on in the thymus if they are agnostic to the microbially derived ligands that are purported to make their way to the thymic medulla[79, 80]? As I will show in the next chapter, it appears that the existence of the EP ligands I have described here may be key in answering some of the questions surrounding the basic biology of MR1T cells.

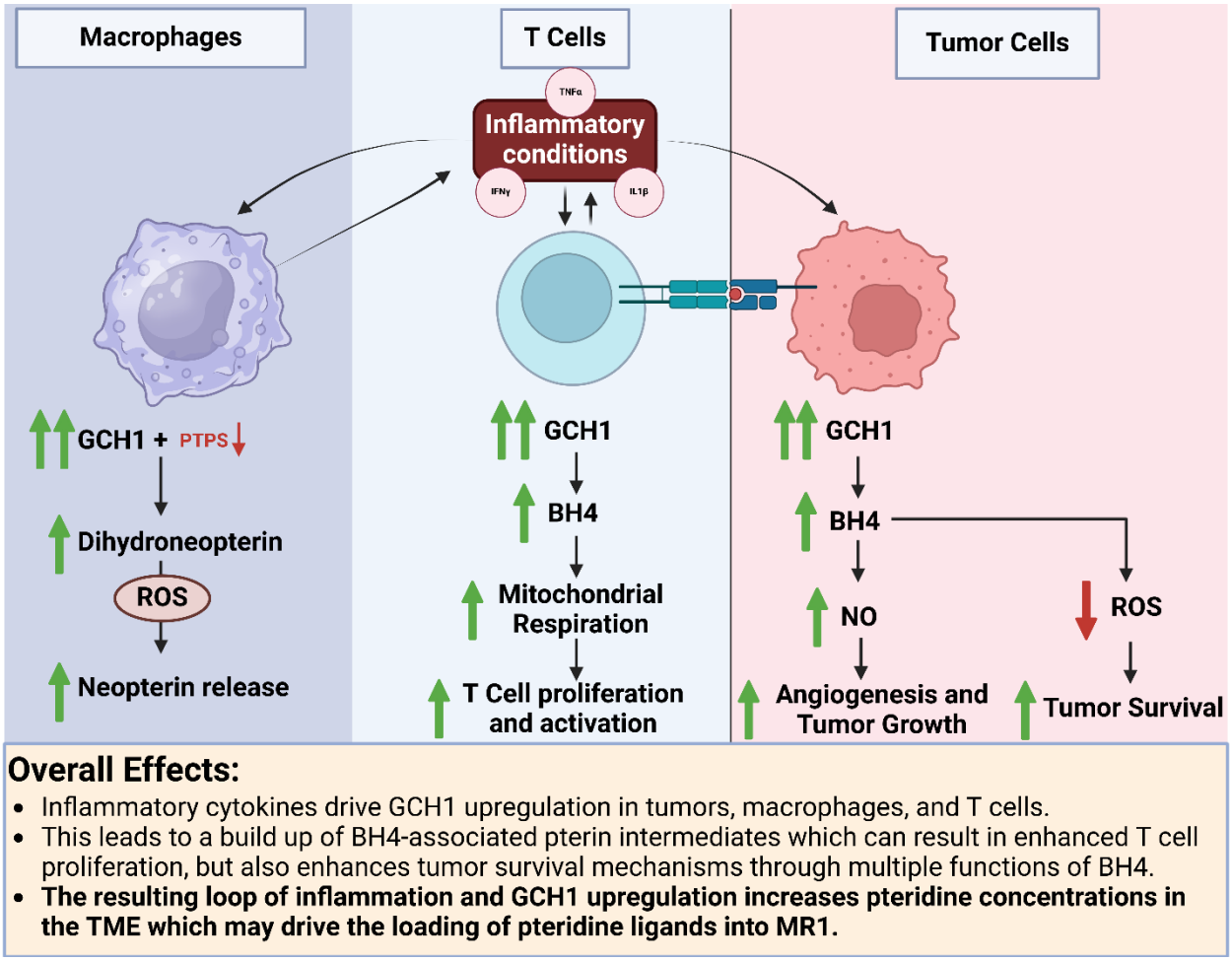
## 2.10 Supplemental Information



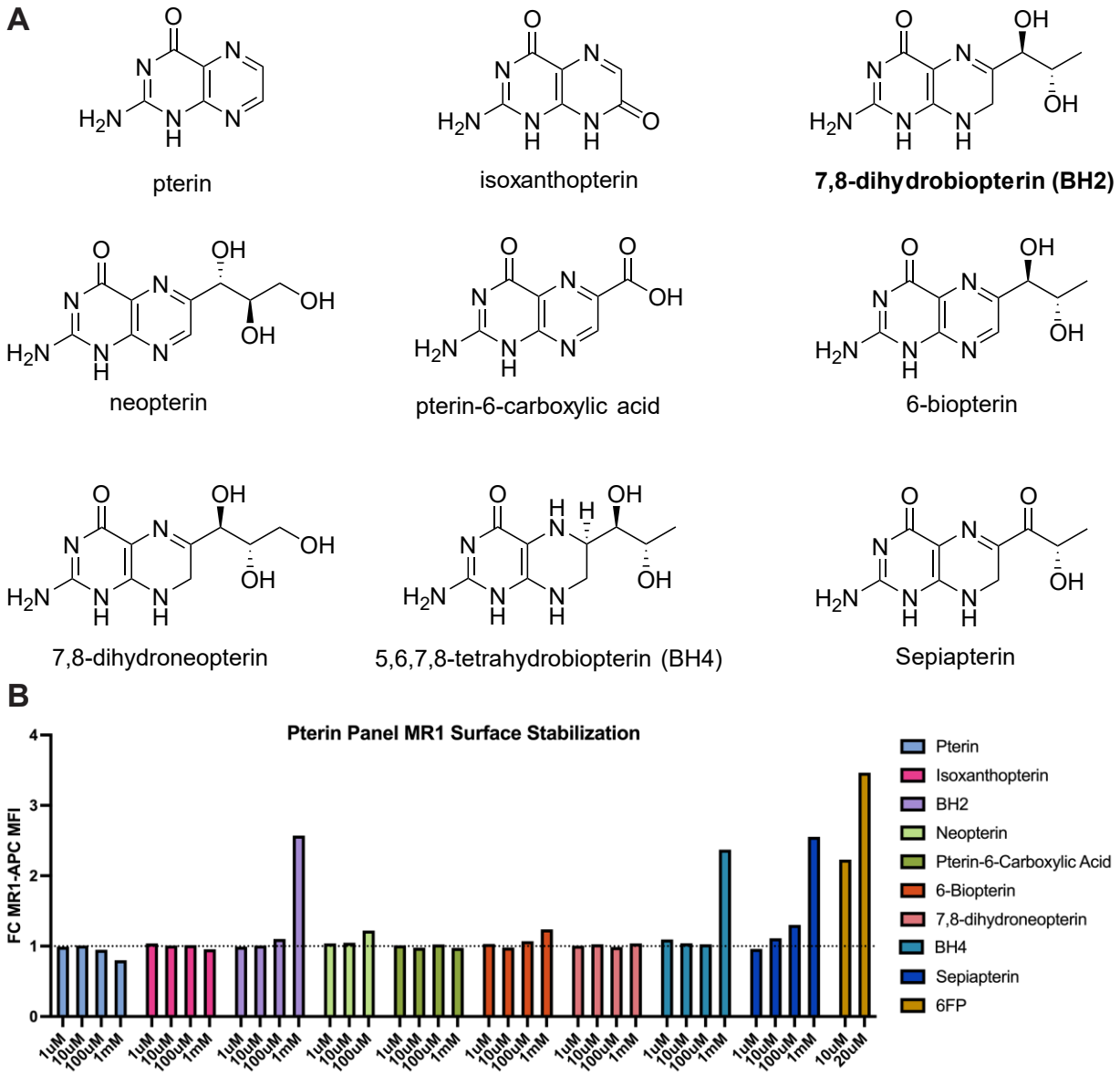

**Supplemental Figure S2.1** Myeloma MR1 ligand identification: experimental set up and data analysis. A) The endogenous ligandome of MR1 in the context of multiple myeloma was surveyed using hpMR1recombinantly produced in myeloma cell lines and hMR1 refolded in the presence of metabolite extracts generated from myeloma cell lines. After purification, MR1 samples had the ligands extracted from them and these samples along with the matched metabolite extract backgrounds were submitted for untargeted metabolomics. B) The resulting MS data set was analyzed by direct comparison of myeloma-MR1 samples to either JY-MR1 or mock condition-MR1 samples to generate a list of significantly enriched ions from myeloma-associated samples. RFe samples had significant ions found to be enriched in JY or Mock refolds subtracted from them before comparison with their matched background sample.




**Supplemental Figure S2.2** Abbreviated list of the 418 significant ions derived from the myeloma-MR1 MS dataset. A) An abbreviated heat map showing each significant ion and the comparison they were found to be significant in. Ions 238.0949 and 220.0831 are highlighted in red. (B-E) volcano plots showing the comparisons where ions 238.0949 and 220.0831 were found. Ion 238.0949 is highlighted in green while ion 220.0831 is highlighted in blue.




**Supplemental Figure S2.3** A summary of the role of BH4 in the tumor microenvironment. Macrophages generate a large amount of neopterin as low expression of PTPS allows for the buildup of dihydroneopterin triphosphate which is converted into neopterin as it is used to absorb aberrant ROS species. This neopterin is released into the TME during macrophage activation along with other effector molecules. T cells have been shown to be reliant on BH4 for effective mitochondrial respiration which is required for their proliferation and effector functions in the TME. As GCH1 is under the control of NFκB, inflammatory signals in the TME drive BH4 production through the *de novo* pathway. Tumor cells, on the other hand, also take advantage of this same axis, as BH4 both protects them from excessive ROS buildup, but also provides survival advantages through its role as a cofactor for many monooxygenase enzymes like NOS.




**Supplemental Figure S2.4** The validation of a panel of EP MR1 ligands. A) The chemical structures and names of compounds included in the initial screen for EP stabilization of MR1 on JY.MR1 cells. B) Results of the initial screening experiments testing each ligand from 1uM to 1mM final concentration. 6FP is included as a reference control for efficient MR1 surface stabilization. Screening experiments were performed once and molecules showing any effect on MR1 stabilization were completed in triplicate at a narrower range of concentrations (see **Figure 2.5 A**).

# Chapter 3. Endogenous Pteridine MR1 ligands are recognized by cancer-associated MR1T cells and atypical MAIT cells

## 3.1 Introduction

As discussed in **section 1.3**, the vast majority of MAIT cells have been shown to be restricted to MR1 when loaded with RBP derivatives like 5-OP-RU. However, work from our lab and others have challenged the assertion that all MAIT cells are only capable of recognizing 5-OP-RU, and have shown that even these semi-invariant MR1Ts are capable of exhibiting preference for one microbially derived antigen over another[61, 68, 70, 86, 100]. Beyond MAIT cells, there exist a class of MR1T cells that are phenotypically similar to MAIT cells, but have access to a diverse repertoire of TCR genes and utilize different docking strategies with MR1 to recognize a wider variety of MR1 antigens[86, 88, 101]. These cells have been observed to react to the canonical MAIT ligand 5-OP-RU as well as the known folate-derived pteridine antigens 6-FP and Ac-6-FP. Others have been described as MR1-autoreactive due to their expansion on MR1+ cells in the absence of exogenous antigens. Interestingly, later studies revealed that the expression of the CD8 coreceptor was shown to be critical for the recognition of MR1 loaded with folate-derived pteridines and boosted the recognition of autoreactive or ligand promiscuous MAIT/MR1Ts as well[78, 88]. In the context of these atypical MR1T cells, the discovery of a novel class of endogenously sourced pteridine antigens as described in **Chapter 2**, is especially exciting. While it has been shown that some of these MR1Ts react to 6-FP and its synthetic derivatives, the physiological relevance of these folate-derived ligands is questionable. The molecular similarities to 6-FP and the endogenous nature of the newly discovered EP MR1 ligands led to the hypothesis that EPs may be a physiologically relevant pool of ligands for these MR1Ts in the body. Using $CD8\alpha^+$ TCR Jurkats, hMR1 tetramer reagents loaded with EP ligands,

and activation assays using APCs pulsed with various pteridines, I was able to show that EP MR1 ligands do represent a cognate antigen for a number of atypical and autoreactive MR1T TCRs. These results indicate that EP ligands play a much broader role in MR1T biology outside of the cancer axis they were discovered in. Studies following up on the role of these ligands in the development and maintenance of atypical MR1Ts are of high importance.

As these EP ligands were discovered to be enriched in MR1 samples derived from myeloma cell lines, it was important for me to interrogate the other remaining class of MR1Ts, those that have been described to be cancer-associated. As discussed in **section 1.3**, these MR1Ts have been shown to be cytotoxic to cancer cells in an MR1 dependent manner and some of these cells have been shown to react to nucleobase adducts generated in cancer cells. In a similar way to the atypical MR1T cells described above, I generated a panel of cancer-associated MR1T JrtB cells to see if they also recognize EP ligands. I show that when the Tc5A87 and DGB129 TCRs are expressed in Jurkat cell lines they can bind to hMR1 tetramers loaded with BH2 and BH4, an interaction that is enhanced greatly by the expression of CD8α. I also show that Tc5A87 and DGB129 TCRs, but not the MG.7.C5 TCR, are activated by APCs pulsed with BH2 and BH4 in an MR1 dependent manner. Taking all these results into consideration, it is clear that these EP MR1 ligands play an important generalized role in the biology of MR1T cells, cancer-associated or otherwise. It is likely that the natural recognition of these ligands occurs outside of the context of cancer, but it is an axis these cells can also leverage when responding to neoplastic tissues.

Finally, I have done some investigation into how broadly applicable these findings are to healthy individuals and myeloma patients. By utilizing the BH2-loaded hMR1 tetramer, I am able to define a subpopulation of MR1T cells in the blood of healthy individuals with a

proportion that corresponds to what has been estimated previously for atypical MR1Ts[99]. The phenotype of these cells is revealed to be diverse with mixed expression of traditional MAIT markers when compared to tetramer negative T cells from the same sample. A pilot study with myeloma patient PBMCs and CD138 negative bone marrow mononuclear cells (BMMNCs) confirm previous reports about MAIT cell depletion in the circulation of myeloma patients, and it appears that BH2-hMR1 tetramers define a small, but substantial population of T cells in the patient that was tested. These exciting initial results show that there is a higher density of BH2-hMR1 tetramer positive cells at the tumor site, as compared to the PBMCs from the same patient. The cells that bind the BH2-hMR1 tetramer in the bone marrow exhibit a more MAIT-like phenotype than when found in the blood. This pilot experiment using samples form one patient is only the beginning of a much more in-depth study of the role EP-reactive MR1Ts are playing in the immune response to multiple myeloma. While more patient samples are needed to generalize further about their specific role in this disease, what is made clear by the results described in this chapter, however, is that EP MR1 ligands play a critical role in MR1T cell biology, both in the context of cancer and at homeostasis.

### 3.2 Development of a β2m KO Jurkat line and endogenous pterin hMR1 tetramer reagents allow for high throughput screening of known MR1T TCRs

While we possess no cancer-associated MR1T cell clones of our own, I was determined to figure out if the EP MR1 ligands described in **Chapter 2** can interact with the TCRs of known MR1T cell family members. To achieve this goal, I was able to successfully reconstruct, clone, and express the TCRs of cross-reactive MAIT, atypical MR1T, and cancer-associated MR1T cells (**Supplemental Figure S3.1 A**). To survey for their ability to bind to MR1 loaded with EP

ligands and their potential for activation, these TCRs needed to be introduced into a partially TCR deficient T cell lymphoma cell line. Many variations exist of the Jurkat cell line, but the one that was selected for this purpose was the JRT3-T3.5 cell line, which is TCRβ deficient and stains negative for TCR and CD3 complex components at steady state[173]. When a TCR is introduced to these cells, generally through the use of a lenti-viral vector, the expression of this TCR can be assayed by flow cytometry staining for surface expression of the TCR and the CD3 complex. However, since some of the cancer-associated MR1T TCRs have been shown to be broadly reactive to cancer cell lines, and in the case of the MG.7.C5 clone, MR1+ Jurkat cells specifically[72], there was a need for further cell line engineering to prevent broad autoreactivity in the population of TCR transformed cells.

To circumvent this potential issue, I was able to engineer a $\beta_2$M KO line of JRT3-T3.5 cells that I have termed JrtB (**Supplemental Figure S3.1 A and B**). This was accomplished with a transiently expressed Crispr plasmid containing guides targeting $\beta_2$M. GFP is also transiently expressed on this plasmid and $\beta_2M^-GFP^+$ cells were sorted as single clones, grown, and then reanalyzed two weeks later for $\beta_2$M surface expression. It was shown that all of the clones were 100% negative for $\beta_2$M surface expression, and the clone E3 was selected as the background JrtB line for the experiments described in this chapter (**Supplemental Figure S3.1 B**). As mentioned in **section 1.3**, recent work has highlighted the importance of CD8αα and CD8αβ engagement with MR1 when MR1T cells are recognizing more diverse or lower affinity antigens[88]. With the help of Andrew Kelly, a technician in the lab, the JrtB cell line was engineered further to create the JrtB.CD8α variant. This is a $\beta_2$M KO line that also overexpresses CD8αα homodimers on the cell surface, which proved to be indispensable for the recognition of EP-MR1 staining reagents (**Supplemental Figure S3.1 C**).

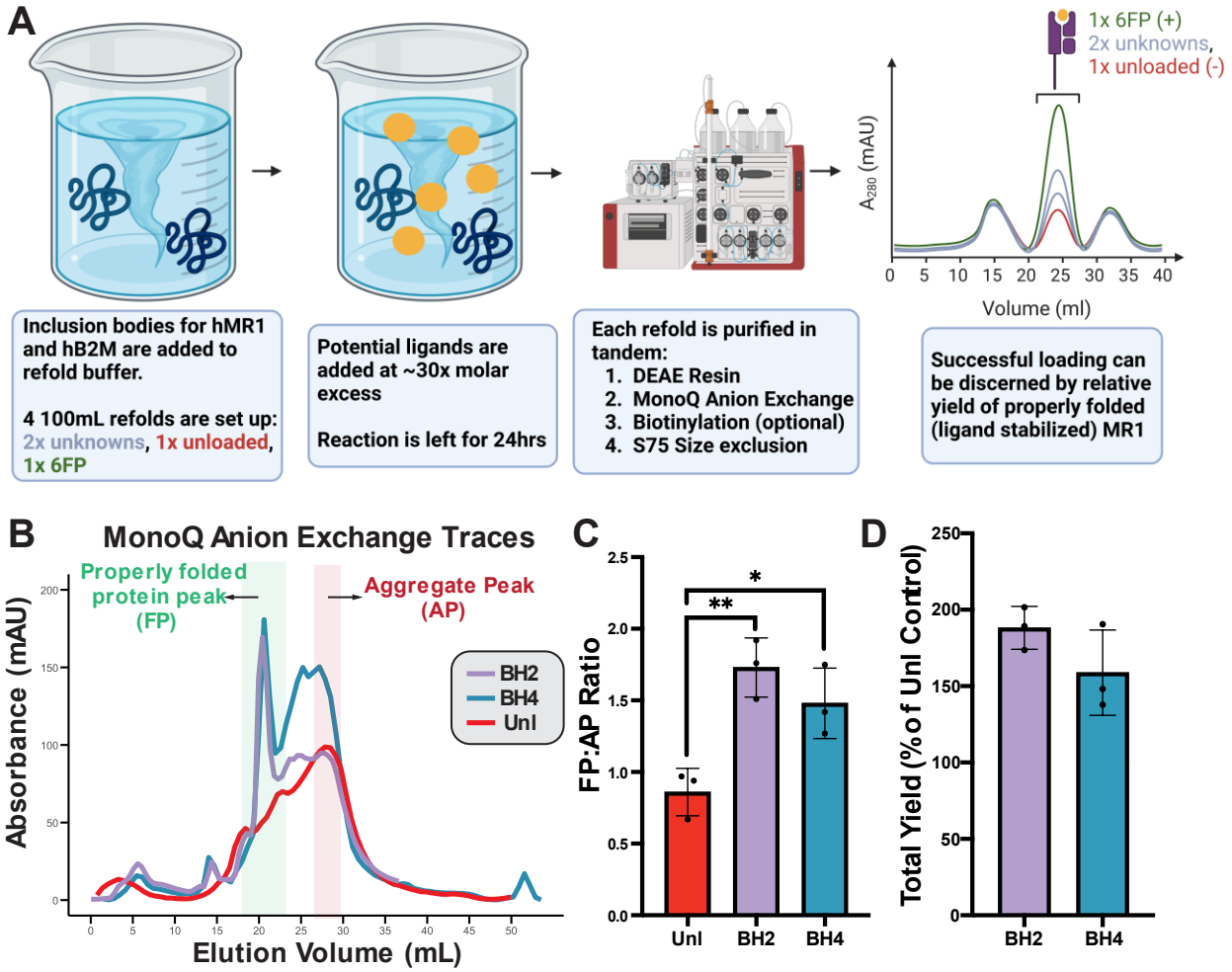



**Figure 3.1** Endogenous pterin MR1 ligands can be refolded with hMR1 to make hMR1 tetramers. A) An overview of the refolding process and purification to generate hMR1 loaded with BH2 and BH4. B) Representative MonoQ anion exchange traces for unloaded (Unl) hMR1, as well as BH2 and BH4 loaded hMR1. Peaks for properly folded and aggregate protein are highlighted in green and red respectively. C) The ratio of these peaks can be used as an indicator for successful refolding. Bar chart shows the results of three hMR1 refolds performed with the ligand indicated on the x-axis. D) Successful refolding is also apparent in the total yield of ligand+ hMR1 refolds relative to the Unl control. Data from three BH2 and BH4 refolds are shown as percent increase in yield over Unl for each respective preparation. In C, significance was ascertained by an unpaired, two-tailed Student's t-test between the indicated groups. P values are indicated for each significant comparison or reported as *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P≤ 0.0001.

A major benefit of using the JrtB and JrtB.CD8α Jurkat TCR expression system was the high throughput manner with which MR1T TCRs could be screened for reactivity to MR1 loaded with EP ligands. The other required half of this screening system, the EP-MR1, was generated via protein refolds of hMR1 and h$\beta_2$M (**Figure 3.1 A**). Through a series of optimization assays it was found that at a ligand molar excess of 30 or more, hMR1 would readily refold with BH2 and

BH4. While the yields of these refolds were modest in comparison to what is observed with a strong, covalent binder like 6-FP, they were in line with what can be expected for a non-covalent MR1 ligand. When using the optimized refold conditions, BH2 and BH4 refolds consistently provided approximately double the final yield and much better protein to aggregate ratios than unloaded (Unl) hMR1 refold controls - indicating that these ligands had a stabilizing effect on the molecule as it refolded (**Figure 3.1 B-D**). Due to the limiting availability of sepiapterin, neopterin, and 6BP, they were not extensively studied for their ability to stabilize MR1 in a refolding assay. Testing these molecules for their ability to stabilize hMR1 for tetramer production is certainly something that should be followed up on, especially in the case of sepiapterin, which was a strong MR1 surface stabilizer (**Figure 2.5 A**).

As an initial validation of these reagents, these TCR JrtB cells and tetramerized EP-hMR1 were used to screen a series of MR1T, MAIT, and other unrelated control TCRs to discern what TCRs may bind EP-MR1, and to ensure that the binding is specific to TCRs that are known to interact with MR1. When testing at a high (1uM) and low (100nM) concentration of APC-labeled Unl, BH2, BH4, 6-FP, and 5-OP-RU hMR1 tetramers, the MR1-unrelated TCRs DP10 (TRGV4 TRDV1), L690 (TRAV14 TRBV7-9), and the TCR-null parental JrtB line had no interaction with any of the MR1 tetramers at any concentration tested (**Figure 3.2**). As shown in **section 2.6**, the canonical, MR1-5-OP-RU reactive MAIT TCR, G11, is not activated by APCs loaded with EP MR1 ligands, and indeed, it was also agnostic to BH2 and BH4 tetramers while binding strongly to hMR1-5-OP-RU tetramer as expected (**Figure 3.2**). These validation experiments proved two things: first, the EP-MR1 tetramers are properly folded and show no non-specific staining of cells without TCRs or bearing MR1-agnostic TCRs, even at high concentrations. Second, these experiments show that the protocol for refolding and

tetramerization of hMR1 as performed here works to create viable MR1 tetramers as evidenced by G11 binding of hMR1-5-OP-RU, which was made concurrently with the BH2 and BH4 tetramers used. What remained to be seen, however, is if there were any MR1T clones that did recognize these EP-MR1 tetramers.

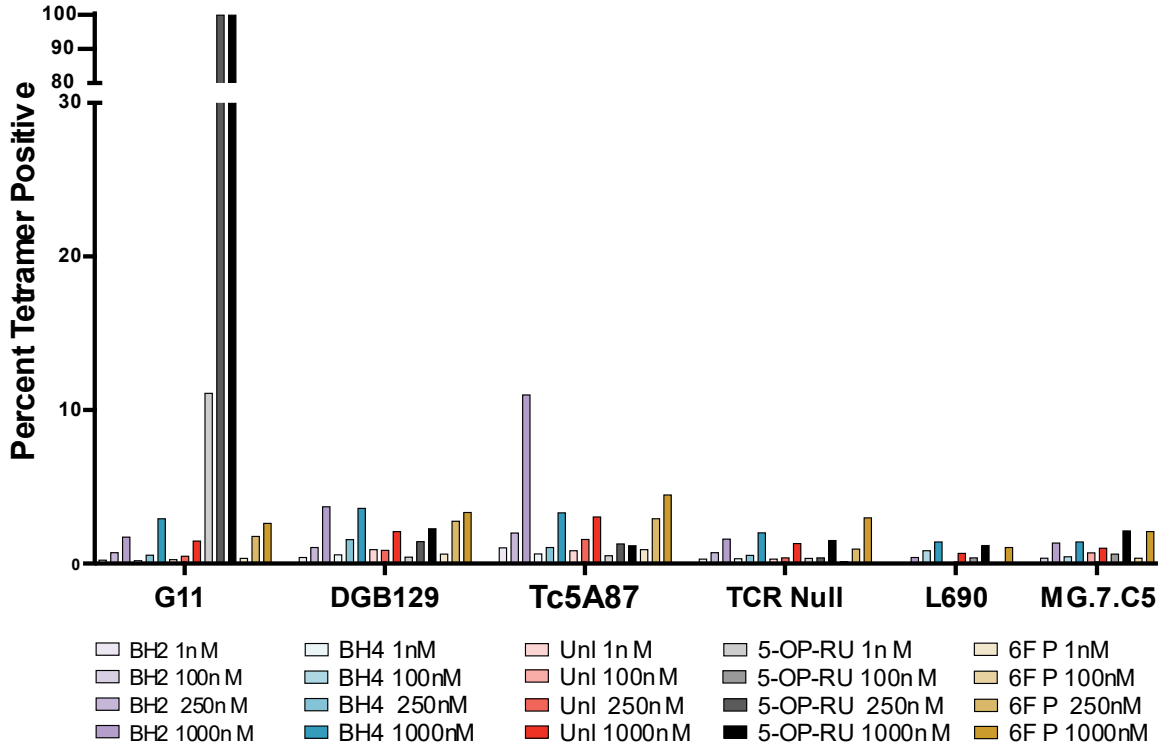



**Figure 3.2** hMR1 tetramer screening of cancer-associated MR1T and unrelated TCR JrtB cell lines. JrtB cells transduced to express the TCR indicated on the x axis were stained with hMR1 tetramers loaded with BH2, BH4, 5-OP-RU, 6-FP, or no-ligand (Unl) as indicated by the colors denoted below the x-axis. Data are represented as the percent of tetramer positive JrtB cells as determined by the APC fluorescence of a no-tetramer control for each JrtB line.

### 3.3 hMR1 tetramers loaded with BH2 and BH4 stain cross-reactive MAIT and atypical MR1T TCR Jurkats

Recent reports in the literature have highlighted the abundance of 6-FP reactive MR1T cells and cross-reactive MAIT cells in healthy individuals, leaving an open question as to the

purpose of these cells in the body and what physiologically relevant ligands they reactive to[78, 86, 88]. Due to the similarity between 6-FP and EP MR1 ligands we hypothesized that 6-FP reactive and ligand cross-reactive MR1Ts would be able to bind to MR1 loaded with endogenously sourced pteridine ligands. To test this hypothesis, we assembled a panel of these MR1T TCRs (**Supplemental Figure S3.1 A**) and tested their ability to bind to BH2 and BH4 loaded hMR1 tetramers (**Supplemental Figure S3.2 A**)[78, 86]. To carry out this test, we chose to express these TCRs in the CD8αα$^{+}$ JrtB.CD8α cell line, as recent reports have highlighted that CD8αα and CD8αβ can bind to the α3 region of MR1 and that co-receptor expression on MR1Ts may be critical for their function[88, 174]. Even more important for our purposes, CD8 expression on MR1T cells was shown to be critical for the specific recognition of MR1-6FP tetramers and therefore, due to the molecular similarities between 6-FP and EP ligands, may be equally critical for the recognition of MR1 loaded with EP ligands[88].

After titrating the BH2, BH4, and unloaded hMR1 tetramers, it was observed that the 6FP-reactive MR1T TCRs, MAV21 and AM3, bound to BH2 tetramers with a heightened affinity as compared to the unloaded and BH4 tetramers (**Figure 3.3 A and B**). The ligand cross-reactive MAIT TCRs, M33.64 and BC75B38, also bound to BH2 and BH4 hMR1 tetramers and, to a lesser extent, the unloaded hMR1 tetramer, which would align with their designation as MR1 “autoreactive” (**Figure 3.3 C and D**). These results indicate that 6-FP reactive MR1Ts and cross-reactive MAIT cells with a previously undefined endogenous ligand repertoire are capable of recognizing EP ligands. Interestingly, in the case of the MAV21 and AM3 TCRs, it is possible that these atypical MR1Ts may be capable of distinguishing between EP ligands presented in MR1. It is appreciated that binding of an MR1T TCR does not always translate to activation. Indeed, it was shown with one of the first MAIT TCR MR1-6FP co-crystals that MAIT TCRs

can bind 6-FP without activation – giving 6-FP the reputation as a MAIT antagonist ligand[59]. Taking this into account and given the similarity between 6-FP and EP MR1 ligands, it was critical that we directly survey their potential for activation of atypical MR1T and cross-reactive MAIT cells.

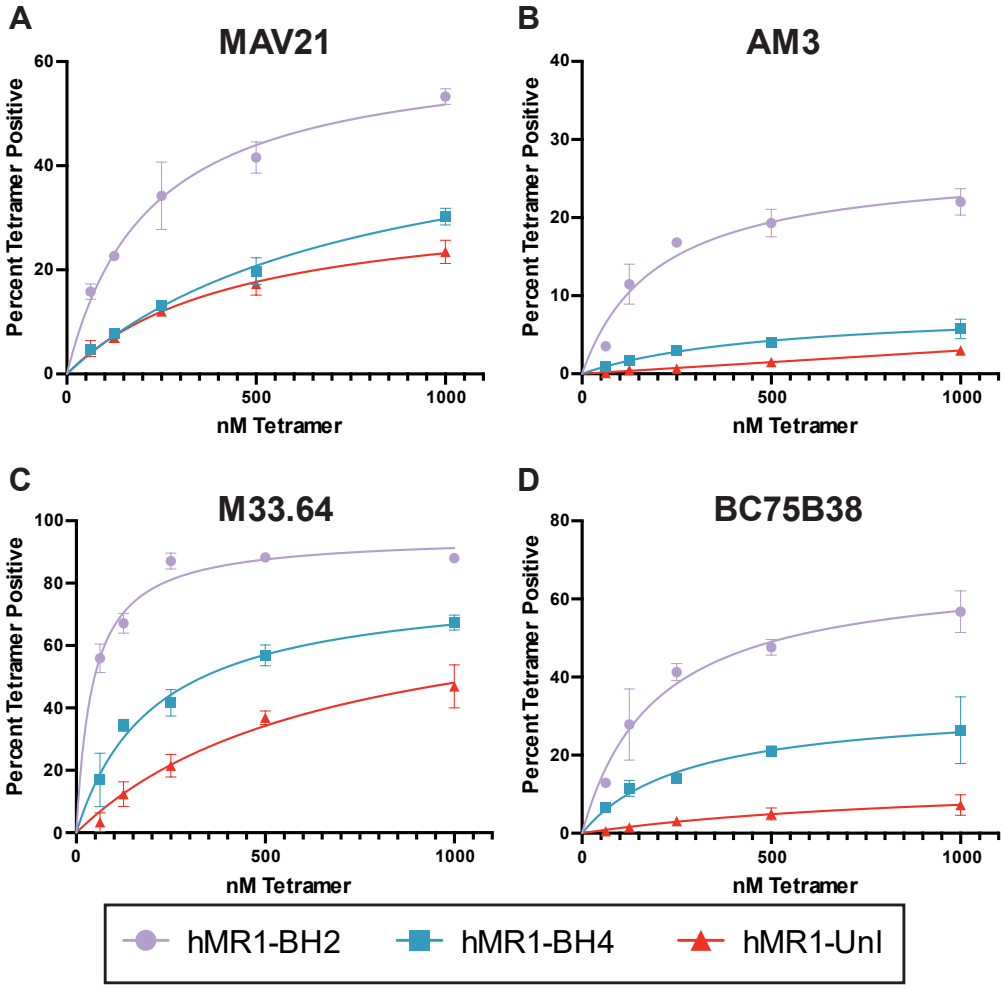



**Figure 3.3** EP-hMR1 tetramer staining of atypical MR1Ts and cross-reactive MAIT JrtBCD8α cells. (A-D) JrtBCD8α cells transduced to express the indicated TCR were stained with a titrated concentration of hMR1 tetramers loaded with BH2, BH4, or no ligand (Unl).

### 3.4 APCs loaded with endogenous pteridines activate cross-reactive MAIT and atypical MR1T TCR Jurkats in an MR1 dependent manner

As was shown in **Figure 3.2**, it is known that the canonical MAIT TCR, G11, does not bind to hMR1 loaded with pteridines, which is in line with its reputation as a highly 5-OP-RU specific canonical MAIT clone[68]. Indeed, it was shown in **Figure 2.5 B** that the introduction of pteridine ligands to a 5-OP-RU based activation assay with this MAIT clone blocked its activation, showing that EP ligands could play a role in dampening canonical MAIT responses. However, it was not clear if the binding of BH2 and BH4 tetramers by the atypical MAIT/MR1T cells described above would lead to productive signaling of their TCRs. To test this, we utilized JY.MR1 cells as APCs to present EP ligands to JrtB.CD8α cells expressing these MR1T TCRs. JY.MR1 cells were co-incubated for four hours with either 5-OP-RU, 6-FP, BH2, BH4, sepiapterin, or a vehicle control prior to the addition of an MR1 blocking (clone 26.5[52]) or an isotype control antibody. After a brief secondary incubation with the antibodies, JrtB.CD8α cells bearing either the AM3, MAV21, M33.64, BC75B38, or control (DP10) TCRs were added to the culture at a 1:1 ratio and allowed to co-incubate overnight. As a positive control for activation, PHA, a lectin that clusters TCRs and causes non-specific but potent TCR stimulation was added to one well for each JrtB.CD8α clone tested. The JrtB.CD8α cells from the culture were analyzed the next day for the early activation markers CD25 and CD69, which are upregulated in Jurkat cells following TCR stimulation (**Supplemental Figure S3.2 B**)[175].

As anticipated, the CD1d-sulfatide specific DP10 TCR expressed in JrtB.CD8α did not react with any preference of the ligand or blocking antibody, and no ligand significantly influenced the upregulation of either CD69 or CD25 non-specifically in this control JrtB.CD8α line (**Supplemental Figure S3.2 C**)[156]. The 6-FP reactive MR1T clones MAV21 and AM3, which each displayed a specific preference for hMR1-BH2 tetramers, were activated in an MR1-dependent manner by BH2 (**Figure 3.4 A and B**). MAV21 was also activated by sepiapterin

and, as previously reported[86], 6-FP while remaining agnostic to APCs loaded with 5-OP-RU and BH4(**Figure 3.4 A**). The ligand dependent activation of the MAV21 TCR implies that this TCR does indeed display a preference for pteridine ligands (folate-derived and EP) and has some mechanism for discerning between them, as it was not activated by BH4. On the other hand, AM3, which was initially reported to be 6-FP reactive (by tetramer staining only[86]), did not react to JY.MR1 cells loaded with 6-FP, BH4, or sepiapterin (**Figure 3.4 B**). Although, it should be mentioned that for the ligands it did react to (BH2 and 5-OP-RU), the observed increase in CD69 surface expression was modest at best, indicating that while this TCR may bind to MR1 tetramers it may not signal efficiently.

The remaining cross-reactive MAIT TCRs, M33.64 and BC75B38, did display some MR1 autoreactivity as evidenced by their high activation baseline when co-incubated with JY.MR1 cells and the vehicle control (**Figure 3.4 C and D**). While displaying a high baseline for activation with these MR1+ APCs, both cell lines did show a higher level of MR1-dependent reactivity to a variety of ligands. The M33.64 TCR's reactivity was boosted with the addition of 6-FP and 5-OP-RU, as previously reported, but also showed a marked increase in CD69 expression when stimulated with APCs loaded with BH2 and BH4 (**Figure 3.4 C**). Interestingly, sepiapterin, the strongest surface stabilizer of MR1 out of the EP panel, did not seem to activate the M33.64 TCR, indicating that the reactivity this TCR was displaying was not solely dependent on the MR1 surface expression of the APC. In our hands, the BC75B38 TCR did not have a significant increase in CD69 expression over baseline when co-incubated with 5-OP-RU loaded JY.MR1 cells, but did show a marked increase in activation when co-incubated with BH2 and BH4 loaded APCs (**Figure 3.4 D**). Taken together, these results highlight that MR1T TCRs which have been observed to sense folate-derived pteridine MR1 ligands can also sense EP MR1

ligands. This phenomenon extends to cross-reactive MAIT cells, with M33.64 and BC75B38 showing a large increase in CD69 expression when exposed to APCs displaying the EP ligands BH2 and BH4.

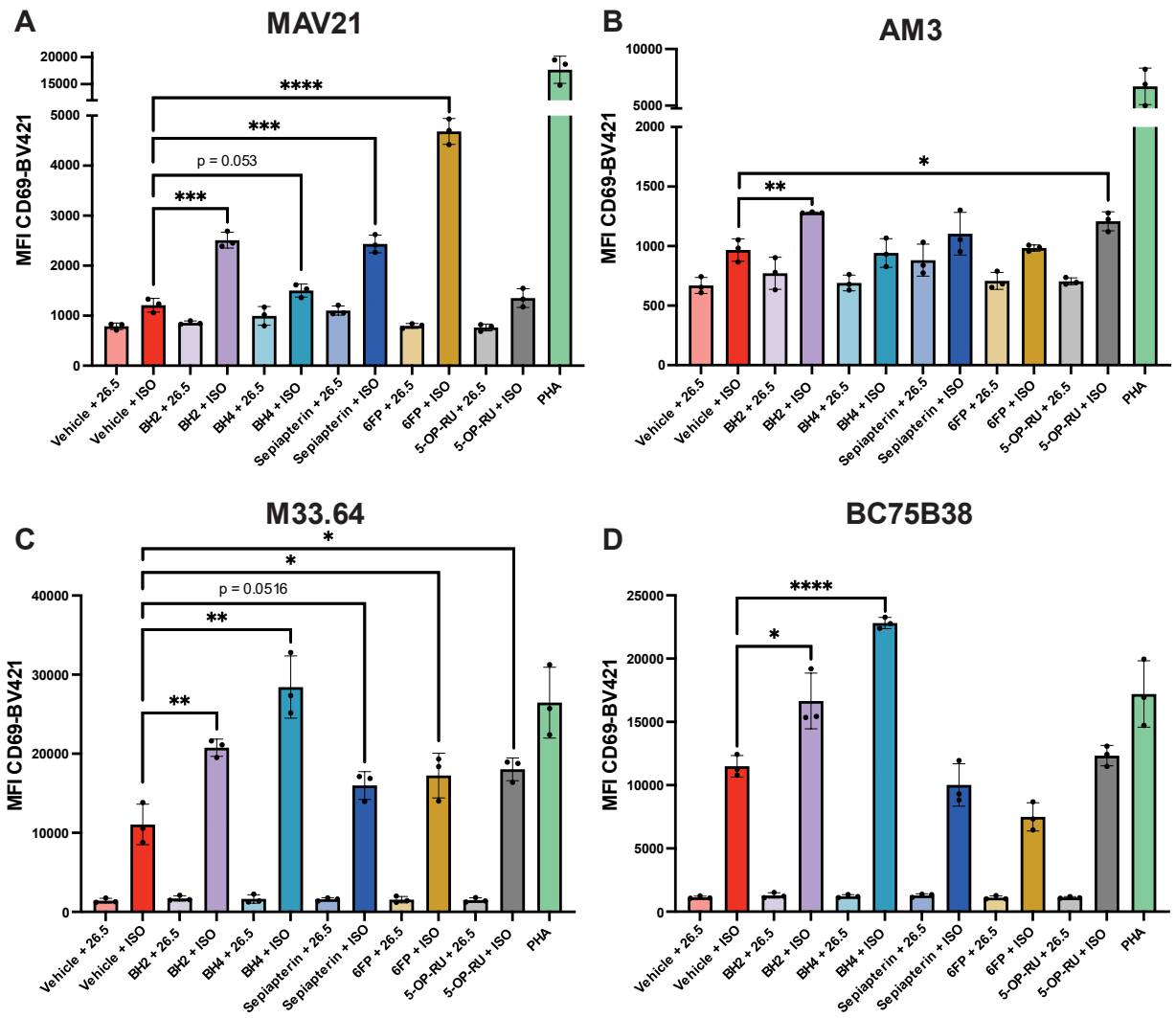



**Figure 3.4** Atypical MR1T cells and cross-reactive MAIT cells are activated by APCs loaded with endogenous pterin MR1 ligands. JrtBCD8α cells bearing the 6-FP reactive TCRs, MAV21 (A) and AM3 (B), as well as the cross-reactive MAIT TCRs M33.64 (C) and BC75B38 (D), were co-incubated with JY.MR1 cells pulsed with the indicated ligand. MR1 blocking antibodies (26.5) or an isotype control (ISO) were added to the culture to ensure reactivity was MR1-dependent. JrtBCD8α cells were analyzed for CD69 expression after 16 hours of coincubation. (A-D) Significance ascertained by an unpaired, two-tailed Student's t-test between the indicated groups. P values are indicated for each significant comparison or reported as *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P≤ 0.0001.

### 3.5 hMR1 tetramers loaded with BH2 and BH4 stain cancer-associated MR1T TCR Jurkats

Our results with the atypical MR1T and cross-reactive MAIT TCRs revealed that EP MR1 ligands play an important role in the activation of 6-FP and autoreactive MR1T cells. As discussed in **section 2.4**, EPs have a propensity to be overproduced in cancer and were discovered as MR1 ligands in cancer cell lines, making it critical to test the EP-reactivity of known cancer-associated MR1T TCRs. We assembled and expressed three previously reported cancer-associated MR1T TCRs, MG.7.C5, DGB129, and Tc5A87, in our JrtB cell lines and screened them for interactions with our panel of hMR1 tetramers[71, 72]. As reported in the literature, these TCRs were agnostic to MR1 loaded with the microbial antigen 5-OP-RU and 6-FP, but we observed modest staining of Tc5A87 with the hMR1-BH2 tetramer when expressed on the JrtB background (**Figure 3.2**). Due to our previous observation with the atypical MR1T cells, and the results of others doing similar assays[174], we hypothesized that CD8 may be critical for the recognition of EP-MR1 for these MR1Ts as well. When expressed in JrtB.CD8α cells, we observed a marked enhancement of both BH2 and BH4 tetramer binding over the hMR1-Unl control for both the DGB129 and Tc5A87 TCRs (**Figure 3.5 A and B**). These data led to two major conclusions. First, CD8 seems to be vital for the recognition of pteridine ligands presented by MR1. Second, it is possible that EP ligands could be an additional ligand source for cancer-associated MR1T cells in the context of neoplastic disease. To ensure that this recognition of EP-MR1 leads to activation through these TCRs, activation assays analogous to those completed for the atypical MR1T and cross-reactive MAIT Jurkats were performed.

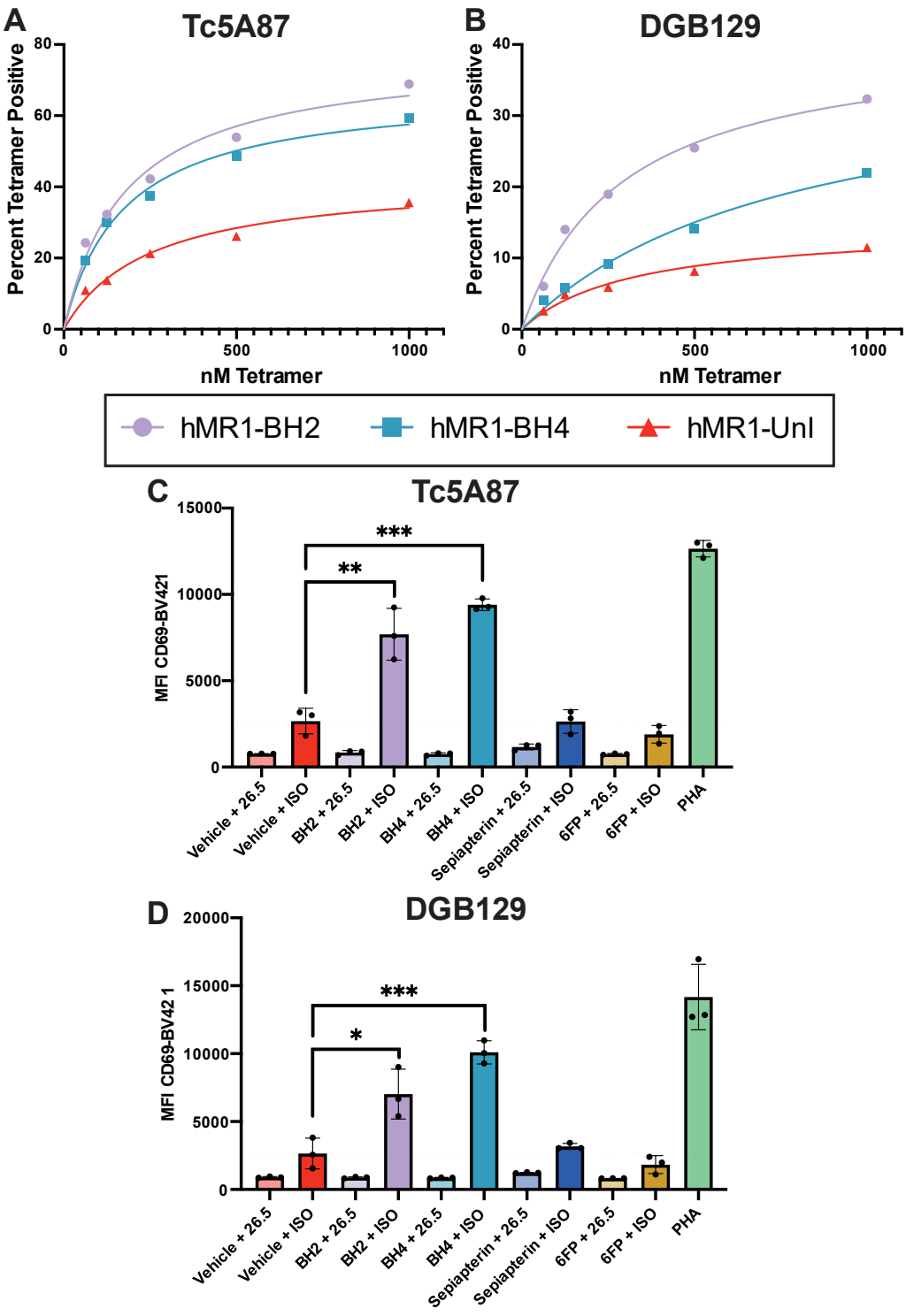



**Figure 3.5** EP-hMR1 tetramer staining and activation of cancer-associated MR1Ts. JrtBCD8α cells bearing the Tc5A87 (A) and DGB129 (B) were stained with titrated EP hMR1 tetramers. JrtB cells bearing the Tc5A87 (C) and DGB129 (D) TCRs were co-incubated for 16 hours with JY.MR1 cells loaded with the indicated ligand in the presence of an MR1 blocking antibody (26.5) or an isotype control (ISO). Activation was assessed by the upregulation of CD69 on the cell surface. (C and D) Significance ascertained by an unpaired, two-tailed Student's t-test between the indicated groups. P values are indicated for each significant comparison or reported as *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P≤ 0.0001.

### 3.6 APCs loaded with endogenous pteridines activate known cancer-associated MR1T Jurkats in an MR1 dependent manner

When considering the role of EP MR1 ligands in the immune response to cancer, I reasoned that these ligands could either be aiding immune evasion of the tumor by blocking the loading of other stimulatory ligands into MR1, much like what was observed with the MAIT TCR G11, or, EP ligands could be aiding the immune response through stimulation of MR1T cells, much like the nucleobase adduct ligands that have already been described[73, 74]. To test if the interaction between hMR1 loaded with EP ligands and these cancer-associated MR1Ts was stimulatory or antagonistic, we assessed the potential of APCs loaded with various EP ligands to stimulate JrtB lines bearing the Tc5A87 and DGB129 TCRs. After co-incubation with APCs bearing EP ligands, it was shown that both the JrtB.DGB129 and JrtB.Tc5A87 cell lines were activated by APCs loaded with BH2 and BH4, but not sepiapterin or 6FP, as assessed by a significant increase in CD69 MFI (**Figure 3.5 C and D**). These results clearly show that EP ligands are not leveraged by cancer cells to block the loading of stimulatory ligands and escape immune surveillance, but rather MR1Ts are capable of recognizing MR1 loaded with EP ligands along with the other products of metabolic stress that are presented on cancer cells and mount a direct immune response.

Taking these results and integrating them with the finding that the atypical and cross-reactive MR1Ts also recognize these ligands, a role for EP ligands in both homeostasis and metabolic dysregulation begins to emerge. It is clear from previous studies that these types of pterin-reactive MR1T cells exist and can be detected (albeit at low levels) in most healthy donors[66, 67, 78, 99]. It now seems possible that these MR1T cells can detect EP-MR1-mediated signals of metabolic distress related to the pteridine buildup associated with

inflammation. This likely plays a role in infection and injury to the mucosa and is something that is coincidentally leveraged in neoplastic disease, as cancer cells and activated T cells generate more of these EP ligands to fuel their growth and expansion. While the results of these *in vitro* assays were exciting, it was important that we confirmed the biological relevance of these cells, both in healthy individuals and individuals with neoplastic disease, to ascertain if they play a role in cancer surveillance *in vivo.*

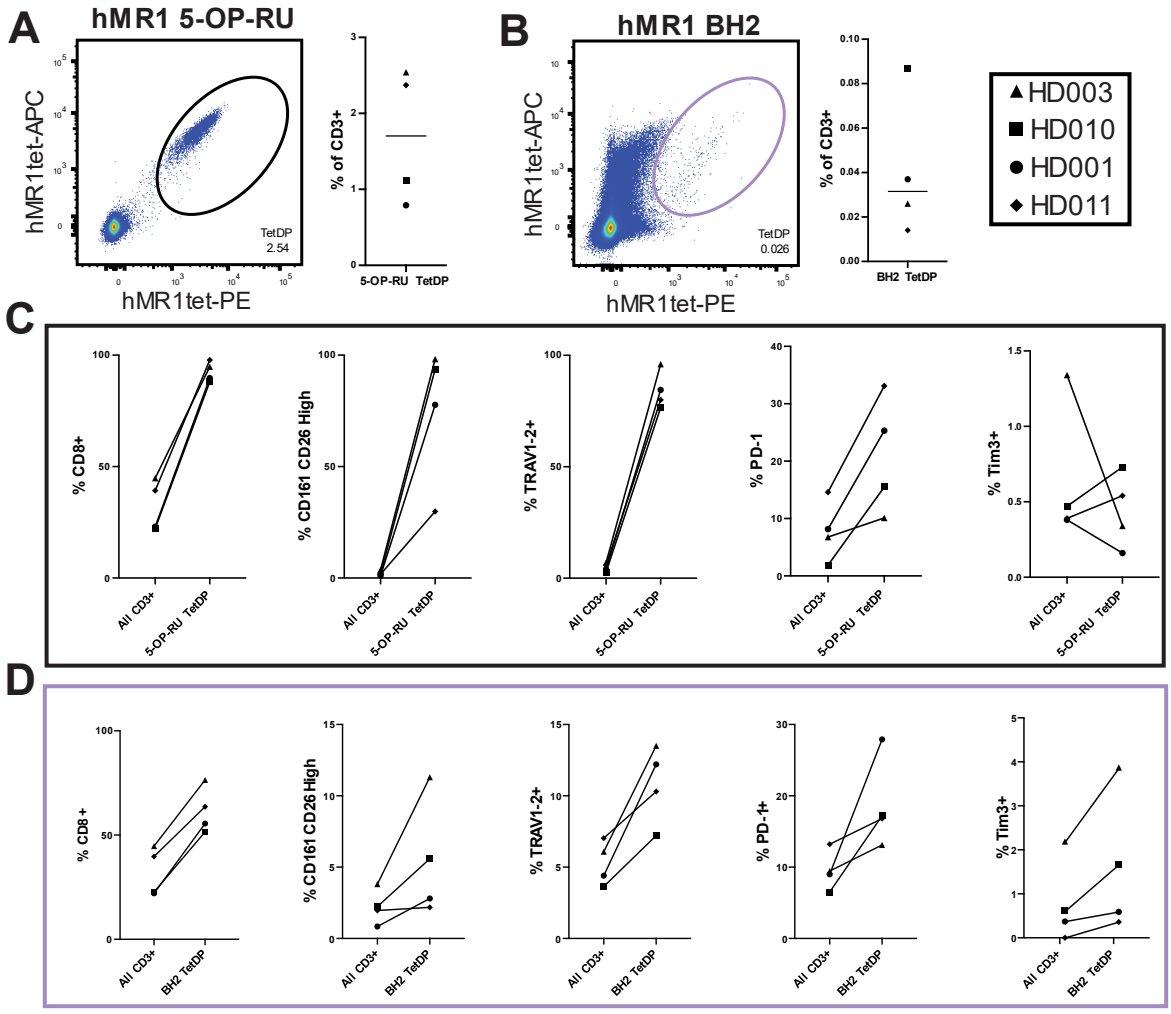



**Figure 3.6** BH2-hMR1 tetramers stain a conserved population of MR1Ts in healthy human PBMCs. A representative flow plot (HD003) for the tetramer dual staining of live, CD14-, CD3+ PBMCs from healthy donors is shown for 5-OP-RU (A) and BH2 (B), with the proportions for all donors indicated to the right of the plot. Paired analyses of the MAIT phenotype markers: CD8, CD161, CD26, TRAV1-2, PD-1, and Tim3 were performed for each healthy donor, comparing the tetramer double positive (TetDP) CD3+ population with all CD3+ in the sample for 5-OP-RU (C) and BH2 (D) hMR1 tetramers. Donor ID is depicted as different symbols throughout the figure. These symbols are denoted in the key on the upper right.

### 3.7 BH2-hMR1 tetramers reveal a population of cognate MR1T cells in healthy volunteers and myeloma patients

While our results have clearly shown a role for EP MR1 ligands in the biology of MR1T cells, it was important to discern the role these MR1 EP-reactive cells may have *in vivo*. Therefore, I devised an experiment to analyze the circulating population of EP-hMR1 tetramer positive cells in healthy individuals and to see if their resting phenotype could be ascertained. The BH2-hMR1 tetramer was selected for these tests as it seemed to bind stronger to MR1T TCRs than the BH4-hMR1 tetramer in previous experiments. The tetramers were included in the panel as both APC and PE conjugates, as to ensure true positive identification of EP-reactive MR1T cells by counting and analyzing only cells that were double positive for both PE and APC (**Supplemental Figure S3.3 A**). Additionally, a panel of antibodies targeting CD14, CD3, CD4, CD8, CD161, CD26, TRAV1-2, PD-1, Tim3, and a live dead stain were included to concurrently ascertain the phenotype of these tetramer positive cells (**Supplemental Figure S3.3 B**). Along with PBMC samples from four healthy donors, we were also able to procure PBMCs and CD138 negative BMMNC from a newly diagnosed myeloma patient. These additional samples allowed us to survey not only the circulating BH2-reactive MR1Ts, but also those that may be present at the tumor site. In addition to our BH2-hMR1 tetramers we included 5-OP-RU-hMR1 tetramers, both as a control for setting the tetramer double positive gates and due to the observation that canonical MAIT cells have been implicated as having both pro and antitumoral roles in multiple myeloma[126, 176-179]. By tracking both the BH2 reactive MR1T populations and the total canonical MAIT cell populations, we were able to draw meaningful conclusions about the roles that both of these cell populations may play in the surveillance, control, and progression of this disease.

As we found that these EP ligands seem to be both important to atypical MR1Ts during homeostasis and to cancer-reactive MR1Ts during the immune response to tumors, it was important to define the overall proportion of these cells and the resting phenotype they exhibit in the blood of healthy donors. PBMC samples from four healthy donors were analyzed with both 5-OP-RU and BH2 hMR1 tetramers, along with the antibody panel described above. It was observed that the canonical MAIT population of these donors ranged between 0.8% and 3% percent of circulating CD3+ T cells, in line with what is known about this population (**Figure 3.6 A**)[62]. Estimates of non-MAIT MR1T cells have been made by multiple groups ranging from 0.01-0.2%[66, 67, 71, 78, 99]. In line with these estimates, our BH2 tetramers were able to define a small, but reproducible, tetramer positive population in the blood of the healthy donors tested, ranging from 0.01-0.08% of circulating CD3+ T cells (**Figure 3.6 B**). A closer look at the phenotype of these cells was performed by comparing the tetramer double positive population of both 5-OP-RU stained and BH2 stained samples with the total CD3+ T cell population from the same sample (**Supplemental Figure S3.3**). The expected results were observed for the 5-OP-RU MR1 tetramer positive cells, showing a strong enrichment of the canonical MAIT markers CD8, CD161, CD26, and TRAV1-2 in the tetramer positive subpopulation (**Figure 3.6 C**). The BH2-MR1 tetramer positive cells showed the same general trends as the 5-OP-RU-MR1 tetramer positive population; enrichment for CD8, CD161, CD26, and TRAV1-2 was generally observed for all samples tested (**Figure 3.6 D**). However, it should be noted that this enrichment was not as stark as with the 5-OP-RU defined population, indicating that circulating EP-reactive MR1Ts are more phenotypically diverse than canonical MAIT cells. The only potential diversion from the similarities between 5-OP-RU and BH2 hMR1 defined populations was the expression of Tim3, which was enriched in the BH2-MR1 tetramer subpopulation over all CD3+ T cells, but

had mixed outcomes on a donor-to-donor basis within the 5-OP-RU MR1 tetramer defined subpopulation. While the small sample size negates any statistical significance to these findings, it is possible that higher Tim3 expression may be a defining characteristic of the BH2-reactive MR1Ts present in healthy PBMCs.

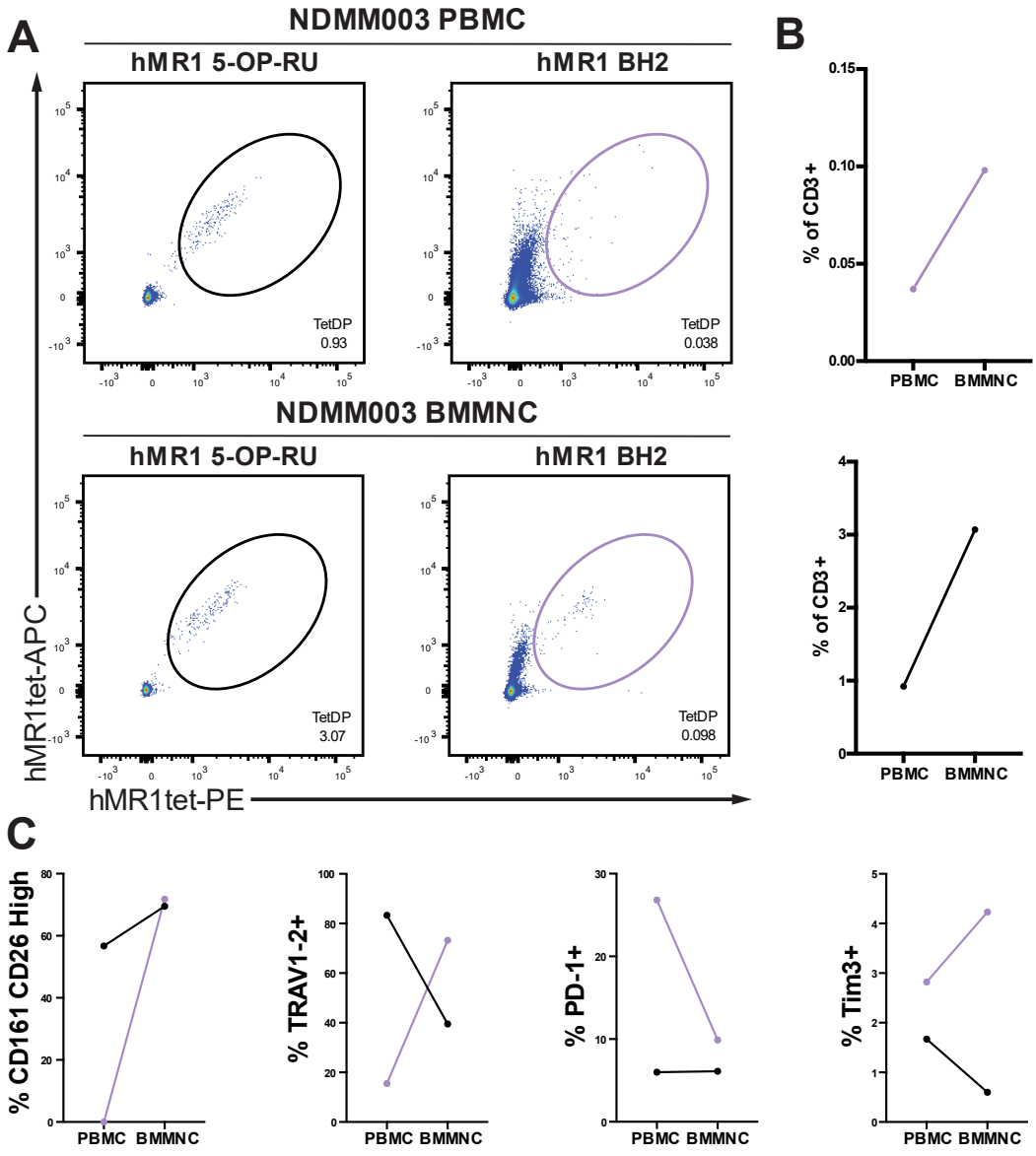



**Figure 3.7** hMR1 tetramer staining reveals an enrichment of BH2 reactive cells in the bone marrow of a newly diagnosed multiple myeloma patient. Tetramer dual staining of 5-OP-RU and BH2 hMR1 tetramers in both the PBMC and BMMNC compartments of a patient are shown (A) with the relative enrichment between compartments shown as well (B). The phenotype of the tetramer double positive cells is shown in (C) comparing between the PBMC and BMMNNC compartments for each marker. BH2-hMR1 tetramer-stained cells are shown in purple. 5-OP-RU tetramer-stained cells are shown in black.

To ascertain the role these EP-reactive MR1Ts may be playing in multiple myeloma, we procured paired PBMC and BMMNC samples from the same patient. These samples were taken during initial biopsy at diagnosis for this multiple myeloma patient; this patient had not yet undergone any treatment for the disease. As has been described previously in multiple myeloma patients, it was also observed here that there were fewer circulating MAIT cells in the blood and a corresponding enrichment in the bone marrow (**Figure 3.7 A and B**)[126, 176]. Similarly, BH2-tetramers stained a relatively low proportion of CD3+ T cells in the blood of this patient, but an over 2-fold increase was observed when assaying the BMMNC sample (**Figure 3.7 A and B**). Interestingly, the tetramer double positive population that was stained in the BMMNC sample had a notably "tighter" grouping, which is indicative of strong tetramer binding and was shown to be generally enriched for traditional MAIT markers when compared to cells stained in the blood (**Figure 3.7 A and C**). It was observed that the cells found in the patient BMMNCs seemed to have lower expression of PD-1, but higher expression of Tim3 - a trend that opposed the 5-OP-RU hMR1 tetramer reactive cells in the same compartments (**Figure 3.7 C**). While firm conclusions cannot be drawn from the data derived from one patient, this is a noteworthy result for a pilot study, and we are working to procure more patient samples for a set of follow up experiments to investigate if these trends hold true in a broader patient population.

### 3.8 Discussion and ongoing work

The data described here and in conjunction with those in **Chapter 2**, highlight the potential purpose of EP MR1 ligands in their ability to stimulate MR1T cells. These pteridine ligands are truly endogenous to the human body and likely bind MR1 as they are produced during inflammation. The MR1T cells that recognize the EP MR1 ligands used in this study were

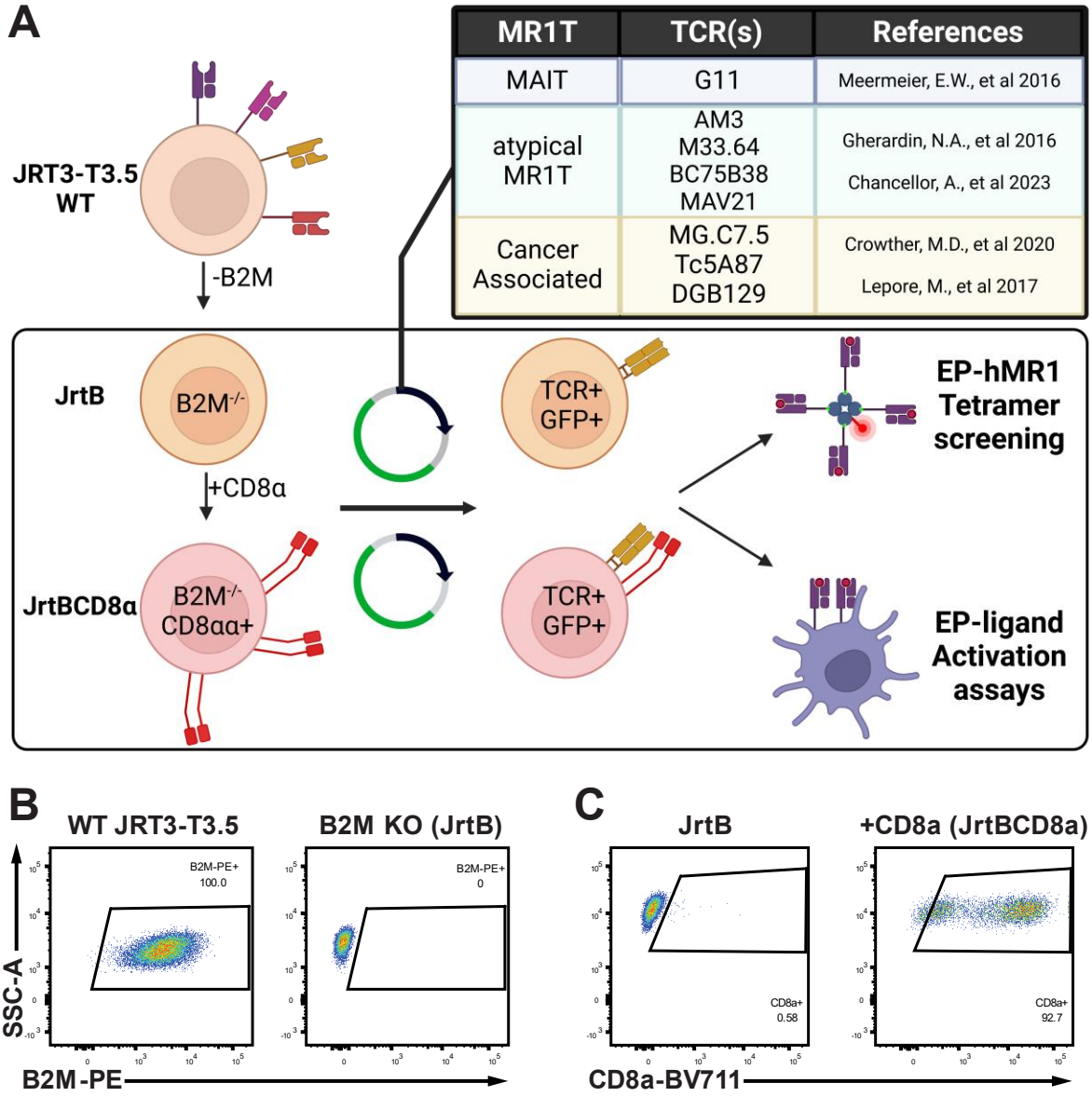
all derived from healthy individuals, and because many of them have been shown to be agnostic to microbially derived ligands, it is implied that the EP ligands they recognize are important for their function in maintaining homeostasis beyond microbial control[71, 72, 78].

This is the first discovery of a pool of endogenous MR1 ligands that many members of the MR1T family can recognize. The discovery of a class of "unifying" endogenous ligands makes it tempting to hypothesize that these cross-reactive MAITs, atypical MR1T cells, and cancer-associated MR1T cells are all much more similar than previously appreciated. It is quite possible they all serve an analogous function at homeostasis, possibly to recognize MR1 when it is sent to the cell surface with EPs - a process that can be controlled by inflammatory signals and the APC's own production of BH4 and its intermediates. Perhaps the MR1T cells that have been described as "cancer-specific" function the same as other MR1T cells at steady-state but have an ability to cross-react between EP ligands and the modified nucleobases that are only present in cancerous tissue, giving them enhanced sensitivity to MR1 in the context of neoplastic disease. In support of this notion, it is important to remember that even these cancer-associated MR1Ts were derived from healthy individuals, it is therefore implied that they are maintained in the body for a purpose that is not solely cancer surveillance. Indeed, helper like functions beyond simply target killing, such as DC licensing or monocyte recruitment, have been demonstrated for many of the cancer associated MR1T clones discovered[71]. Is it possible that their primary function is to enter inflamed tissues, recognize the tissue is stressed via MR1-EP complexes, and then signal to recruit cells from the innate immune system? Does the ligation of their TCR with MR1-EP complexes give these cells more nuanced effector profiles? Perhaps favoring the expression of wound healing factors in addition to the helper functions that have been ascribed to them. Certainly, more work needs to be done with EP reactive primary cells to see if these MR1

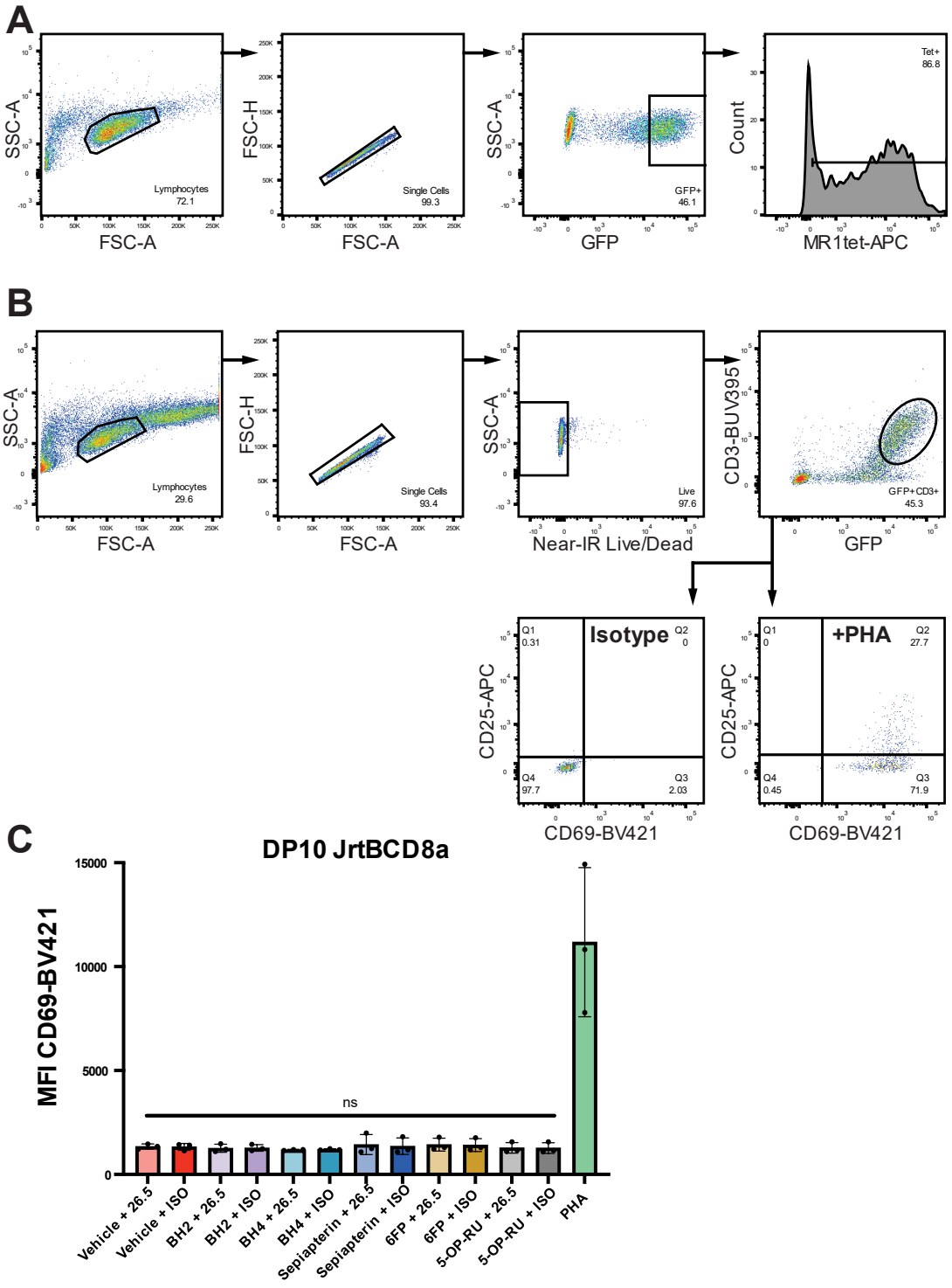
ligands can induce a different effector response than stimulation with microbial or even nucleobase-adduct ligands.

The study of MR1-BH2 reactive cells derived from healthy and myeloma patient samples provides much needed biological context for the results obtained using MR1T Jurkat cell lines. In line with how EP MR1 ligands were able to interact with both non-MAIT and MAIT MR1T cells, it was shown that the BH2-hMR1 tetramer stains a population that exhibits moderate expression of canonical MAIT markers. These data support the hypothesis that EP-ligands are a class of endogenous "bridge" ligands that can interact with cell types from across the MR1T cell family. Taking this broad reactivity into account, it is tempting to speculate that these EP ligands may play a role in the development of MR1T cells and perhaps even MAIT cells. It is worth noting that the thymus is full of proliferating thymocytes, and previous work has clearly shown that EP molecules are important mediators of T cell expansion[156]. This could potentially provide an environment that is rich with EP ligands for MR1 that double positive thymocytes can use to test their TCRs for MR1 binding. An additional body of work would be needed to investigate this hypothesis, but it is a clear future direction that the study of EP MR1 ligands could take. When considering all the data presented in the previous two chapters, it is evident that these EP ligands are important to the majority of MR1T cell subclasses outside of canonical MAIT cells. I am confident that further study of these ligands will help answer many of the remaining questions about the development, general biology, and roles of non-MAIT MR1T cells in health and disease.

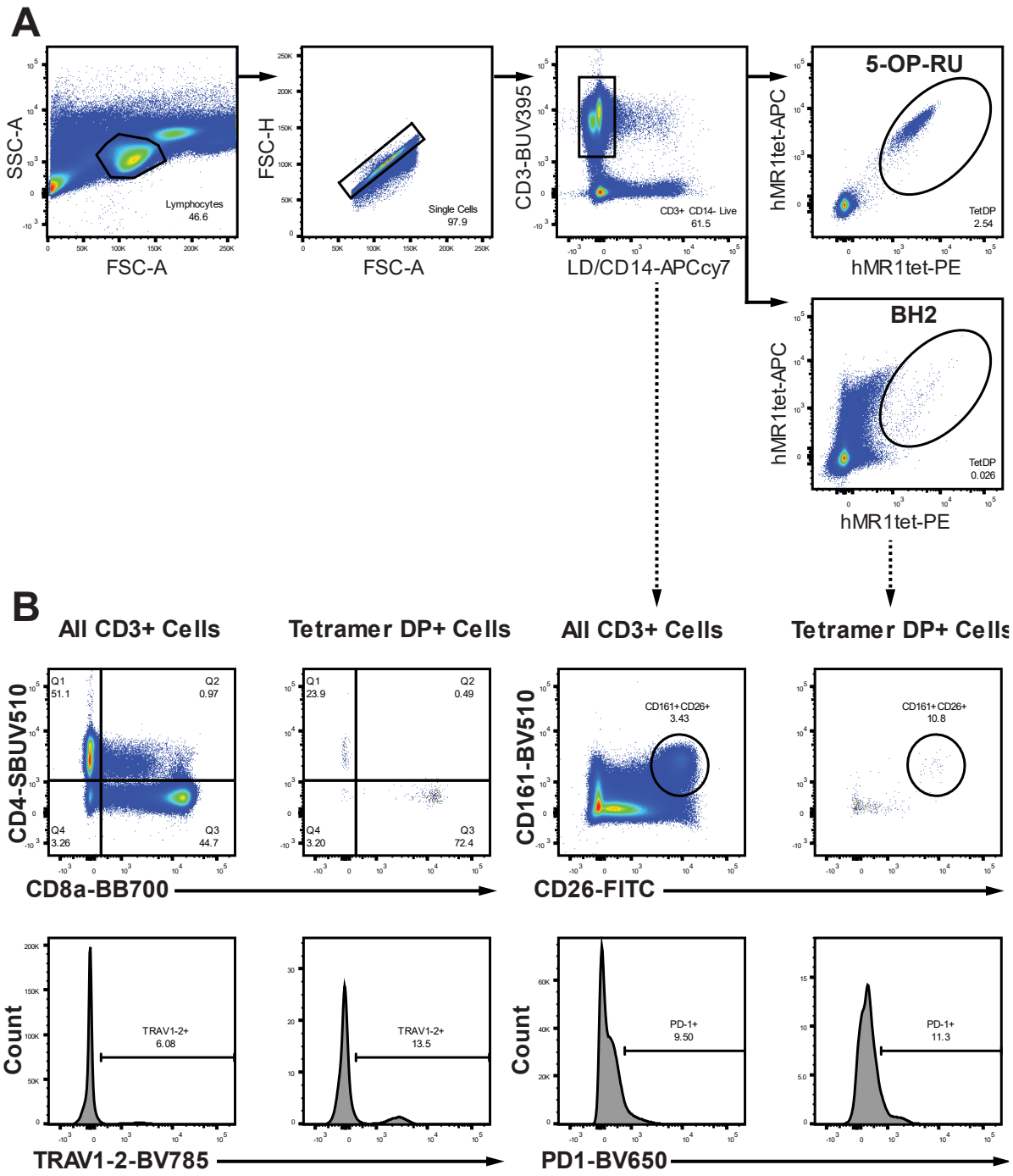
### 3.9 Supplemental information




**Supplemental Figure S3.1** The generation of β2M KO and CD8α+ Jurkat lines. (A) A schematic shows how the JrtB and JrtBCD8α cell lines were made and summarizes the published MR1T TCRs used in this study. (B) Flow plots show the efficacy of the β2M KO performed on WT JRT3-T3.5 cells in order to generate the JrtB line. (C) Flow plots show the further modification of the JrtB line with a lentiviral vector containing the DNA sequence for human CD8α to create the CD8α+ β2M- JrtBCD8α cell line. Data in (C) was generated by Andrew Kelly.




**Supplemental Figure S3.2** Gating strategies for tetramer staining and activation assays. (A) The gating strategy used for all tetramer staining assays with the BH2-hMR1 staining of M33.64 at 1μM shown as a representative plot. (B) The gating strategy used for all activation assays with JrtB variants and JY.MR1 cells. The staining of DP10 TCR expressing JrtBCD8α cells is shown as a representative plot under either isotype antibody staining or full staining with cells treated with PHA. (C) The unrelated, CD1-d-sulfatide-restricted γδ TCR, DP10 is not activated by JY.MR1 APCs loaded with EP or traditional MR1 ligands and is not non-specifically activated by the ligands themselves.




**Supplemental Figure S3.3** The gating strategy used for hMR1 tetramer dual staining of PBMC and BMMNC samples. (A) The gating strategy used to exclude CD14+ monocytes, dead cells, and other CD3- cells prior to tetramer gating is shown. (B) Representative flow plots are show (HD003) for how positive populations were gated for the phenotype features studied in **Figures 3.6 and 3.7**. All gates were drawn using an FMO sample for each color.

# Chapter 4. γδ T cells recovered from asthmatic lung tissue bind MR1 in an antigen-dependent manner

## 4.1 Introduction

As discussed in **Section 1.3.3**, γδ MR1T cells are a relatively new addition to the MR1T family of T cells. To date, there have only been two major publications on the observed interactions between γδ TCRs and MR1, both of which detail the interactions of TCRs derived from the same panel of MR1 5-OP-RU tetramer positive γδ T cells generated from the blood of healthy individuals[110, 111]. The first of these studies revealed one of the most unusual binding modes ever described for an interaction between a TCR and an antigen presenting molecule[110]. With CDR residues from the Vγ9Vδ1 G7 TCR binding primarily to the membrane proximal α3 domain of MR1, and a select few interactions between the TCR and the underside of the antigen binding pocket, the G7 TCR binds completely perpendicular to MR1[110]. This discovery, along with activation and binding kinetics data from the whole panel of TCRs, highlighted an antibody-like and metabolite-independent recognition of MR1 by these γδ TCRs. Structural data from the follow up study pushed this paradigm forward. This study focused on another γδ MR1T TCR (G83.C4, Vγ8Vδ3) that bound MR1 in a more "canonical" way, by making contact with the α1/α2 platform rather than the membrane proximal α3 region, yet still did not interact with the antigen in the binding pocket. Together, these studies have provided foundational evidence for the interactions between γδ T cells and MR1, adding γδ MR1Ts to the MR1T family, but at the same time, have argued that γδ MR1T interactions occur only in an "MR1 autoreactive" manner.

In this chapter, I will provide evidence that challenges the paradigm that γδ T cell recognition of MR1 is limited to antibody-like protein-protein contacts. Through the work of two

previous graduate students in the lab, Dr. Augusta Broughton and Dr. Nicole Ladd, it was discovered that there are γδ MR1T cells present in human lung tissue. I was able to isolate, clone, and express one of these γδ MR1T TCRs in the JrtB cell line and was surprised to find that it exhibits exceptional antigen specificity in its interactions with MR1. I discovered that this γδ MR1T TCR would not bind MR1 tetramers loaded with any canonical or heterogenous mixture of ligands but instead had a strong preference for the MR1 ligand PLIII. This ligand was described through the work of our lab and others[68], and is found in MR1 samples generated in the presence of the mycobacterium M smegmatis. A closer analysis of the scRNA-seq dataset that uncovered the antigen-specific γδ MR1T TCR, GDM95, revealed that there were other γδ MR1T cells that bound to the hpMR1-Msmeg tetramers used in the original experiment. These γδ MR1T cells were found almost exclusively in the lung tissue of patients who reported having asthma during their lifetime. When their gene expression was compared to the other T cells in the dataset, they revealed signs of microbial antigen exposure, activation, and possible participation in the type 2 asthmatic immune response. It was also observed that these lung-resident γδ MR1T cells favor the use of certain TCR genes and an inspection of the CDR3δ loops of these TCRs revealed a conserved motif imparting specific biophysical properties to these TCRs. Current work is focused on describing the structural details of how the antigen specificity of the GDM95 TCR is achieved and if this type of MR1-PLIII recognition is commonplace among lung-resident γδ MR1Ts. The conclusions drawn from this study imply that, while antibody-like recognition of MR1 is certainly a mode of recognition for γδ MR1Ts, it is also possible for these cells to exhibit antigen dependent recognition as well. Ongoing investigation may help us understand how mycobacterial infection can impact the γδ MR1T

repertoire of the lung and identify if these cells have broader impacts in the pathogenesis of asthma or mycobacterial infection.

### 4.2 Barcoded MR1 tetramers loaded with antigens from M. smegmatis bind to γδ T cells recovered from human lung samples

Our lab has been interested in investigating antigen specific interactions between antigen presenting molecules and γδ T cells since the initial discovery that γδ T cells can interact with CD1d in a lipid permissive manner[41, 109]. A former graduate student, Dr. Augusta Broughton, was involved in exploring this further by investigating the CD1d-restricted γδ T cell subset present in human lungs and the potential role these cells may have in asthma pathogenesis. To do this, Dr. Broughton and Dr. Ladd generated CD1d and MR1 tetramers labeled with DNA-barcodes – allowing them to be used analogously to CITEseq antibodies[180], surveying the TCRs of labeled cells for antigen reactivity (**Figure 4.1 A**). These tetramers were used to sort out lymphocytes extracted from both asthmatic and non-asthmatic explanted lung tissue, and these tetramer positive cells were subsequently used to generate a scRNA-seq data set on the 10x Genomics Chromium Platform™. The goal of the initial experiment was to capture the transcriptome, paired TCR repertoire, and antigen specificity of CD1d reactive γδ T cells in these tissues. The hpMR1 tetramer, loaded with antigens from M. Smegmatis (hpMR1-Msmeg), was included as a negative control – hypothesizing that a microbially loaded hpMR1 tetramer would mainly extract αβ MAIT cells from the lung tissue, providing a disparate population of αβ T cells to compare the recovered γδ T cells to. After the resulting data was analyzed, it was revealed that this hpMR1-Msmeg tetramer bound to several γδ T cells along with the anticipated group of MAIT cells (**Figure 4.1 B**). Surprisingly, one of the most highly expanded γδ T cells in the data

set, referred to from here on as GDM95, had a high read count for the hpMR1-Msmeg barcode and a greatly reduced read count for the CD1d tetramer barcode (**Figure 4.1 C**).

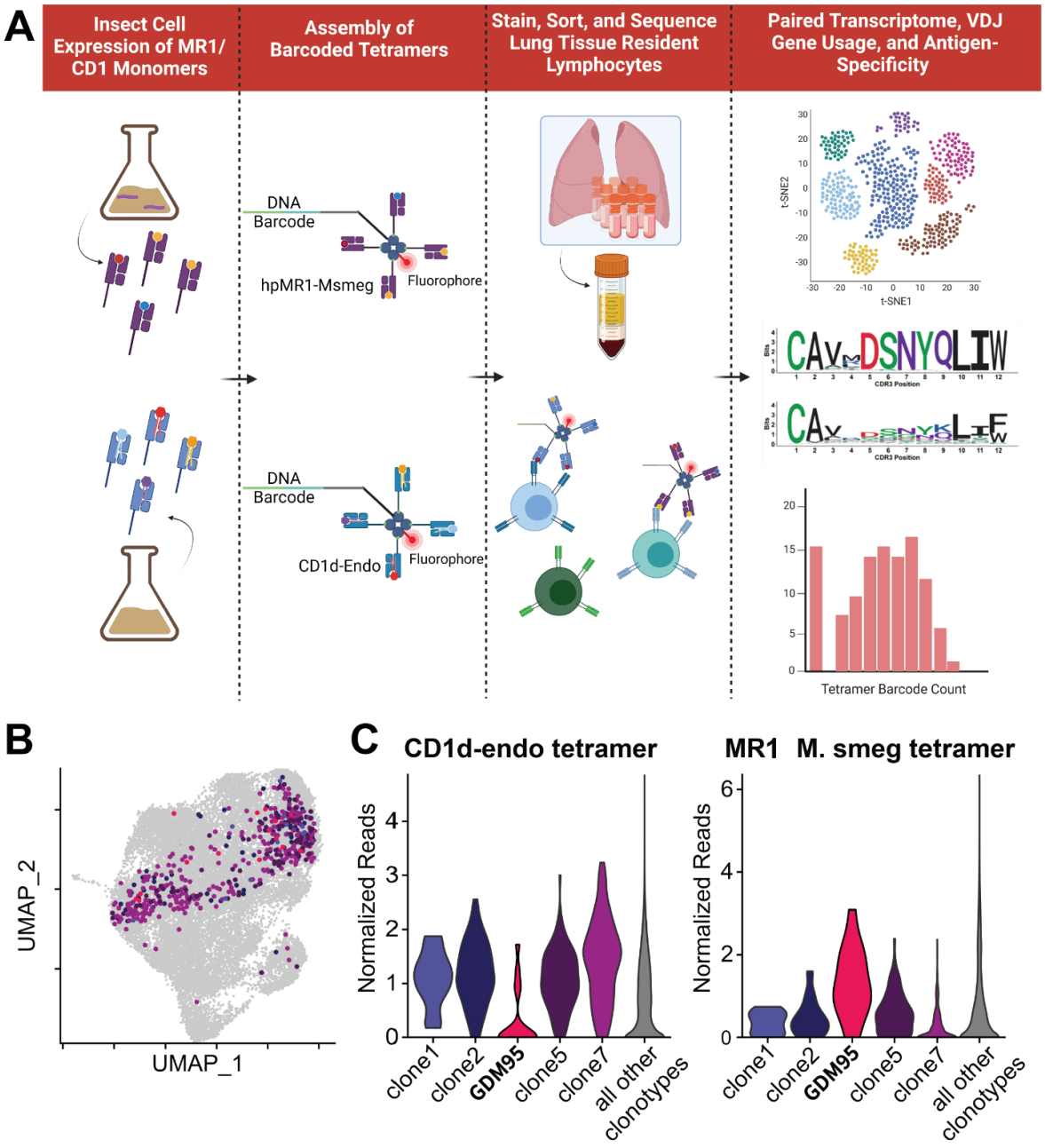



**Figure 4.1** The discovery of γδ MR1T cells in explanted lung tissue. A) An overview of the scRNA-seq experiment that was utilized to isolate tetramer reactive T cells from the tissue of explanted lungs. B) A UMAP projection of all T cells recovered in this analysis. The colored dots belong to the γδ T cell clones shown in C. C) The comparison of different barcoded tetramer reads between the γδ T cells with the highest expansion state in the scRNA-seq dataset. The γδ MR1T clone, GDM95, is highlighted in red. Data in B and C were collected by Augusta Broughton and adapted for this figure.

### 4.3 γδ MR1T cells from the lung display similar TCR gene usage as well as similarities in the sequence and biophysical properties of their CDR3δ loops

In total, nine unique γδ T cell clones were found with a high read count for the MR1 tetramer and a low read count for the CD1d tetramers (**Figure 4.2 A**). Interestingly, all but one of these clones were derived from the lung samples that were from patients who reported having asthma. The one γδ T cell clone that was derived from the "healthy" lung tissue sample was found to be a Vγ10 TCR through VDJ sequencing. As briefly discussed in **section 1.3.3**, in humans, Vγ10 is considered to be a pseudo gene, meaning that it does not code for a productive protein due to mutations incurred during evolution[102]. Due to the questionable nature of the sequencing assignment, this TCR was excluded from downstream analysis, meaning all of the γδ MR1Ts that were derived from this experiment were found in the lungs of patients who reported having asthma. These γδ MR1T clones seemed to highly favor Vδ1 gene usage, this is not necessarily surprising given the fact that these are tissue resident γδ T cells, which are known to primarily express Vδ1 (**Figure 4.2 B**)[102, 181]. As far as gamma chain usage was concerned, these cells were more diverse, but did show a preference for Vγ4, Vγ2, and Vγ8 (**Figure 4.2 B**). A few clones were Vγ8 and Vγ9(Vδ2), but these were only found as unexpanded, single clones and when taking this into account, their overall contribution to the total TCR gene distribution is negligible. Analysis for sequence similarities in the CDR3 loops of these γδ MR1Ts revealed no real consensus in untemplated amino acids in the CDR3γ loops for these TCRs (**Figure 4.2 C and Supplemental Figure S4.1 A**). However, the CDR3δ loops did seem to have conservation of a certain hydrophobic/basic motif near the center for their CDR3 loop (**Figure 4.2 D)**. This motif, FL(P/H)W(G/K)G, is partially derived from the TRDD genes shared by these TCRs, but the contribution of the more basic residues are from untemplated N and P additions, implying

possible directional selection for this motif (**Supplemental Figure S4.1 A and B**). The expansion state and presence of this CDR3δ motif in these γδ MR1Ts led us to investigate the CDR3δ in more detail using the Automated Immune Molecule Separator (AIMS) software package.

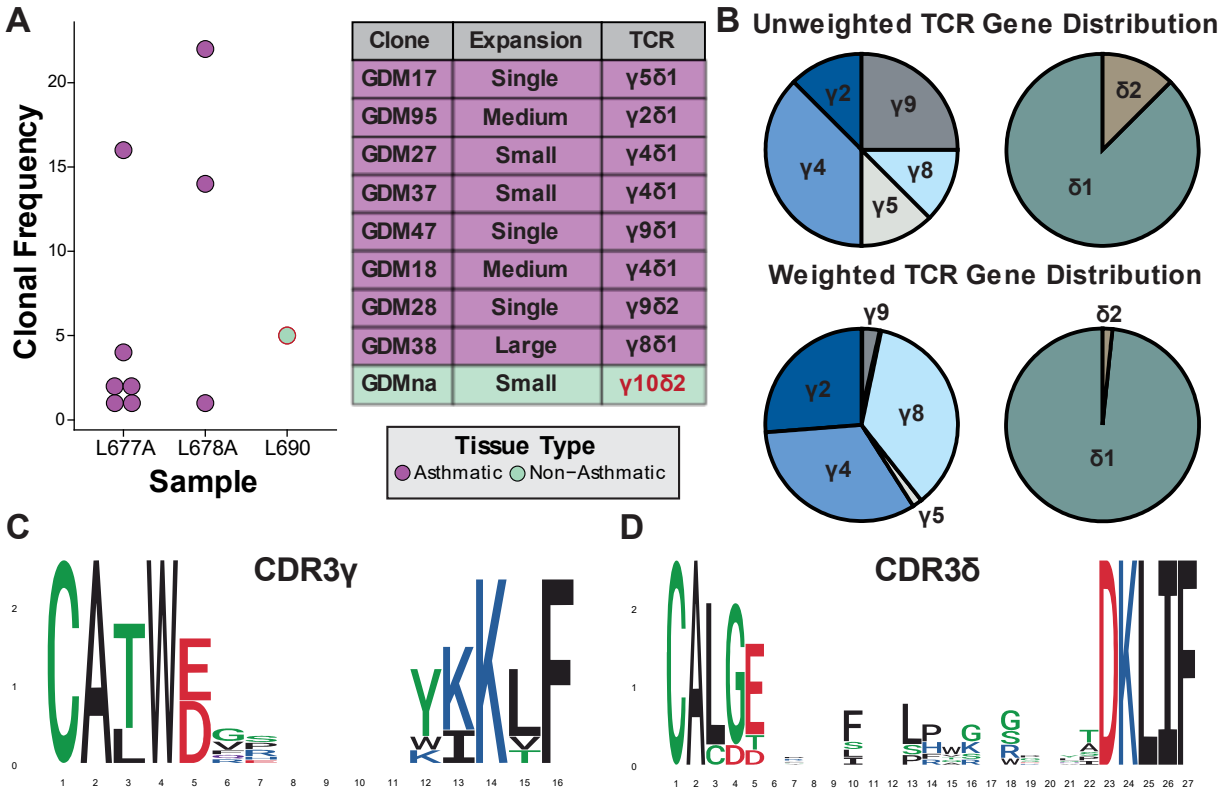



**Figure 4.2** Overview of the γδ MR1T subpopulation found in explanted lung tissue. A) Distribution of the γδ MR1T cell clones found to have a high read count for only the hpMR1-msmeg tetramer. The expansion state of each clone is denoted as the number of cells recovered bearing the exact same TCR nucleotide sequence: single =1 cell, small = 2-5 cells, medium 6-20 cells, and large = 20+ cells. B) The TCR gene usage of the γδ MR1T cells found in this data set. Weighted TCR gene distribution figures take into account the expansion state of each clone. C) A sequence logo derived from the aligned CDR3γ loops. D) A sequence logo derived from the aligned CDR3δ loops.

This software package, which was developed by a previous graduate student in the lab, Dr. Chris Boughter, allows for the high throughput analysis and comparison of immune receptors through the characterization of their biophysical properties[182, 183]. It works by considering

the positions, biophysical properties, and local environment of each residue in the context of the entire receptor or CDR loop that is being analyzed. It then uses these properties to make generalized predictions about the behavior of the receptor, accomplished through the generation of a high-dimensionality matrix detailing all the intrinsic and relative biophysical properties of each residue. This is beneficial as some of the structural information is maintained (i.e. the sequence position and relative distance between amino acids), but the focus is on the positional and grouped biophysical properties made when considering the entire CDR loop or receptor. This approach has allowed for experimentally confirmed predictions about antibody polyreactivity, SARS-COV2 binding epitopes, CDR1/2 mediated MHC recognition, and the TCR repertoire that can be observed in different cancers[184-187]. Due to the recurring semi-untemplated motif that was observed in the expanded γδ MR1T cell CDR3δ loops, I hypothesized that the biophysical properties of the γδ MR1T CDR3δ loops found in this dataset may differ in comparison to all the other γδ T cells that have little to no MR1 tetramer barcode reads.

After pruning the list of MR1-tetramer negative γδ T cell clones to include only those with one paired TCR receptor and then collapsing the data to look at only one instance of each unique CDR3δ, I generated a list of unique 214 non-MR1 γδ T cell clone CDR3δ sequences. These CDR3δ sequences were compared directly to the eight unique, non-pseudo gene containing γδ MR1Ts derived from the same dataset. After sequence alignment and the generation of a matrix summarizing the biophysical properties of each sequence, the AIMs software performs a dimensionality reduction step and a subsequent clustering analysis that groups CDR3 sequences by similarities in their biophysical characteristics. This is done in an unbiased fashion not taking into account which group (MR1 tetramer positive or MR1 tetramer

negative) the CDR3 sequence came from. It was decided to use the DBscan clustering algorithm for these data as this algorithm will specifically remove any "noise" CDR3 sequences that do not fit neatly into the clusters found, allowing me to focus on truly disparate clusters[188]. I found that 100% of the γδ MR1T CDR3δ sequences cluster together in the largest of the five clusters that were generated after PCA dimensionality reduction (**Figure 4.3 A and B**). Next, I utilized AIMs to calculate position specific fluctuations in charge, bulkiness, hydropathy, and main-chain flexibility for each group of sequences. As anticipated from the fluctuating range of basic, hydrophobic, large, and small residues that was observed in the γδ MR1T CDR3δ motif (**Figure 4.2 D**), the AIMS software revealed a significant fluctuation in charge and steric bulk when comparing the γδ MR1T CDR3δ sequences to those from the MR1 tetramer negative group (**Figure 4.3 C**). It should be noted that these observations cannot be attributed to any relative CDR3δ length discrepancies between the two data sets, as no significant difference in CDR3δ length was observed between the MR1 tetramer positive and negative datasets and the "budge" alignment feature used in the initial processing step automatically corrects for any differences in sequence length (**Supplemental Figure S4.1 C and D**)[183]. When averaging these biophysical properties over the entire CDR3δ sequence and comparing between datasets, a significant difference in overall charge can be observed, meaning that the net charge from the γδ MR1T CDR3δ sequences is generally more basic than in the MR1 tetramer negative γδ T cell group (**Figure 4.3 D**). While the bulkiness of these residue side chains fluctuates on a positional basis as shown in **Figure 4.3 C**, the overall average of this property is not significantly different from the MR1 tetramer negative γδ T cell group (**Figure 4.3 D**). This makes logical sense when considering that these large positional differences fluctuate equally in either direction across the positional average of the MR1 tetramer negative γδ T cell group (**Figure 4.3 C**).

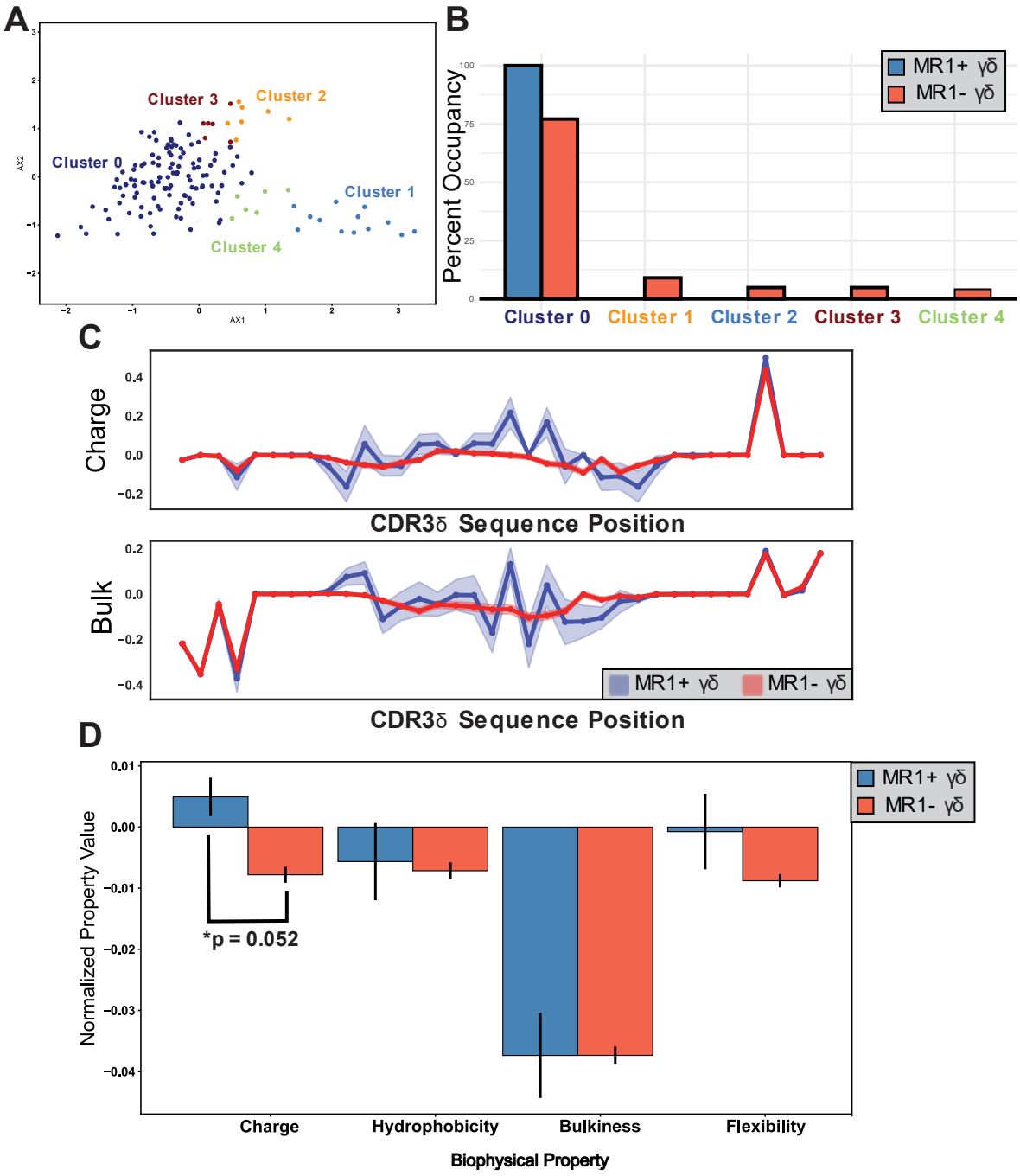



**Figure 4.3** AIMS results predict biophysical similarities in γδ MR1T TCRs. A) PCA dimensionality reduction and DBScan clustering of the γδ MR1T and MR1 tetramer negative γδ T cell CDR3δ loops included in this analysis. B) The composition of each CDR3δ cluster is broken down by whether the sequence came from a γδ MR1T or a MR1 tetramer negative γδ T cell. C) Positional variance in charge (top) and steric bulk (bottom) for each residue in the analyzed CDR3δ loops. D) The averaged value for the entire CDR3δ loop sequence for the given biophysical properties. Significance shown in D is from Welch's t-test with degrees of freedom set to 1, the p-value is indicated on significant comparisons.

This analysis of the biophysical properties of the γδ MR1T CDR3δ sequences reveals that not only is there a conserved motif that seems to be utilized by these TCRs in their recognition of MR1, but this motif imbues these γδ MR1T TCRs with unique biophysical properties that may allow for this interaction. It seems that the fluctuation between large and small residues, as well as positively charged and hydrophobic residues, may be the most critical of these characteristics. It is tempting to speculate that these fluctuations may allow for the γδ MR1T CDR3δ loop to insert larger or more charged residues into harder to reach positions, perhaps within the binding pocket of MR1, during target recognition. Direct biochemical and structural experimentation will be needed to fully test this hypothesis.

### 4.4 γδ MR1 T cells in the lung show signs of bacterial activation, have a higher expression of NK receptors, and show more sign of participating in allergic responses

The nature of the scRNA-seq dataset that these γδ MR1Ts were found in allowed for profiling of their phenotype, as well as their TCR repertoire, through analysis of the transcriptome. Gene expression data for the γδ MR1T cell subset was compared to all other αβ and γδ T cells recovered in the data set. This comparison revealed that the γδ MR1T subset appeared to be activated showing higher than average expression of TOX, a transcription factor associated with prolonged TCR stimulation, and effector molecules like granzyme-A (GZMA) and granzyme-K (GZMK), as well as the chemokine XCL2 (**Figure 4.4 A)**[189]. Upon activation, γδ T cells have been shown to upregulate a "professional" APC-like program which was evidenced in these data as well - with the γδ MR1T population showing high RNA expression of MHC-II molecules as well as HLA-F[190]. The γδ MR1T population also had significantly less expression of the IL-7 receptor which is associated with a less naïve and more

effector-like phenotype (**Figure 4.4 A**)[191]. Another striking difference found in the γδ MR1T population when compared to all other T cells, was the heightened expression of NK receptors (**Figure 4.4 B**). While γδ T cells are known to have a higher-than-average expression of NK receptors[181, 192], the amount of KIR, LIR, and KLR genes found to be significantly upregulated in the γδ MR1T cell population implies that they must be important for the function of this specific subset. Many of these specific receptors (primarily the KIR and LIR families) are inhibitory and their heightened expression may be directly tied to the previous observation that these cells seem to have markers for cytotoxic effector cells that have experienced prolonged activation.

To narrow down which genes may separate the phenotype of the lung tissue-resident γδ MR1T population from the tissue-resident MR1 tetramer negative γδ T cell population, a similar analysis was performed by first excluding all αβ T cells from the comparison. This comparison maintained that some of the effector molecules like GZMA and GZMK are still heightened in the γδ MR1T population when compared to all other γδ T cells, implying that these granzymes are heavily utilized by this specific subset to carry out their effector functions (**Figure 4.4 C**). Interestingly, the γδ MR1T population also showed a significant upregulation in genes that are related to the response to mycobacterium. These genes included factors known to be made by γδ T cells during the cellular response to tuberculosis to recruit macrophages, CCL4L2 and CCL3L1, as well as TLRs and TLR accessory proteins that are known to respond to bacterially derived PAMPs (**Figure 4.4 C**)[192, 193]. These findings are of particular interest as the barcoded MR1 tetramers that were bound by these cells were loaded with mycobacterial antigens from *m. smegmatis*. Another interesting correlation found during this analysis was that the expression profile of the γδ MR1T population, which were found exclusively in the asthma

associated samples, had heightened signs of participating in the asthma response in comparison to other tissue resident γδ T cells in the dataset (**Figure 4.4 D**). The receptors for cytokines involved in the pathogenesis of asthma, IL23R and IL17R, were significantly upregulated on the γδ MR1T population in comparison to other γδ T cells. Interestingly, the accessory protein IL21-AS1 which has been shown to drive type 2 cytokine signaling and subsequent airway inflammation was also found to be at a much higher expression level[194, 195]. Furthermore, in comparison with the other γδ T cells recovered from the lung tissue, the γδ MR1T population was revealed to have significantly lower expression of IL32, which has recently been shown to act on Th2 and IL-10 producing cells to limit the inflammation incurred during the asthma response[196]. Taking these results into consideration, there is a clear correlation between the samples that the γδ MR1T population was found in, the tetramers that they were identified with, and their gene expression profiles. These data show that this tissue resident γδ MR1T T cell subset seems to be actively participating in both the response to mycobacterium and in asthmatic airway inflammation. Further work will focus on proving a causal relationship between these correlations.

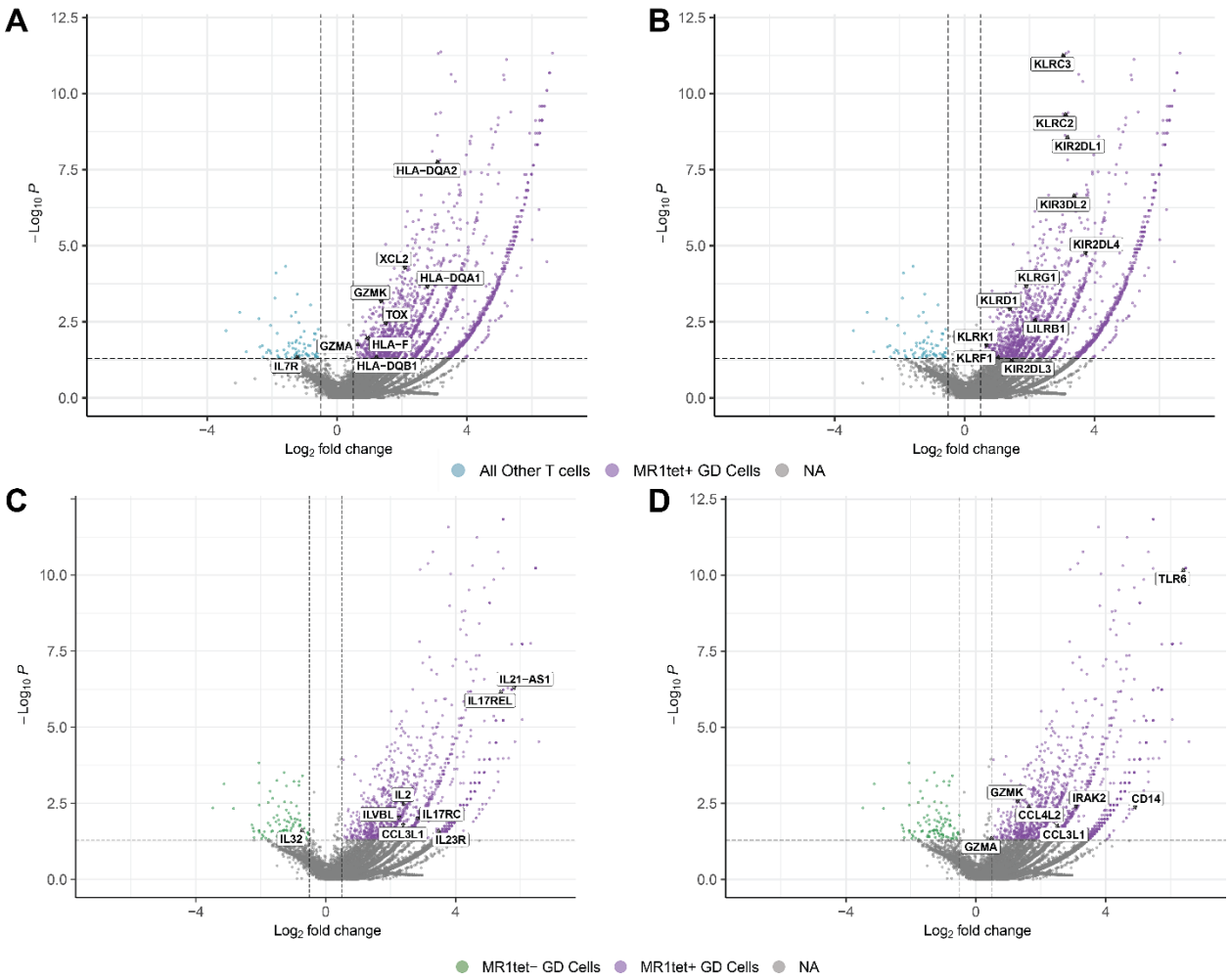



**Figure 4.4** Relative gene expression of γδ MR1T cells and other lung-resident T cells. A) and B) Volcano plots showing the relative fold change and significance of genes expressed in γδ MR1T cells over the expression values found for all other T cells in the data set. C) and D) Volcano plots showing the relative fold change and significance of genes expressed in γδ MR1T cells over the expression values found for MR1 tetramer negative γδ T cells. Significance was determined using a two tailed students t-test with FDR correction. Dotted lines are plotted at p-values lower than 0.05 and $\log_2$(FC) values of greater or less than 1. Genes of interest are labeled in each panel.

## 4.5 The lung-resident γδ MR1 T cell TCR GDM95 displays specificity for MR1 loaded with PLIII

While these correlative observations about the TCR gene usage, MR1 specificity, and effector phenotype of these γδ T cells made a promising case that we had uncovered a novel population of lung resident γδ MR1T cells, it was necessary to validate their MR1-reactivity *in vitro*. To accomplish this, I expressed the TCR of one of the γδ MR1T cells observed to have

high MR1-tetramer reads in the scRNA-seq dataset, GDM95, in the JrtB cell line to test its ability to bind to a panel of MR1 tetramers. Along with GDM95 I used two TCRs with highly specific antigen requirements as controls. As a reminder from **Chapter 3**, G11 is a canonical αβ MAIT TCR with high specificity for MR1 loaded with 5-OP-RU, while DP10 is a Vγ4Vδ1 TCR highly specific for CD1d loaded with sulfatide (**Supplemental Figure S4.2 A**). I tested both the GDM95 and DP10 TCR JrtB lines against a panel of heterogeneously loaded and homogeneously loaded MR1 tetramers. This tetramer panel covers a range of loading conditions, expression systems, and MR1 ligands, which allowed me to survey for a wide variety of potential ligand specificities; a special thanks to Idongesit Ekpo, a graduate student in the lab, who graciously let me borrow some of the PL3 tetramers she had recently made (**Supplemental Figure S4.2 B**).

The other γδ MR1T cells described to date recognized MR1 in a ligand-independent manner, some showing specificity for the α3 domain of MR1 and others for the side of the α1/2 platform. Therefore, if the GDM95 was targeting the platform domain in a ligand independent manner, one would anticipate equivalent binding to all of the MR1 tetramers regardless of the ligand loaded or α3 domain origin. If the GDM95 TCR was targeting the α3 domain like the previously described G7 TCR, it should bind equivalently to all of the fully human MR1 tetramers in the panel (hMR1) but may have a lower affinity for the hpMR1 tetramers made with a bovine α3 domain. Surprisingly, the GDM95 TCR exhibited very specific binding only to the hMR1 tetramer that was homogenously loaded with PLIII, a microbially derived metabolite that we have shown previously to be a ligand for MR1 (**Figure 4.5 A**)[68]. This result was particularly interesting as the GDM95 TCR was agnostic to the other fully human MR1 tetramer in this panel, unloaded hMR1, which implied ligand specificity of the interaction.

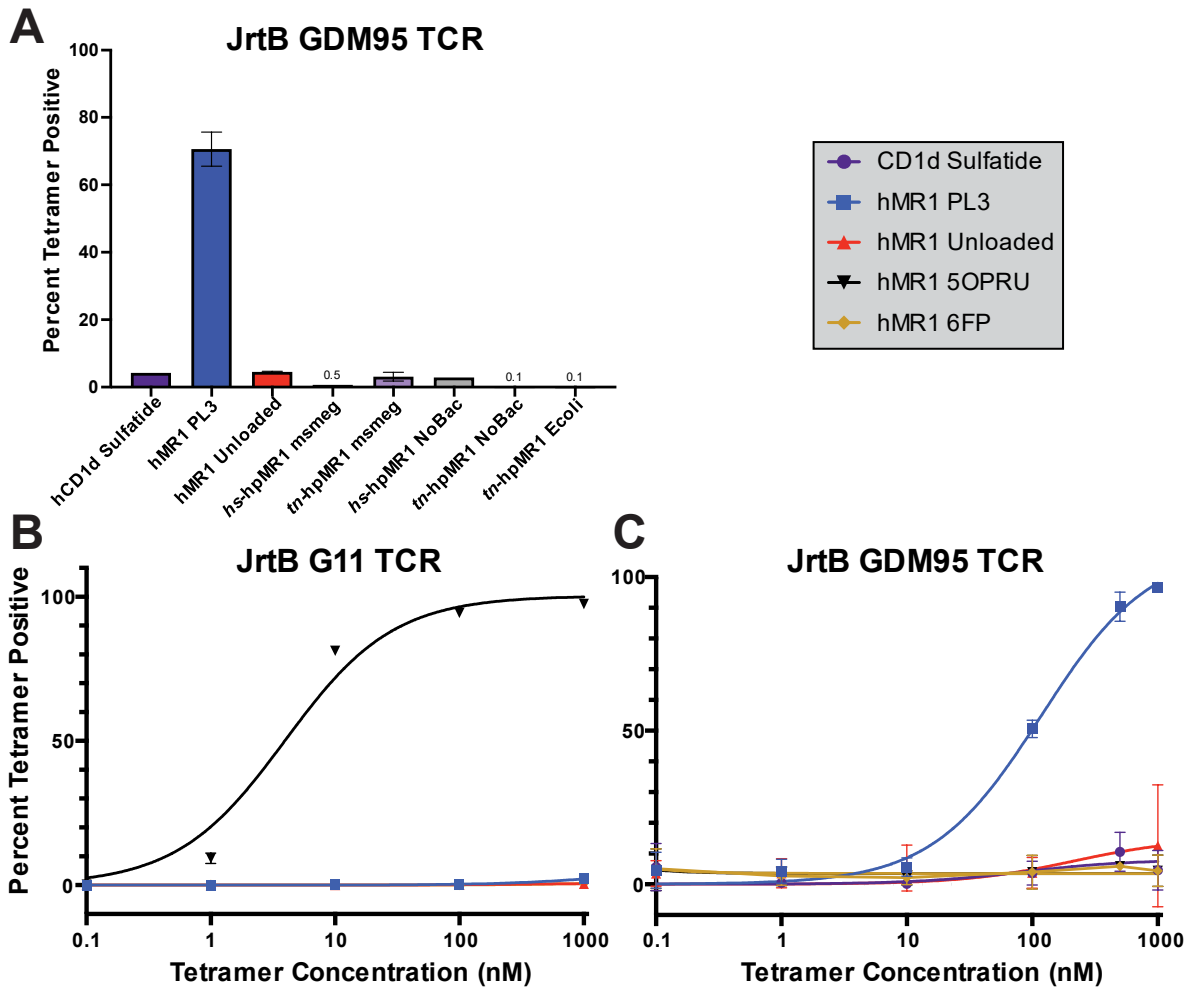



**Figure 4.5** The GDM95 TCR is specific for hMR1 tetramers loaded with PLIII. A) Results of tetramer panel screen with the GDM95 TCR expressed in the JrtB Jurkat cell line with tetramers tested at 200nM. B) Results of a ligand variable hMR1 tetramer titration with the canonical MAIT TCR G11. C) Results of a ligand variable hMR1 tetramer titration with the GDM95 TCR.

It is important to note that the heterogeneously loaded tetramer that was barcoded and used to identify this clone in the original scRNAseq experiment, *tn*-hpMR1 msmeg, did a relatively poor job of staining the GDM95 JrtB cell line at the concentration tested in this screen (200nM). When considering an explanation for these results, it was important to consider the work of a previous graduate student in the lab, Dr. Nicole Ladd, who in collaboration with the Hildebrand Lab at OHSU, identified that hpMR1 made in the presence of *M. smegmatis* contains primarily photolumazine molecules, including PLIII (**Supplemental Figure S4.2 C**). It is

possible that the low affinity interaction we observe with the heterogeneously loaded tetramer in this screen could be due to the GDM95 TCR being very specific for MR1 loaded with PLIII and the relatively low occupancy of PLIII in this tetramer (approximately 20% by Dr. Ladd's estimates (**Supplemental Figure S4.2 C**)). If approximately one out of every four hpMR1 molecules used in the tetramer is loaded with the ligand this TCR is specific for, it would make sense that the heterogeneously loaded tetramer does not stain as well as the homogenously loaded hMR1-PLIII tetramer when used at the same concentration. The observation that the original GDM95 T cell clone had reads for the barcoded *tn*-hpMR1 msmeg tetramer is likely due to DNA sequencing being a much more sensitive technique than flow cytometry. A lower threshold of surface staining would be detectable with a barcoded tetramer in a sequencing experiment in comparison to the same level of staining with a fluorescently labeled tetramer in a flow cytometry experiment. Another possible explanation, which is not mutually exclusive with the first, is that the heterogenous loading process does not always produce identical compositions of loaded protein, making it likely that these different preparations had varying amounts of PLIII occupancy which would impact the binding of GDM95.

To fully validate the interaction between the GDM95 TCR and the hMR1 PLIII tetramer, I generated MR1 tetramers in the same human MR1 construct as our PLIII tetramer, but with canonical, homogeneously loaded MR1 ligands. One would anticipate that if the GDM95 TCR was recognizing PLIII directly in the hMR1 tetramer that it was likely doing so as a MAIT TCR would - making direct contacts with the exposed ribityl group that is found on 5-OP-RU and PLIII (**Supplemental Figure S4.2 D**). As anticipated, the 5-OP-RU specific MAIT TCR, G11, bound strongly to only the hMR1 5OP-RU tetramer with an EC50 of about 10nM (**Figure 4.5 B**). Surprisingly, despite the structural similarities between 5-OP-RU and PLIII, the GDM95 JrtB

cell line did not bind either the 5-OP-RU or 6-FP hMR1 tetramers and remained highly specific for the hMR1 tetramers loaded with PLIII over a range of concentrations (estimated EC50 of 100nM) (**Figure 4.5 C**). These initial experimental results went beyond the validation of the MR1-reactivity of the GDM95 TCR, but instead, revealed a potentially novel mode of recognition for MR1 by a γδ MR1T cell. The ligand specificity shown by GDM95 in these tetramer staining experiments is potentially something akin to DP10, which revealed that αβ TCR-like recognition was not only possible for γδ T cells but could also be incredibly antigen specific. To test this system further, I decided to ensure that PLIII was an activating ligand for the GDM95 TCR.

### 4.6 γδ MR1 TCR Jurkats are activated by APCs loaded with PLIII, but not other MR1 ligands

To test the reactivity of the GDM95 TCR to APCs loaded with PLIII, I utilized the same JY.MR1 cell line employed in the activation assays performed on the MR1T cells studied in **Chapter 3**. Initial validation experiments showed that this MR1 expressing BLCL cell line can capture and present PLIII at low μM concentrations when added to the cell culture medium (**Supplemental Figure S4.3 C**). I was able to test the GDM95 JrtB cell line and two control JrtB lines with known ligand specificity for their ability to be activated by either the canonical MR1 ligands, 5-OP-RU and 6-FP, or the mycobacterial antigen PLIII. As expected, the CD1d-sulfatide specific γδ TCR DP10 was agnostic to the JY.MR1 cell line regardless of the MR1 ligand added (**Supplemental Figure S4.3 D**). Consistent with previous findings, the G11 JrtB line reacted strongly only to APCs loaded with 5-OP-RU and this interaction could be blocked by the introduction of the MR1 blocking antibody 26.5 (**Figure 4.6 A**). Finally, the GDM95 TCR, which was expected to be specific for MR1 loaded with PLIII, reacted only to JY.MR1 cells in

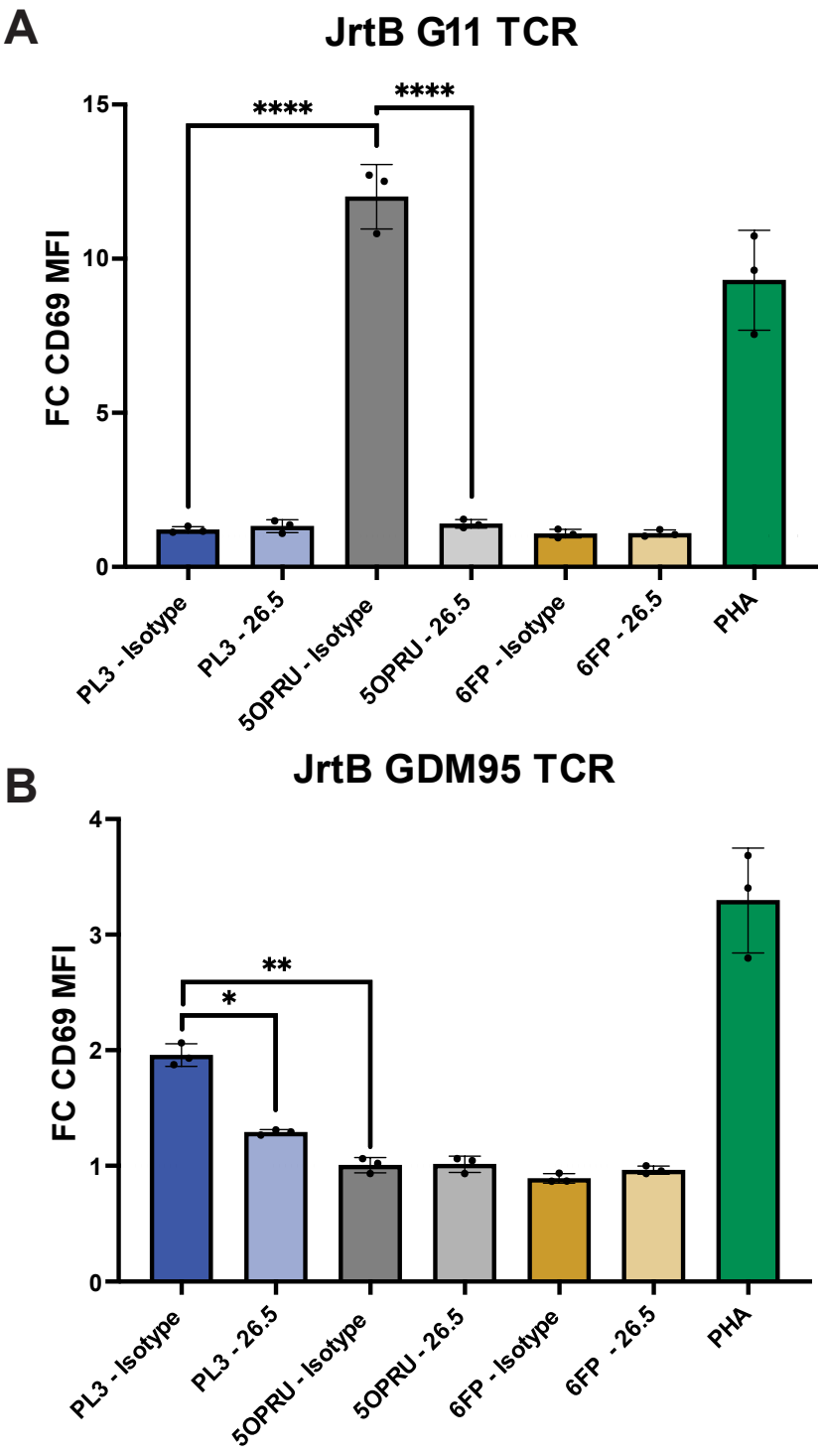



**Figure 4.6** The GDM95 TCR is activated by JY.MR1 cells loaded with PLIII. A) Results of overnight activation assays using JrtB cells expressing the G11 MAIT TCR. B) Results of overnight activation assays using JrtB cells expressing the GDM95 TCR. Significance ascertained by an unpaired, two-tailed Students t-test between the indicated groups. P values are indicated for each significant comparison or reported as *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, and ****P≤ 0.0001.

an MR1 dependent manner when they were loaded with PLIII (**Figure 4.6 B**). When combined with the tetramer staining data shown in the previous section, it is clear that the GDM95 TCR does indeed exhibit selective binding and reactivity to the mycobacterial antigen PLIII when presented by MR1 - a novel finding for the γδ MR1T cell field.

### 4.7 Discussion and ongoing work

Through a combination of sequencing, computational, and biochemical techniques, we identified and validated a novel population of tissue resident γδ MR1T cells that exhibit remarkably antigen specific binding to MR1. These γδ MR1Ts were found almost exclusively in the tissue samples derived from the explanted lungs of individuals who reported having asthma during their lives. This fascinating result was shown to correlate with the gene expression data, as the γδ MR1T cell subpopulation of the γδ T cells found in this data set had increased expression of cytokine receptors shown to be critical in the Th2 mediated asthmatic response. Furthermore, these cells appeared to be both activated and cytotoxic, as they had increased expression of NK receptors and granzymes when compared to the MR1 tetramer negative γδ and total T cell populations. This gene expression program correlated well with the mycobacterial-antigen loaded tetramer (*tn*-hpMR1 Msmeg.) that they were reported to be bound to. A closer look at the TCRs of these clones revealed that they primarily used the Vδ1 gene segment and a select few Vγ chains, including Vγ2, Vγ4, and Vγ8. While the CDR3γ chain did not show any evidence of a conserved motif among the γδ MR1T cell subpopulation, the CDR3δ chain did show the conservation of a partially-untemplated motif. This motif was analyzed further and the CDR3δ of the γδ MR1T cell subpopulation was shown to be significantly different from the other lung resident γδ T cells in this dataset. The motif seems to utilize fluctuating positively

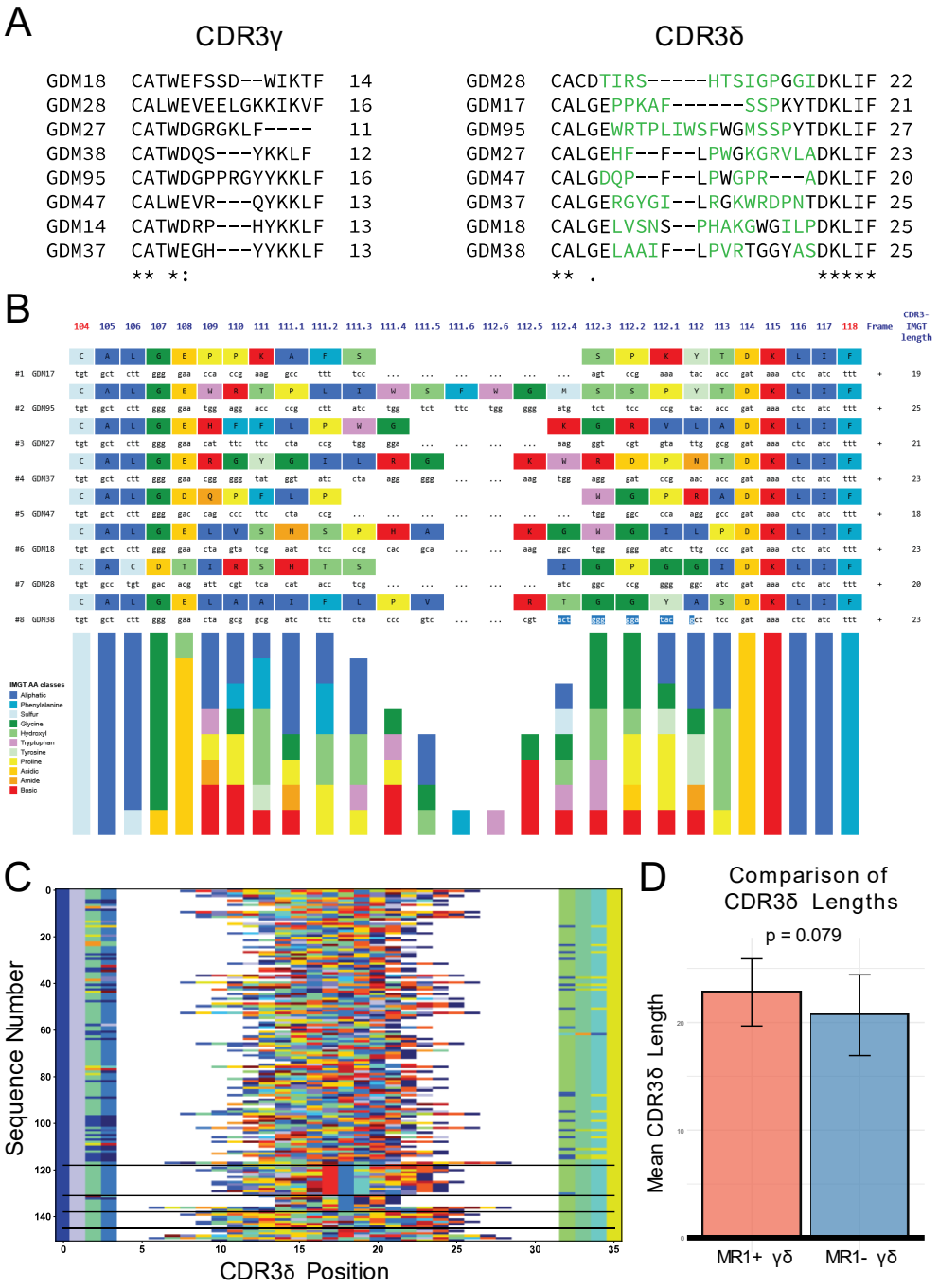
charged and sterically large residues to mediate its recognition of MR1. This recognition was validated experimentally with tetramer staining and activation assays that revealed one of these TCRs, GDM95, to be specific for hMR1 loaded with PLIII, a mycobacterial MR1 antigen.

These results paint an interesting picture for what the function of lung-resident γδ MR1T cells may be. The gene expression data shows a phenotype of a bacterially focused cytotoxic effector that has been influenced by the allergic response in asthma. In support of this, the biochemical testing of a γδ MR1T TCR from this population revealed a high degree of specificity for MR1 loaded with only a specific microbially derived photolumazine molecule. Taken together, these data seem to indicate γδ MR1T cells found in the lung have evolved to sense directly for microbes that make photolumazine molecules. This hypothesis is well grounded in the findings presented here and a large body of research already exists implicating lung-resident γδ T cells in the response to mycobacterium infection, including tuberculosis[192, 197, 198]. On the other hand, it is harder to make sense of what these γδ MR1Ts may be doing in the asthmatic response or why they are so expanded in the lungs of people who reported having this autoimmune disease. It could be that the phenotypic programing of these mycobacterium specific γδ MR1T cells allows them to be particularly susceptible to the inflammatory conditions present in asthma, but much more experimentation will be needed to fully flush out this correlative observation.

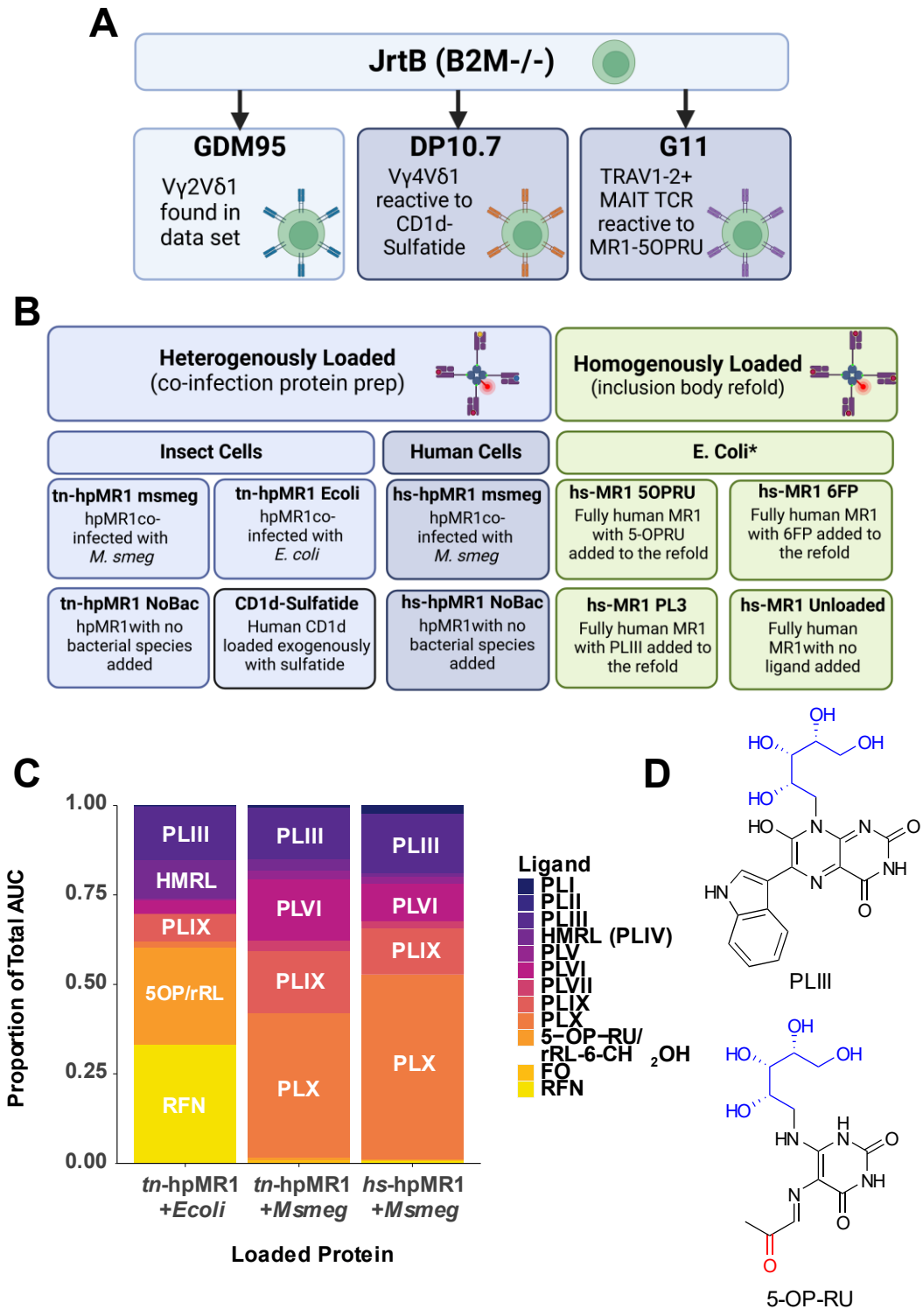
Further work with this project will involve the recombinant expression of the GDM95 TCR for use in biophysical and structural experiments to explore the modality of its PLIII specificity and interrogate the computational results of **Figure 4.3**. It should be noted that the progress of this work has been hindered by the small quantities of PLIII that we possessed at the start of our investigation. We were working for some time to get more PLIII synthesized to

continue the work on this project, and I am happy to note that during the preparation of this dissertation, we have received newly synthesized PLIII in a quantity needed for these types of assays. In the meantime, I worked closely with a technician in the lab, Hortencia Graves, to clone out and test other members of the panel of γδ MR1T cells identified in our scRNA-seq dataset. The newly acquired PLIII will revitalize our efforts to test a broader pool of γδ MR1T cells and to see how generalizable the photolumazine specificity is for these lung-resident T cells. If successful, this study could lead to a deeper understanding of the role of MR1 in the lung and could generalize the rules of engagement for the γδ T cells that recognize it in the context of bacterial infection.

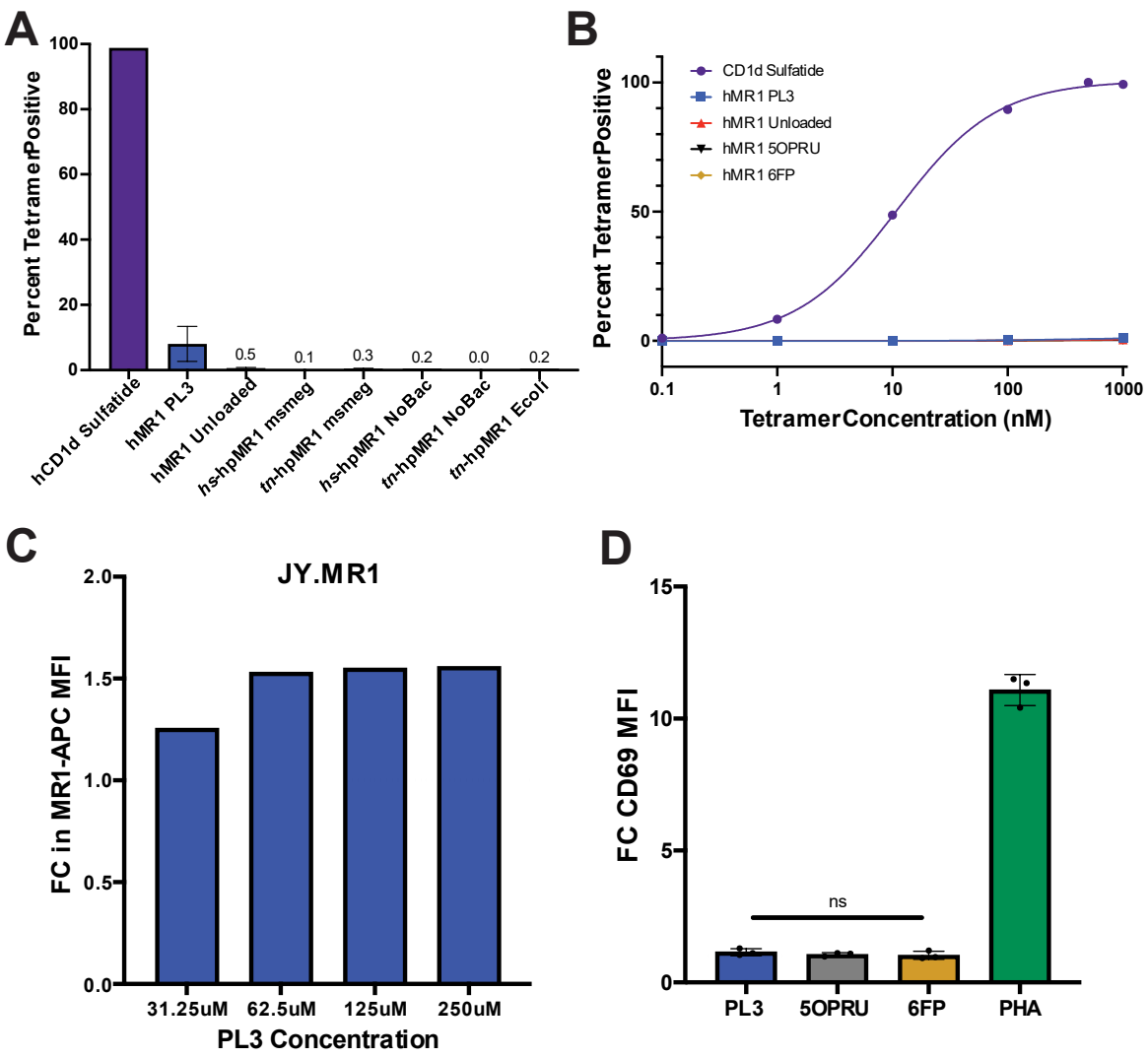
## 4.8 Supplemental Information




**Supplemental Figure S4.1** Analysis of the CDR3 loops of γδ MR1Ts. A) Multiple sequence alignments for the CDR3γ and CDR3δ loops of the γδ MR1Ts recovered in this analysis. For the CDR3δ loops, green lettering denotes amino acids that derive from at least one untemplated nucleobase as determined by IMGT junction analysis[199]. C) results of the IMGT junction analysis highlighting similar residues in the compared CDR3δ loops. C) Budge alignment from the AIMS software for the CDR3δ loop sequences analyzed. D) a comparison of the average CDR3δ loop length between the MR1 tetramer negative γδ T cells and the γδ MR1T cells analyzed. Statistical insignificance determined by an unpaired two tailed students t-test, p -value determined is displayed in panel D.




**Supplemental Figure S4.2** MR1 tetramer composition. A) An overview of the JrtB TCR Jurkats used in this chapter. B) An overview of the tetramers used in the analysis covered in this chapter. The expression system and method of loading are detailed for each. C) The relative ligand composition of different heterogeneously loaded MR1 tetramers. The data and the figure (C) was generated by Dr. Nicole Ladd. D) The chemical structures of PLIII and 5-OP-RU. The shared ribityl moiety is highlighted in blue while the carbonyl group that makes a covalent bond with MR1 is highlighted in red for 5-OP-RU.




**Supplemental Figure S4.3** Analysis of the control TCR JrtB cell line DP10. A) the screening results of the DP10 JrtB cell line with MR1 and CD1d tetramers. B) A tetramer titration of DP10 with various hMR1 and CD1d tetramers. C) The results of overnight incubation of JY.MR1 cells with varying concentrations of PLIII, displayed as fold change in the detected MFI of an MR1-APC antibody. D) The results of overnight activation tests with DP10 JrtB cells and JY.MR1 cells loaded with various MR1 ligands. Non-significance was determined by an unpaired, two tailed students t-test performed between each ligand group.

# Chapter 5. Conclusions, Significance of Findings, and Future Directions

## 5.1 Endogenous pteridine metabolites are likely the tip of the iceberg for native metabolites that can interact with MR1

**Chapter 2** of this dissertation provides a close look at the work I was able to accomplish in moving our understanding of the basic biology of MR1 forward, as well as the concrete steps I made towards identifying new therapeutic modalities that target MR1 in cancer. The EP MR1 ligands that I describe are the first instance of a truly endogenous, unmodified class of metabolites that bind MR1 and can block the activation of MAIT cells. I show that these metabolites comprise a native pool of MR1 ligands through the augmentation of their biosynthetic pathway and the direct effect this augmentation has on MR1 surface stability in myeloma and BLCL cell lines. The potential significance of these findings is far reaching, confirming decades long held hypotheses that MR1 can interact with the native metabolic environment of its host cells. Considering the content of **Chapter 3** and my investigation of the cells that can recognize MR1 in an EP bound state, the characterization of these ligands provides potential answers to questions around the development, maintenance, and purpose of MR1T cells in both homeostasis and cancer. The discovery of this class of endogenous MR1 ligands is undoubtedly the tip of the iceberg for the study of the native metabolome that can interact with MR1 given the right context, and I am excited to see how others will follow up on this work.

While our discovery that these EP metabolites can act as a native pool of MR1 ligands in myeloma cell lines clearly shows a role for them in cancer, the question remains as to why this system of EP presentation by MR1 would be useful for contexts outside of this disease. One potential explanation based on the data found presented in **Chapter 2** is that these EP ligands work to block the aberrant activation of MAIT cells in the periphery. As it is well known that

GCH1 expression increases under inflammatory conditions, hypothetically, an injury to the mucosa or the incursion of a microbial species would, as part of the inflammatory response induced by MAIT cells and other members of the immune system, cause an increase in BH4 metabolism within the local environment (**Figure 5.1**)[155]. As has been described in the literature, the balance of BH4 is strictly maintained by healthy cells, but it is a long-standing observation that during infection or general inflammatory conditions the intermediate and by products of its metabolism drastically increase locally and systemically[148, 152, 153]. It is quite possible that as inflammation persists, and these molecules continue to build up within the cells presenting microbial MR1 ligands, the EPs would start to outcompete the activating microbial ligands as a way to quell the activation of MAIT cells in the inflamed tissue. The MAIT JrtB activation blocking experiments would certainly seem to support this hypothesis, as even an increase to the low μM range for some of these ligands began to out compete 5-OP-RU and dampen the activation of the MAIT JrtB line.

Other homeostatic functions for the EP-MR1 axis could exist as well, and although these explanations are not as closely supported by the data, I feel they are still plausible and worth mentioning. Recent work has shown that MAIT cells are key players in the wound healing response. Being able to secrete factors like VEGF, IL-17, IL-22, and amphiregulin, these cells have been shown to accelerate wound closure and induce a proliferative effect on keratinocytes and other epithelial cells[89, 90]. While this is a well described phenomenon, the factors that drive MAIT cells to perform this function over a cytotoxic or pro-inflammatory phenotype are unclear. More recent work has shown that TCR signaling is critical for the access to this program and that the length or intensity of signaling may be instructive as to which program to activate[90, 91]. When considering the known ligands for MAIT cells, this may sound

counterintuitive. If MAIT cells can only recognize the potently activating ribitylpyrimidines or ribityllumazines made by B-vitamin autotrophic microbes, how can they reproducibly make the "right" decision of when to choose a tissue healing program over an infection-controlling cytotoxic one? What if there is not a meaningful level of microbial metabolites in the vicinity of the wound to induce TCR signaling? Recent work has suggested that inflammatory conditions in the gut lead to increased permeability of microbially derived ligands for MR1[92]. I propose the inflamed tissue of the wounded area would be equally adept at making EP ligands as it is at capturing any microbial ligands that breach the mucosa. It could be that these EP ligands provide the lower level or shorter duration of TCR signaling necessary to access the tissue repair effector profile. Another more "out there" hypothesis for the role of EP ligands in this axis would be that MAIT TCRs can directly "sense" the difference between microbially derived and endogenously derived antigens in the context of MR1, informing the decision they make between effector phenotypes (**Figure 5.1**). Certainly, an inspection of the surface presented by MR1 with a stimulatory ligand like 5-OP-RU and an "inhibitory" pteridine like 6-FP shows a very different biophysical landscape for the TCR to inspect. Could it be possible that this difference can be sensed and reported to the cell through the duration or potency of signaling? More *in vitro* or *in vivo* work with MAIT clones and EP MR1 ligands would be necessary to test this hypothesis, but it is one that deserves further investigation.

The findings described in **Chapter 2** provide not only a novel class of MR1 ligands, but a novel approach for identifying more endogenously sourced MR1 ligands. The mass spectrometry pipeline that was utilized to identify EPs in myeloma samples can certainly be leveraged in its unaltered state for use on other cancer types or other tissue samples as well. Indeed, even from the relatively narrow scope of our search on three myeloma cell lines, I was

inundated with a wealth of potential ligand identifications that I did not have the time to fully validate and, as evidenced in **section 2.8**, many of these are still attractive targets. Using this system with other cancer types or even simply using the metabolite refold enrichment methodology, which provided the majority of significant ligands to the downstream analysis, one could even apply this same system to primary tissue samples.

However, a more attractive approach for future studies may be achieved by making simple modifications to the original pipeline that would drastically improve the quality of the results. As discussed in **Chapter 2**, there were many assumptions that had to be made along the way when planning this experiment, most of which centered around the mass spectrometry methodologies used. Our focus on finding endogenous ligands for MR1 led us to use a simple MS1 methodology, but we needed to compromise when it came to deciding between the breadth of our search and the depth of our search. We chose to utilize direct injection for our samples and only looked for ions in negative mode - decisions that were made based on previous work done in our lab[68, 69]. However, one could imagine that adding a liquid chromatography step prior to injection would give much more information about the hydrophobicity of the ions and could provide better resolution of each ion from the bulk mixture. Better yet, running the instrument in switch polarity to capture molecules that are either ionized well in negative mode and positive mode would also increase the breadth of the dataset. The biggest downside to our current pipeline, which was mentioned throughout **Chapter 2**, was the MS1 methodology we used. Using only MS1 in our analysis meant that we could only match ions based solely on their m/z and calculated chemical formula – no structural information could be gained by further fragmentation. While this approach was sufficient for the identification of the EP ligands, there are many more significant ions in this data set that we cannot accurately identify. If future

experimentation were to include a fragmentation step using an MS2 or even an MS3 methodology, the structural information gained would be critical in narrowing down the thousands of possible chemical structures that can be attributed to a single chemical formula. To include these methodologies, one would have to sacrifice some breadth of the analysis to gain this additional depth. However, by using the information we gained here, perhaps focusing fragmentation on ions with the core pteridine structure, a targeted analysis of even the same samples could provide critical information to help us decipher the identity of our most significant ions.

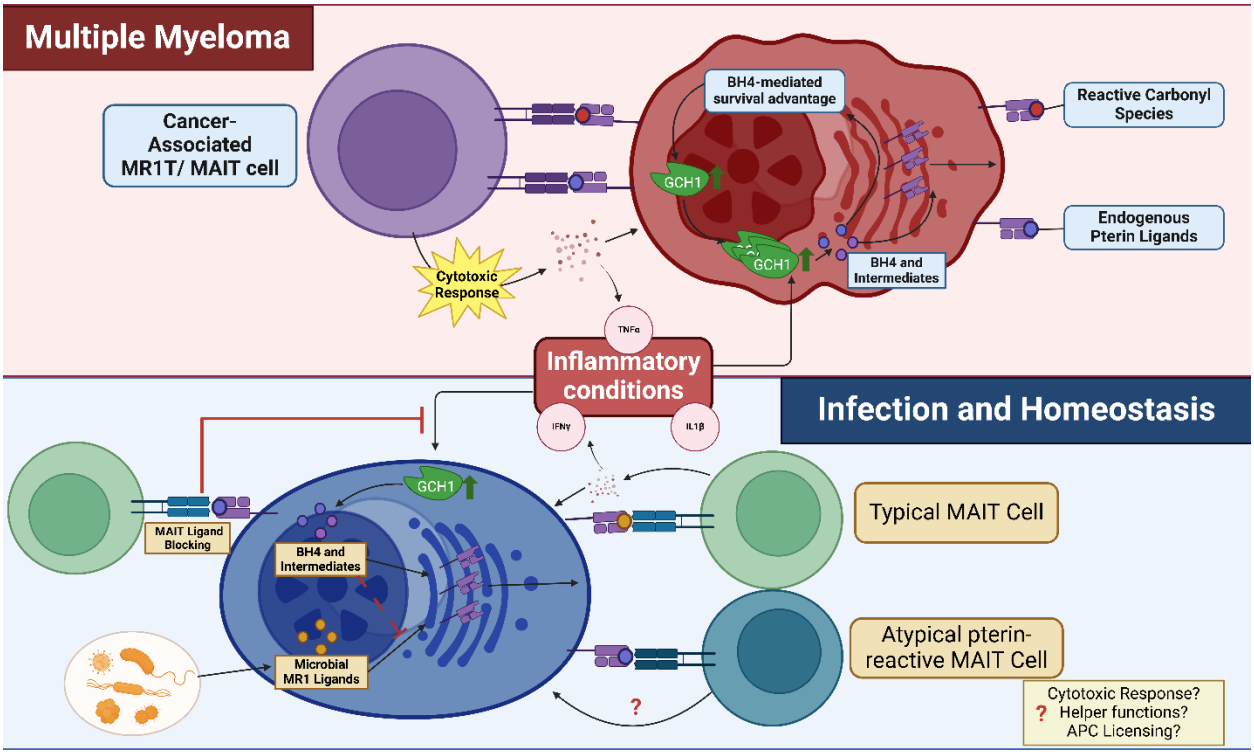



**Figure 5.1** An overview of the findings described in **Chapters 2 and 3** of this dissertation. EP ligands play a role in both cancer surveillance and homeostatic sensing of infection. We have shown that EP ligands can block the loading of microbially derived MAIT ligands but have also shown these ligands to be stimulatory to certain cancer-associated and atypical MR1T/MAIT cells.

In full support of the assertion I made at the beginning of this dissertation, MR1 has continued to prove itself to be an immunological Swiss army knife, and while initially perceived as a static molecule with limited functions, its further study continually proves it anything but. The addition of endogenous metabolite presentation is just another feature that this molecule can utilize to direct a response from the immune cells that sense it and, as I explored in **Chapter 3**, these MR1T cells are equally full of surprises.

## 5.2 The recognition of endogenous pteridines by cancer associated MR1T cells and atypical MAIT cells implies interconnection between these cell subsets

**Chapter 3** is a critical extension of the work presented in **Chapter 2**, lending biological relevance to discovery of EP ligands for MR1. The ability to generate both tetramer reagents and variations of the Jurkat cell line proved to be critical for this analysis providing a relatively high throughput way to test the reactivity of multiple previously published MR1T and MAIT TCRs. The hypothesis that folate-derived pteridine reactive MAIT and MR1T cells would also recognize the EP class of ligands proved to be correct. Interestingly, we were able to show that these atypical MR1Ts and "auto-reactive" MAIT cells were capable of discerning between EP and canonical ligands in the context of MR1, which likely involves unique biochemical mechanisms of TCR engagement. Importantly, we were able to show that these EP ligands stimulate certain cancer-associated MR1T cells as well (**Figure 5.1**). This finding is relevant not only to the fact that we discovered these ligands to be present in myeloma cell lines, but also interesting when considering that I was able to stain a population of MR1T cells in the bone marrow of a patient with newly diagnosed multiple myeloma. These findings bind cancer-associated and atypical MR1Ts into one closer related family, as this work has proven they share

reactivity to a novel class of ligands. This discovery also lends itself to interesting questions we can now ask about the origin and general biology of these cells, in both the context of cancer and at homeostasis.

The discovery of EPs as MR1 ligands and their broad applicability to members of the MR1T family is an exciting discovery, but as with most discoveries, it leaves us with more questions than answers. It has been shown that certain MR1Ts, cancer-associated or otherwise, do not have the same cytotoxic phenotype as MAIT cells and have been shown to display T cell helper functions in vitro[71]. Do EP MR1 ligands play a role in inducing this type of response and can the recognition of different ligands within MR1 lead to different effector functions? If so, this paradigm would not be too far off from how MAIT cells have been shown to function within the human body – being able to integrate multiple signals and provide either a type 1 or type 17 effector response depending on the context of the environment. Perhaps the presence of EP ligands are signals of an inflammatory response that has gone on too long, and when sensed by the atypical MR1T or cross-reactive MAIT TCR, they favor wound healing or anti-inflammatory responses.

The discovery of truly endogenous MR1 ligands that are expressed in all the tissues of the body allows for tempting speculation on further roles they might play outside of the paradigms described above. As the MR1T cells described to be reactive to them are found (albeit at low percentages) in healthy people, it is likely that some sort of antigen exposure is required for their development and maintenance in the periphery. If MR1 loaded with EP ligands provides this signal, then it is possible that this signal would be important for their development in the thymus as well. The thymus is rich with developing thymocytes and naïve T cells which undergo phases of rapid expansion during the selection process. As it has been shown that BH4

metabolism plays a critical role in helping to support the rapid cell division of T cells[156], indicating that the thymus is likely a microenvironment rich with EPs. The development of MAIT cells in humans is still under active investigation, but a few things are known for sure. It seems that they are selected on other double positive thymocytes expressing MR1 and they do continue to persist, albeit at reduced numbers, in a germ-free setting[80, 200]. Is it possible that MR1 expressed in other developing thymocytes is capturing the large quantities of EPs being made during expansion and development and thus selecting other MAIT or MR1T cells for development? Significant work would have to be done to know for sure, but it is a tempting hypothesis, nonetheless.

Furthermore, these data seem to suggest that EPs acting as MR1 ligands likely have roles in the body acting to aid (or perhaps quell in the case of blocking MAIT ligands) the "normal" inflammatory response that occurs due to microbial incursion or injury. Yet, they also have been shown to be stimulatory to cytotoxic cancer-associated MR1Ts. It is tempting to draw parallels between this system and others used by the immune system that serve important roles in host defense yet can be leveraged in the immune response for cancer as well. One does not have to look far past the innate-like T cell family to find a precedent for a system of this nature, as the recognition of butyrophilin (BTN) molecules from the conserved Vγ9Vδ2 T cell family mirrors this type of dual utility. It is well understood that the Vγ9Vδ2-BTN system likely evolved to recognize tissue infected by phosphoantigen (pAg) generating bacteria, as the intracellular domain of BTN 3A1 binds to these small molecules within the cell, causing it to dimerize with BTN 2A1 and stimulate a cytotoxic T cell response[102, 201, 202]. Coincidentally, due to their metabolic needs pAg builds-up within the cytosol of cancerous cells as well, allowing for subsequent detection by Vg9Vd2 T cells[102, 203]. While more work is needed to definitively

prove this, I believe it is highly likely that something similar is happening with MR1, EP ligands, and MR1T cells. It seems that the MR1T cells that are not known to be cancer reactive recognize EP ligands which are present outside of cancer as well, but due to the well documented advantages BH4 biosynthesis provides cancer cells[127, 128, 133, 134, 158, 163], the same system could be coincidentally leveraged as a form of tumor surveillance. To further draw this parallel between this EP-MR1-MR1T system and the pAg-BTN-Vg9Vd2 system, it has been observed that pAg sensing by Vg9Vd2 T cells is critical for both the control of mycobacterial infection and the control of plasma cell cancers. It is well known that MR1T cells are capable of sensing mycobacterium via the RBP ligands they produce and, as we have described here, certain MR1T cells are capable of sensing plasma cell cancers through their generation of EP ligands for MR1 as well [55, 68, 204, 205]. The presence of a similar system within the same T cell family lends credence to the idea that EP MR1 ligand sensing likely has multiple roles in the body, both during homeostasis and during cancer surveillance.

The findings described in **Chapters 2 and 3** tie together a cohesive story about the discovery, validation, and broader role that EP MR1 ligands play in the body. These findings add to the validity of atypical MR1T cells as an important population of circulating T cells in the body and the finding that they react to EP ligands helps to further explain their roles. I am confident that the further study of these cells, perhaps through their isolation from patient samples with EP-hMR1 tetramers, will only continue to yield powerful insights that will allow us to consider novel therapeutic approaches for cancer, wound healing, and infection.

### 5.3 The antigen-dependent activation of lung-resident γδ MR1T cells implies a conserved role for γδ MR1T mediated surveillance of the mucosa for PL-generating microbes

**Chapter 4** of this dissertation provided evidence for the existence of a novel tissue-resident γδ MR1T cell population in the human lung and a novel modality for the recognition of MR1 by γδ MR1T cells. This discovery was possible through the collaborative effort between myself and previous graduate students in the lab Augusta Broughton and Nicole Ladd, who devised and executed the initial sequencing experiment that allowed for these observations. I was able to show that these cells can be found reproducibly in multiple donors, that they are highly enriched in donors who likely had a history of inflammatory autoimmune disease, and that these cells share common TCR features that may mediate their recognition of MR1. These cells appear to be activated and cytotoxic with hallmark traits of participating in an anti-microbial and asthmatic immune response. The closer inspection of a TCR from one of these γδ MR1T cells revealed a high degree of ligand specificity for photolumazine molecules. This is a very interesting finding in light of the insights gained through the in depth *in silico* biophysical analysis done on the CDR3δ loops from the entire population of γδ MR1Ts. The total of the work detailed here presents an exciting new paradigm in MR1 and γδ T cell biology, and these novel findings open up new avenues for future experimentation that will investigate the impact of this new immune axis.

When considering the role of antigen-specific γδ MR1Ts that may be in the lung tissue at homeostasis, interesting hypotheses can be generated by analyzing the transcriptomes of these cells. As discussed at the end of **Chapter 4**, a solid hypothesis based on the MR1 antigens they seem to recognize and their effector phenotype as described by the gene expression data, is that these cells play a role in barrier defense from photolumazine generating microbes like

mycobacterium and many others (**Figure 5.2**). Their expression of granzymes, NK receptors, and TLRs paint a picture of cells that are primed to detect microbial PAMPs, while their high expression of MHC-II molecules indicate that they are using these receptors to bridge the innate response with the adaptive (**Figure 5.2**). The fact that their TCRs seem to recognize MR1 when loaded with microbially loaded metabolites also supports this hypothetical classification.

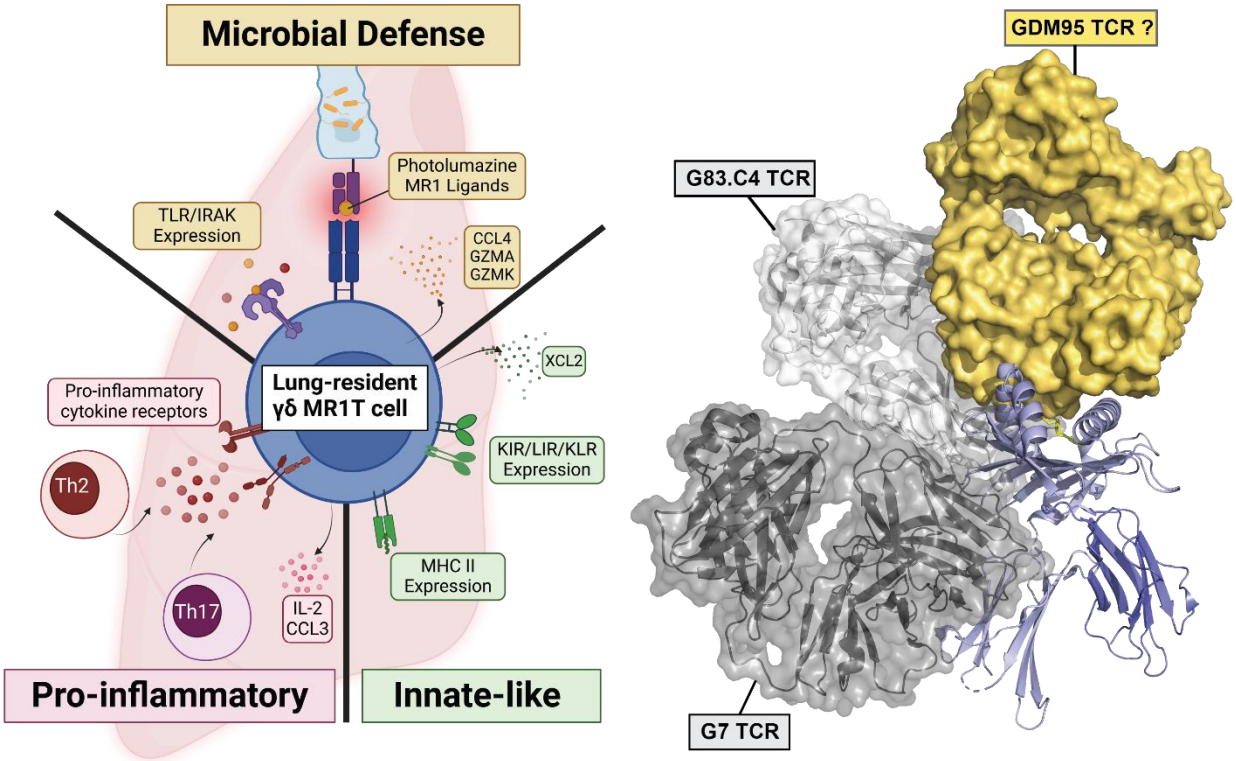



**Figure 5.2** An overview of the findings detailed in **Chapter 4**. (Left) Analysis of the gene expression data derived from the lung-resident γδ MR1T cell population in comparison to other lung-resident T cells described the γδ MR1T cell population to have three main effector phenotypes: First and foremost, they appeared to be focused on microbial defense – a finding supported by the PLIII MR1 restriction of GDM95. Second, they were shown to express the hallmark NKR and MHC-II molecules that aid their function as innate-like T cells. Finally, there was some evidence for these cells' participation in the asthmatic response. (Right) A cartoon representation of MR1 (light blue) being bound by the two known MR1 auto reactive γδ TCRs, G7 (gray) and G83.C4 (white), and a hypothetical representation of GDM binding is represented by a MAIT TCR shown in yellow. PDB: 6MWR, 7LLI, and 4LCW

It is important to note that these cells were found to be enriched in asthmatic lungs, resulting in a major outstanding question: are these cells helping to drive the pathogenesis of this

disease or are they simply drawn into and activated by the inflammatory signals associated with the pathology of asthma? While this is very much an open question, the transcriptome data does seem to have some answers. The expression of certain interleukin receptors show that these cells are primed to receive Th2 and Th17 mediated proinflammatory signals from the environment (**Figure 5.2**). While they seem to be producing fewer anti-inflammatory molecules than the other γδ T cells in these samples, it seems less likely that they are driving asthma pathogenesis, as they do not appear to be high producers of Th2 cytokines themselves. More work is needed to prove this to be true, but I would hypothesize that these cells are drawn into the inflamed tissue and partially activated by the signals present. As other groups have shown[92], inflammation of the mucosa can lead to greater permeation of microbially-derived flavins and other MR1 antigens related to photolumazines. It remains possible that these cells could be activated through their TCR as MR1 captures these antigens, which would explain their expanded numbers and activated effector phenotypes in asthma-associated samples.

As mentioned in the discussion of **Chapter 4**, work on this project is very much on going. Our recent procurement of more PLIII will revitalize our efforts to broaden the scope of this project. Through the help of Hortencia Graves, I have been able to clone out and express additional γδ MR1T TCRs from the dataset on the JrtB background. The ability to refold MR1 with PLIII and once again be able to make hMR1-PLIII tetramers will allow us to perform a myriad of critical outstanding experiments. First, we will take a closer in-depth look at GDM95. The generation of more hMR1-PLIII will finally allow us to study the biochemistry of the interaction between MR1 bound to PLIII and the GDM95 TCR through SPR and structural experimentation. The creation of hMR1 PLIII tetramers will also allow us to return to the lung tissue samples and survey for more direct interactions between γδ MR1Ts and MR1 that is

homogenously loaded with this molecule. As discussed in **Chapter 4**, the interaction between a PL-reactive γδ MR1T like GDM95 and a heterogeneously loaded tetramer that only contains a small amount of PLIII is relatively weak. When considering the difference in binding the GDM95 TCR exhibits between the PLIII hMR1 tetramer and the heterogeneously loaded hpMR1-M smeg tetramer, it is highly possible that we missed the vast majority of γδ MR1T cells in the initial scRNA-seq experiment. Flow cytometry experiments can confirm if this is truly the case, as I anticipate returning to the lung-tissue samples with a homogenously loaded PLIII tetramer will reveal a much higher frequency of γδ MR1T cells.

An outstanding line of inquiry that will likely be more difficult to answer but is still worth discussing would be confirming that the reactivity of the GDM95 TCR and others like it are solely constrained to PLIII. Our lab, in collaboration with others, has identified the structure of multiple photolumazine molecules from MR1 samples including PLI, PLIII, and the multiple isomers of PLV[68, 69]. Through the work of Dr. Nicole Ladd, we have also been able to identify the existence of many other photolumazines that make up a large proportion of the total ion content of hpMR1 made in the presence of various microbes (**Supplementary Figure S4.2**). Is it possible that these photolumazines also stimulate lung-resident γδ MR1T cells? I think it is highly probable, given that these molecules have the same ribityl chains that 5-OP-RU and PLIII have but are structurally much more like PLIII in their steric bulk. The major issue here is the limiting nature of these reagents. As we have found through collaboration with multiple chemists, these molecules are difficult to generate *de novo*. We have been successful at isolating bulk photolumazine molecules from culture, but isolating these highly related photolumazine molecules from one another remains an elusive goal.

The work described in **Chapter 4** allowed for the discovery of this novel γδ MR1T population and is just the beginning for this field of research. With improved tools at our disposal, the study of these cells will continue to yield exciting new insights for years to come - potentially generating new ways to treat mycobacterial infections or the pathogenesis of asthma along the way. Finally, when taking into consideration the total breadth of the work described in this dissertation, I truly hope that the reader can appreciate how the basic study of a single antigen presenting protein can lead to wide array of discoveries about the human immune system. These findings not only have long-term implications for how we think about the basic biology of the immune system, but also how we should consider treating diseases like cancer or infection in the future. The continued study of these avenues can only provide more of these insights, and I cannot wait to see what new surprises MR1 and MR1T cells have in store.

# Chapter 6. Materials and Methods

## 6.1 Materials and Methods for Chapter 2

### Cell Lines:

The L363 (DSMZ #ACC 49) and JY (ATCC #77441) cell lines were maintained in R10 media: RPMI 1640 (ATCC #30-2001) with 10% fetal bovine serum (Bio-Techne #S11150), Pen Strep [100 U/mL Penicillin 100 μg/mL streptomycin] (Gibco #15140122), and 1x L-glutamine (Gibco #A2916801). The Molp8 (DSMZ #ACC 569) and U266 (ATCC #TIB-196) cell lines were maintained in R20 media: RPMI 1640 with 20% fetal bovine serum, Pen Strep [100 U/mL Penicillin 100 μg/mL streptomycin], and 1x L-glutamine. LentiX cells (TakaraBio #632180) were maintained in DMEM (Corning #10-013-CV) with 10% fetal bovine serum, Pen Strep [100 U/mL Penicillin 100 μg/mL streptomycin], and 1x L-glutamine. All cells were grown in stationary incubators kept at 37°C and 5% $CO_2$ unless explicitly noted and cell lines were routinely checked for mycoplasma using the Universal Mycoplasma Detection Kit (ATCC #30-1012K) per the manufacturer's instructions. All stable MR1, CD8α, GCH1, and TCR expressing cell lines were made using lentiviral transduction. Lentivirus was generated by co-transfection of pMD2.G, psPAX2, and transfer plasmid (pHR_CMV or pHIV) containing the relevant protein into the LentiX cell line using Lipofectamine 3000 (Thermo Scientific #L3000001) per the manufacturer's instructions. After 72hrs, the lentiviral supernatant was harvested and 1 x $10^6$ recipient cells in 500uL growth media supplemented with 10ug/mL polybrene (Milipore #TR-1003-G) were transduced with 500uL of virus at 33°C and 850g for 2hr. Cells were allowed to rest 48 hours at 37°C and 5% $CO_2$ before assessing the expression of the relevant florescent reporter and were subsequently sorted on expression of this reporter unless otherwise noted.

Generation of myeloma hpMR1 and T22 inducible cell lines were performed as described[144]. In brief, Molp8, L363, U266, and JY cell lines were transduced with lentivirus containing the plasmids for both the TetR protein with a puromycin resistance cassette (pHR_CMV_Tetr_Puro) and the pHR_CMV_GFP-B2M-hpMR1 or pHR_CMV_GFP-B2M-T22 single chain construct proceeded by tetO2 binding sites between the construct and the promoter and followed by an IRES sequence and an mRuby reporter (pHR_CMV_GFP-3C-B2M-hpMR1_IRES-mRuby and pHR_CMV_GFP-3C-B2M-T22_IRES-mRuby). After transduction, cells were selected with 2ug/mL puromycin (Gibco #A1113803) over 10 days and subsequently sorted on mRuby expression. The resulting cells were frozen and thawed prior to use. The JY.MR1 and L3.MR1 cell lines were made via lentiviral transduction with the pHR_CMV plasmid containing the full length human MR1 protein sequence (GenBank accession number NM_001531) followed by an IRES mRuby reporter. These cells were sorted by MR1 and mRuby dual expression 48hrs post transduction. GCH1 overexpressing JY, L363, Molp8, and U266 lines were engineered in a similar fashion using the pHR_CMV plasmid containing the full length GCH1 monomer protein followed by an IRES mRuby sequence.

**Preparation of Myeloma-hpMR1 Samples:**

L363.Tetr.hpMR1-GFP, Molp8.Tetr.hpMR1-GFP, U266.Tetr.hpMR1-GFP, JY.Tetr.hpMR1-GFP, and JY.Tetr.T22-GFP cell lines were grown to 600mL before induction with 1μg/mL doxycycline (Sigma-Aldrich #D3447) and 2mM Sodium Butyrate (Millipore Sigma #8175000100). After 10 days of expression, cells were separated from the media by centrifugation and the resulting supernatant was brought to a final concentration of 20mM Tris and 100mM NaCl pH8.0 and then co-incubated with αGFP resin (made in-house, αGFP fab

protein plasmid was graciously gifted to us by the Kossiakoff Lab) for 4 hours at room temperature on an orbital shaker set to 100rpm. The resin was extracted from the supernatant by vacuum filtration and was washed with 40mL of PBS pH8.0. The hpMR1 protein was released from the αGFP resin by coincubation with the 3C enzyme (made in-house) at 4°C for 48hrs and was collected in the flowthrough as well as 3 x 2mL washes of the column with cold PBS pH8.0. Excess GFP was removed and the column regenerated by washing with 10mL of 0.1M glycine pH 2.6. Protein was pooled, concentrated, and purified further by size exclusion chromatography before ligand extraction and mass spectrometry analysis.

**Metabolite Extraction from Myeloma Cell Lines and MR1 Protein Samples:**

For the refold capture and refold enrichment samples included in the mass spectrometry ligand identification experiments, the metabolite extracts of Molp8, L363, U266, and JY cells were used. Cells were grown to 250 mL at a concentration of $1x10^6$ cells/mL before transfer to 50mL conical tubes (Fisher Scientific #1443222). Cells were pelleted in a pre-cooled 4°C centrifuge at 350g for 5 min and were washed twice with sterile ice-cold PBS pH7.4 (Corning #21-040) before being resuspended in 1mL of a solution of 80% methanol (Thermo Scientific #044571K7) 20% HPLC grade water (Sigma-Aldrich #576905). Cell suspension was transferred to a 1.7mL microcentrifuge tube (Dot Scientific #RN1700) and vortexed at the max setting continuously at 4°C for 10 minutes. Tubes were spun down for 10 minutes at 21,000g at 4°C. Extraction supernatant was removed carefully and dispensed to a new pre-weighed, microcentrifuge tube and the sample was dried under nitrogen stream before being weighed and then stored at -80°C until use. The total mass of the metabolite extraction was calculated by subtracting the starting weight of the tube to the final weight of the tube after complete drying.

“Mock” extractions followed the same exact protocol but started with 250 mL of growth medium instead of 250 mL of cell culture.

**Preparation of hMR1 Refolds:**

Soluble recombinant MR1 monomers were generated as previously described[68, 69, 206]. Briefly, nucleotide sequences coding for the soluble portions of the mature human β2m (GenBank accession number NM_004048.4) and human MR1 (GenBank accession number NM_001531) including an N terminal biotinylation sequence tag were cloned into the bacterial expression vector pET28a. Transformed Escherichia coli BL21(DE3)pLysS (Promega, catalog no. L1195) were then grown to optical density >0.6 at 600nm before induction with 1mM IPTG (GoldBio #I2481C100). After 4 hours of additional culture, the cells were lysed, and inclusion bodies were cleaned, purified, and fully denatured in 8 M urea, 10 mM EDTA, and 0.1 mM dithiothreitol (Thermo Scientific #R0861) for subsequent storage at −80°C.

Inclusion bodies for hB2M and hMR1 were thawed on ice and 1mM fresh dithiothreitol was added to each. A refolding buffer comprised of 0.1 M Tris–HCl pH 8.5, 2 mM EDTA pH 8.0, 0.4 M l-arginine, 5 mM reduced glutathione (GSH), 0.5 mM oxidized glutathione (GSSG). Was made fresh for each refold and kept at 4C. Inclusion bodies for hB2M were added to the refolding buffer while stirring at high speeds. After 30 minutes, directly before the addition of the hMR1 inclusion bodies, either purified ligands (e.g 5-OP-RU, BH2, 6-FP) or, in the case of the refold enrichment for mass spectrometry analysis, 4mg extracted metabolites diluted in 1mL HPLC grade water, were added to the refold mixture. The refold was allowed to incubate at 4C while stirring for 24 hours before transfer into 10kDa dialysis tubing (Thermo Scientific #88245) and dialyzed into 10x the total refold volume of 10 mM Tris–HCl pH 8.0. The

dialysis buffer was exchanged at least four times over the next 48 hours yielding at least a 1:10,000-fold final dilution. The dialyzed refold is then purified by DEAE resin, MonoQ™ anion exchange, and size exclusion chromatography (SEC). If being used for tetramers, the refolded hMR1-hB2M complex is biotinylated with the BirA enzyme (made in-house) for 48 hours at 4°C between the anion exchange and SEC steps of the purification. Biotinylation efficiency is tested by the addition of a molar excess of traptavidin (made in house) to a small aliquot of the protein and the corresponding shift to high molecular weight multimers visualized on an SDS-PAGE gel. See "**Generation of Homogenously Loaded Tetramers**" in section 6.2 for more information on tetramer reagents.

**Preparation of Protein Samples used in Mass Spectrometry Analysis**

As the last step of each protein purification for all protein that was sent for mass spectrometry ligand analysis, ligands were extracted and separated from the MR1 protein. All protein concentrations were normalized to be 0.5mg/mL prior to ligand extraction which was carried out by a protein crash with hot 70% EtOH (added at 100x volumetric excess) lightly shaking for 3 minutes. The sample was then spun at 21,000g for 10 minutes and the resulting supernatant was stored at -80°C until analysis. Samples were prepared in triplicate (separated by batches), immediately frozen after extraction, and stored at -80°C until all samples were prepared for submission to mass spectrometry analysis. Each metabolite lysate sample was resuspended to a concentration of 4mg/mL in HPLC grade water before being added to the refold mixture. 10uL of this was taken and diluted to 200uL in 70% EtOH to be used for the corresponding metabolite background sample.

**LC-MS Analysis and Ligand Identification:**

Untargeted metabolomic analysis was performed by General Metabolics (https://generalmetabolics.com/). The method described here is adapted from Fuhrer et al 2011[145]. Metabolome profiles of the sample extracts were acquired using flow-injection mass spectrometry. The instrumentation consisted of an Agilent 6550 iFunnel LC-MS Q-TOF mass spectrometer in tandem with an MPS3 autosampler (Gerstel) and an Agilent 1260 Infinity II quaternary pump. The running buffer was 60% isopropanol in water (v/v) buffered with 1 mM ammonium fluoride. Hexakis (1H, 1H, 3H-tetrafluoropropoxy)-phosphazene) (Agilent) and 3-amino-1-propanesulfonic acid (HOT) (Sigma Aldrich). The isocratic flow rate was set to 0.150 mL/min. The instrument was run in 4GHz High Resolution, negative ionization mode. Mass spectra between 50 and 1,000 m/z were collected in profile mode. Prior to analysis extracts were diluted further 1:2 in a chilled solution of 80% methanol in water. 5 μL of each sample was injected twice, consecutively, within 0.96 minutes to serve as technical replicates. The pooled study sample was injected periodically throughout the batch. Samples were acquired randomly within plates. Chemical formulae were matched against HMDB, KEGG and CHEBI metabolite databases resulting in 1,199 annotated ions within a 1 mDa annotation threshold. Ion intensities of technical (injection) replicates were averaged prior to statistical analysis.

**Analysis of Mass Spectrometry Data:**

The resulting table of annotated m/z values obtained for each sample was analyzed with the MetaboanalystR package for R (4.0.0)[207]. All comparisons were done where samples in the same group were normalized to a gaussian distribution prior to an unpaired t-test. FDR correction and foldchange analysis were performed next in order to create the data necessary to

generate each volcano plot. 12 of these comparisons were from the refold capture and recombinant expression data groups where myeloma samples were compared directly to either the RF-JY/UnL or the RE-JY/T22 samples respectively. The refold enrichment sample comparisons were performed by first standardizing each ion in the refold and background metabolite samples as a percent of total TIC, next the significantly enriched ions in either the mock or JY enrichment volcano plots were removed from the myeloma samples, finally the ions that were enriched in the refold as compared to the corresponding background ions were found via pairwise analysis. Further analysis of the data by averaging the fold change and p values for each significant ion allowed for identification of ions that were found to be significant in more than one condition or more than one cell line across samples and groups.

**MR1 Surface Stabilization Assays:**

JY.MR1 cells ($1x10^5$ cells per well) were tested for MR1 surface expression after incubation with either endogenous pterins, 6-FP, or a matched vehicle control for 16 hours at 37°C. 6-FP (20 μM) was used as a positive control for MR1 surface up-regulation. The cells were washed once with FACs buffer (Sterile PBS + 2.5% fetal bovine serum) and then blocked with Human Fc Block (BioLegend # 422301) for 20 min at 4°C before staining with LIVE/DEAD™ Fixable Near-IR Dead Cell Stain (ThermoFisher #L34975) and APC labeled anti-human MR1 mAb (clone 26.5, Biolegend # 361106) or with APC-labeled mouse IgG2a isotype control (clone MOPC-173, Biolegend #400211) antibodies for 20 min at 4°C, then washed three times in FACS buffer and analyzed by flow cytometry. For each condition, the fold change in MR1 expression was calculated by dividing the MFI of MR1-APC recorded for the

pterin treated cells by that of the same cells treated with a vehicle control added at the same volume.

**MR1 Ligands and Small Molecules:**

The source of MR1 potential and known MR1 ligands used in this study are summarized in the following table:

| Chemical Name | Manufacturer | Part number |
|---|---|---|
| (6R)-5,6,7,8-Tetrahydrobiopterin dihydrochloride | Sigma Aldrich | T4425 |
| 7,8-Dihydro-L-biopterin | Sigma Aldrich | 37272 |
| D-(+)-Neopterin | Sigma Aldrich | N3386 |
| Pterine-6-carboxylic acid | Sigma Aldrich | 82553 |
| 6-Biopterin | Sigma Aldrich | B2517 |
| Nicotinic Acid Mononucleotide | Sigma-Aldrich | N7764 |
| S-Nitrosoglutathione | Sigma-Aldrich | N4148 |
| 6-formylpterin | Schirks Co. | 11.415 |
| Pterin | Schirks Co. | 11.903 |
| Isoxanthopterin | Schirks Co. | 11.703 |
| L-Sepiapterin | MedChemExpress | HY-112234 |
| 11α-Hydroxyprogesterone β-D-Glucuronide | Santa Cruz Biotech | 208852 |
| Estrone 3-(β-D-glucuronide) sodium salt | sigma Aldrich | E1752 |
| Glycodeoxycholic Acid, Sodium Salt | sigma Aldrich | 361311 |
| Taurolithocholic acid 3-sulfate disodium salt | sigma Aldrich | T0512 |
| Cholic Acid 7-sulfate | Cayman Chemical | 9002532 |
| Chenodeoxycholic acid 3-sulfate disodium salt | Avanti Lipids | 700320 |
| 5-A-RU | In-house[68] | N/A |
| 5-OP-RU | In-house[68] | N/A |
| Photolumazine III | In-house[68] | N/A |

**Table 6.1** Origin of utilized MR1 ligands. This table details the manufacturer and part number for each chemical used. For chemicals "made in-house" the reference that details their synthesis is cited in the manufacturer column

**MAIT activation blocking assays:**

JY.MR1 APCs were plated at $5 \times 10^4$ cells per well in a 96 well plate. Endogenous pterin MR1 antigens or 6-FP were added to the indicated concentrations. APCs were incubated at 37°C

for 4 hours before washing with sterile PBS pH 7.4 and resuspension in fresh R10 media. 5-OP-RU (generated by the reaction of methyl-glyoxal and 5-A-RU at mixed at a 1:1 molar ratio immediately prior to use[65]) was added to each well at a final concentration of 10pM. After 30 minutes at 37°C, MR1 blocking (Ultra-LEAF Purified clone 26.5, Biolegend, #361110) or mouse IgG2a isotype control mAbs (Ultra-LEAF Purified clone MOPC-173, Biolegend, #400263) were added to the culture at 25ug/mL. APCs were incubated for an additional 30 minutes at 37°C before the addition of $5 \times 10^4$ JrtB.G11 cells. After 16hrs at 37C, cells were washed once with FACs buffer (Sterile PBS + 2.5% fetal bovine serum) and then treated with Human Fc Block (BioLegend # 422301) for 20 min at 4°C before staining with NearIR-L/D (Thermo Scientific #L34975), BV421-labeled anti-human CD69 (clone: FN50, BioLegend # 310929), and APC-labeled anti-human CD25 (clone: BC96, BioLegend # 302610) antibodies for 20 min at 4°C in the dark, washed three times in FACS buffer, and immediately analyzed by flow cytometry.

**GCH1 Modulation Assay**s:

The indicated cell lines indicated for each analysis were plated at $5 \times 10^4$ cells per well in 200uL fresh growth medium in a 96 well plate. Either 2,4-Diamino-6-hydroxypyrimidine (DAHP) (Thermo Scientific Chemicals, #B21240-22) or the same volume of a vehicle control (DMSO) was added to the cell culture. After a 5-hour incubation, the cells were washed and stained for expression of MR1 with APC-labeled anti-human MR1 mAb (clone 26.5, Biolegend # 361106) and for living cells using LIVE/DEAD™ Fixable Near-IR Dead Cell Stain (ThermoFisher #L34975). The fold change in MR1 expression was calculated by dividing the MFI of MR1-APC recorded for the DAHP treated cells by that of the same cells treated with the matched vehicle

control. For GCH1 over expression assays, the MR1 expression was compared between cell lines that were engineered to over express GCH1 and their parental cell line. In all GCH1 modulation experiments the surface expression of HLA-A,B,C (HLA-Ia) was monitored by co-staining with BV421-labeled anti-human HLA-A,B,C (clone W6/32, BD Biosciences #567862) to ensure the effect was specific to MR1.

## 6.2 Materials and Methods for Chapter 3

### Additional Cell Lines:

The JrtB cell line was generated by nucleofection of JRT3-t3.5 cells (ATCC #TIB-153) with the pSpCas9(BB)-2A-GFP (PX458) plasmid containing guides for human $\beta_2$M (guide 5′ CACCGGCCGAGATGTCTCGCTCCG 3′). Nucleofection was carried out on an Amaxa Biosystems Nucleofector II using the Cell Line Nucleofector Kit V (Lonza #VCA-1003) per the manufacturer's instructions. Crispr KO of $\beta_2$M was confirmed by flow cytometry with anti-human β2M-PE antibodies (clone: 2M2, BioLegend #316305) and genomic DNA sequencing. The JrtB cell line was modified further to over express CD8α to create the JrtB.CD8α cell line through lentiviral transduction with the pHR_CMV_CD8α_IRES-mRuby plasmid and subsequent sorting on CD8α and mRuby dual expression. To introduce TCRs into the JrtB and JrtB.CD8α cell lines TCRs were cloned into the pHIV vector as a single chain separated by a P2A cleavage site (TCRα_p2a_TCRβ). The pHIV_ TCRα_p2a_TCRβ vector was introduced to the JrtB and JrtB.CD8α cell lines via lentiviral transduction. Transduction efficiency was assessed at 48-72 hours post transduction. Transduced JrtB or JrtB.CD8α cells were assessed for TCR and plasmid expression by staining with anti-human CD3-APC antibody (clone: UCHT1, BioLegend # 300412) and LIVE/DEAD™ Fixable Near-IR Dead Cell Stain (ThermoFisher

#L34975) as well as expression of cytoplasmic GFP from the pHIV plasmid. Cells were gated on size, singlet status, and Live/Dead negative before being assessed for GFP and CD3-APC. GFP and APC double positive cells were considered to be successfully transduced and TCR+.

**Generation of Homogenously Loaded Tetramers:**

The functional monomer of hMR1 loaded with a given ligand is obtained from hMR1 refold as outlined in **section 6.1**. This hMR1 monomer was biotinylated using a BirA enzyme (in-house) overnight at 4°C. Excess biotin was removed using S75 10/30 (GE Healthcare, 29148721) gel filtration before tetramerization with APC-conjugated streptavidin (Prozyme, catalog no. PJ25S) or PE-conjugated streptavidin (Prozyme, catalog no. PJRS25) at a 4:1.1 molar ratio over 8 additions. Each batch of tetramers were tested for quality before use by titrating them with the WT-TCRnull JrtB line and (when applicable) a JrtB line bearing a TCR known to recognize the specific antigen.

**Tetramer Staining Experiments:**

JrtB or JrtBCD8α cells were plated at $1x10^5$ cells/well in a 96 well plate. Cells were washed once with FACS buffer prior to staining with the indicated concentration of hMR1-APC tetramer. hMR1-APC Tetramer dilutions were made in FACs buffer supplemented with 10% human serum (GeminiBio #100-512-100). After washing, cells were stained with the tetramers at a volume of 25uL for 45 minutes at 4°C in the dark. After staining, cells were washed three times with FACS buffer prior to analysis by flow cytometry. Cells were gated on size, singlet status, and GFP expression prior to being analyzed for tetramer binding (sample gating strategy shown in **Supplemental Figure 3.2 A**).

**Jurkat Activation Assays:**

JY.MR1 APCs were plated at $5 \times 10^4$ cells per well in a 96 well plate. Endogenous pterin MR1 antigens were added to a final concentration of 250uM. Control MR1 ligands 6-FP and 5-OP-RU were added to 20uM and 20pM respectively. APCs were incubated at 37°C for 4hrs before washing with sterile PBS pH7.4 and being resuspended in fresh R10 media. MR1 blocking (Ultra-LEAF Purified clone 26.5, Biolegend, #361110) or mouse IgG2a isotype control mAbs (Ultra-LEAF Purified clone MOPC-173, Biolegend, #400263) were added to a final concentration of 25ug/mL. APCs were incubated for 30 minutes at 37°C before the addition $5 \times 10^4$ of the indicated JrtB or JrtB.CD8a cell line. All assays shown were done at a 1:1 effector to target ratio. After 16hrs at 37°C, cells were washed once with FACs buffer, stained as described in **section 6.1 "MAIT activation blocking assays",** and then immediately analyzed by flow cytometry. Cells were gated on size, singlet status, live dead stain negative, and CD3 GFP double positive expression prior to being analyzed for activation markers (sample gating strategy shown in **Supplemental Figure 3.2 B**).

**Human Samples:**

Deidentified healthy donor PBMCs were harvested from fresh blood draws from anonymized, consented donors into heparin-coated tubes. They were separated from other blood components by Ficoll-Paque (Cytiva # 17144002) before freezing in fetal bovine serum supplemented with 10% DMSO (Corning #25-950-CQC). Deidentified myeloma patient samples were obtained frozen from a biobank ran by Dr. Andrzej Jakubowiak at the University of Chicago Medical Center.

When ready for analysis, samples were warmed in a 37°C water bath until almost completely thawed. Warm R10 media was dripped into each sample container prior to the whole sample being transferred dropwise to 10mL of warm R10 media. The cells were spun down and counted using a LUNA-FL™ Automated Fluorescence Cell Counter (Logos Biosystems #L20001) and propidium iodide live dead dye (Logos Biosystems # F23003). Cells were washed once in FACs buffer and then stained with Near IR-L/D (Thermo Scientific #L34975) at a ratio of 1:1500 for 10 minutes at 4°C in the dark. Tetramers dilutions were prepared in FACs buffer supplemented with 10% human serum (GeminiBio #100-512-100) Human Fc Block (BioLegend # 422301). PE and APC 5-OP-RU hMR1 tetramers were added at 15nM each while PE and APC BH2 hMR1 tetramers were added at 100nM each. The 5-OP-RU tetramers were added at a lower concentration as it was noted in pilot experiments that they began to block other TCR complex targeting antibodies when included at concentrations higher than 25nM. 50uL of the desired tetramer mix was added to each cell sample and allowed to incubate for 45min at 4°C in the dark. The antibody panel was assembled during this time in FACS buffer. The antibodies used are summarized in **Table 6.2**. FMOs for each sample and each color used were prepared alongside the full panel mixture. FMO controls from each sample were used for all gating. After staining with tetramers, the cells were spun down and resuspended directly in the appropriate antibody mixture for the given sample. Cells were allowed to stain 20 minutes at 4°C in the dark in this cocktail. The cells were then washed three times in FACS buffer before analysis by flow cytometry (sample gating strategy shown in **Supplemental Figure 3.3 A**).

| Antibody Target | Fluorophore | Clone | Manufacturer | Part number |
|---|---|---|---|---|
| CD3 | BUV395 | UCHT1 | BD Biosciences | 563546 |
| CD4 | SBUV510 | RPA-T4 | BioRad | MCA1267SBUV510T |
| Tim3 | BV421 | F38-2E2 | BioLegend | 345007 |
| CD161 | BV510 | HP-3G10 | BioLegend | 339921 |
| PD-1 | BV650 | EH12.2H7 | BioLegend | 329949 |
| TRAV1-2 | BV785 | 3C10 | BioLegend | 351721 |
| CD26 | FITC | BA5b | BioLegend | 987502 |
| CD8 | PerCP-eFlor710 | SK1 | Thermo Scientific | 46-0087-42 |
| CD14 | APCcy7 | 63D3 | BioLegend | 367107 |

**Table 6.2** Antibodies used in the human sample flow cytometry panel. Target, conjugated fluorophore, antibody clone, manufacturer, and part number are show for each antibody.

## 6.3 Materials and Methods for Chapter 4

### Generation of bacterially loaded hpMR1:

The generation of heterogeneously loaded hpMR1 was done primarily by Nicole Ladd. Methods describing the production of hpMR1 loaded with heterogeneous ligands from bacteria have been described in detail in the literature and in published protocols[68, 69, 206]. Briefly, a recombinant, secreted, single-chain hpMR1 construct composed of the bovine α3 and β2-microglobulin domains and the human α1 and α2 domains is generated into a baculovirus. Hi5 cells are infected with the hpMR1 baculovirus at a density of $2 \times 10^6$ cells/ml. M. smegmatis is added to the Hi-5 culture at a density of $4 \times 10^4$ cells/ml. After 72 h, the supernatant is separated from the cells by centrifugation, filtered, and buffer exchanged into HBS pH 7.4. Protein is purified by Ni-NTA resin (Thermo Fisher #88223) binding of a 12x His tag on the hpMR1 protein. After cleavage of the 12x His tag by 3C protease (in-house), the protein can be biotinylated with the BirA enzyme (if needed) prior to final purification by size exclusion chromatography.

**Lung mononuclear cell isolation:**

All lung tissue processing and cell staining for scRNA-seq was performed by Augusta Broughton as described in her dissertation: "Cells were isolated from dissociated lung tissue as described previously. Briefly, human lung samples were obtained from Gift of Hope Regional Organ Bank of Illinois, from organ donations that were not used for transplantation. Samples were denoted as "asthmatic" or "nonasthmatic" based on self-reported asthma status. Therefore, "nonasthmatic" samples cannot be considered "healthy controls" because they were not verified to be free of all lung disease. Lung tissue was perfused with FBS and PBS. Lymph nodes attached to the lungs dissected and pooled. Lymph nodes were mechanically dissociated into a single cell suspension and cryopreserved. Likewise, lung lobes were mechanically dissociated, enzymatically digested, and cryopreserved. For use in experiments, the lung and lymph node samples were thawed and subjected to density gradient centrifugation with Ficoll to remove dead cells and debris before proceeding to flow cytometry staining or sample preparation for single-cell RNA sequencing."

**Generation of barcoded tetramer reagents:**

The generation of oligo barcoded MR1 and CD1d tetramers was performed by Nicole Ladd and Augusta Broughton. In brief, DNA oligos compatible with the Chromium Single Cell 5' v2 workflow (containing barcodes from the Feature Barcode Whitelist) were obtained from IDT with a 5' C12 Amino Modifier. Streptavidin PE (Agilent Technologies, PRJS34) was modified with each oligo using the Vector Laboratories Protein-Oligo Conjugation Kit (Vector Laboratories #S-9011-1) per the kit instructions. The final modified Streptavidin-PE was purified by size-exclusion chromatography to remove excess oligo. MR1 or CD1d tetramers were

produced by adding labeled Streptavidin-PE to the MR1 or CD1d protein a 4:1 molar ratio over 8 separate additions.

**Sequencing:**

After sorting based on tetramer staining, the 10X Genomics Chromium Next GEM Single Cell 5' Reagent Kits v2 (Dual Index) with Feature Barcode Technology User Guide was used as instructed to generate DNA libraries (CG000330 Rev D). 5 ng of cDNA was put toward γδ TCR transcript amplification, which was amplified with custom primers and then processed per the instructions found within the Reagent Kits v2 (Dual Index) with Feature Barcode Technology User Guide. After verifying quality of the libraries, samples were submitted to the University of Chicago Genomics Facility for sequencing on the NovaSeq 6000. Paired end 26-10-10-90 bp runs with unique dual indices were performed for each library.

**scRNA-seq Data Analysis:**

FASTQ files were preprocessed using the Cellranger pipeline (3.0 or 7.0) developed by 10X Genomics and all reads were aligned to the GRCh38 reference genome. For alignment of the γδ sequences, a custom reference gene set generated from IMGT consisting of only TRG/TRD sequences was used. Unfiltered TCR contigs were paired, quantified, and analyzed using scRepertoire (v 1.7.2). scRepertoire was also used to quantify clonal expansion by cell count and diversity indices. Potential doublet GEMs were removed based on the number of genes per cell and UMIs per cell while low-quality dying cells were removed based on the percent of expressed genes being of mitochondrial and ribosomal nature. Only cells with paired TCR sequences were included in repertoire analyses; rare cells with both αβ and γδ TCR

sequences called were excluded. Processed count matrices were analyzed with Seurat (4.3.0.1). Quality control steps were performed to filter out low-quality cells based on total gene counts, unique molecular identifiers (UMIs), and mitochondrial gene expression. Data from each sample was normalized with SCTransform (0.3.5) and integrated using Harmony (0.1.1) to correct for batch effects from samples processed on different days. Only cells with a tetramer signal ten times higher than background were considered to be true tetramer positives. MR1 positive γδ T cells were only considered for analysis if they were single positive for the MR1-M smg tetramer. γδ T cells that were positive for the MR1 tetramer and one or more of the CD1d tetramers were not considered to be MR1-resticted and were therefore not included in the γδ MR1T analysis. Graphs were made using ggplot2 (3.5.1), cowplot (1.1.1), ggseqlogo (0.2) and enhancedVolcano (1.22.0)

**CDR3 Analysis with AIMS:**

The AIMS software was used to perform detailed biophysical analysis of the CDR3δ loop sequences from the γδ T cells recovered in the scRNA-seq experiment[183]. First, a list of non-MR1 tetramer reactive and MR1 tetramer positive cells was generated by filtering for these parameters within the metadata of the Seurat object generated for this analysis. Just the CDR3δ sequences were retrieved from these lists and were reformatted in order to be fed into the AIMs graphical user interface (v0.9). CDR3δ sequences from MR1 tetramer positive and MR1 tetramer negative γδ T cells were aligned using budge alignment and then analyzed via PCA and DBScan clustering. The figures generated for this dissertation were made by choosing to directly compare the MR1 tetramer positive and MR1 tetramer negative γδ T cells that resulted from this analysis

rather than comparison by cluster. All figures were generated following the standard AIMs workflow from that point forward (https://aims-doc.readthedocs.io/en/latest/AIMS_GUI.html#)

**Flow Cytometry:**

All flow cytometry experiments were carried out on a BD Aria A3 cytometer utilizing the BD FACS Diva software. All antibodies and tetramers were titrated on relevant cell lines prior to use and gating was performed using isotype or FMO controls. Flow data was analyzed on FlowJo (V10.0) prior to statistical analysis in GraphPadPrism (Version 9.1.0)

**Statistical analysis**

An unpaired two-tailed Student's t test was performed adjusted for multiple comparisons using the Benjamini-Hochberg method (FDR < 0.05) while other analyses were done using a one-way analysis of variance (ANOVA) allowing for multiple comparisons as indicated for each assay in the figure legends. All statistical assessments were done using GraphPadPrism (Version 9.1.0, GraphPad Software Inc.). For graphs that show statistical significance, asterisks denote the level of statistical significance (*P < 0.05, **P < 0.01, ***P < 0.005, and ****P < 0.001). using GraphPad Prism version 9.1.0.

**Word Processing and Graphics Design:**

Figures were made using Adobe Illustrator (29.3), Biorender, Prism (9.1.0), ChemDraw (23.1.2), Pymol (2.5.4), FlowJo (v10.0), or the R package ggplot2 (3.5.1). Many figures contain images that were generated using a combination of these software packages complied in Adobe Illustrator. Figures that utilized Biorender images are 1.1, 1.2, 1.5, 1.6, 2.1, 2.2, 2.4, S2.1, 3.1,

S3.1, 4.1, S4.2, 5.1, and 5.2. This dissertation was drafted and completed using Microsoft Word (Microsoft Office 2024 v2412).

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