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Mucosal-associated invariant T (MAIT) cell contributions to mucosal immunity

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Abstract

Mucosal-associated invariant T (MAIT) cell contributions to mucosal immunity

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Mucosal-associated invariant T (MAIT) cells are a semi-invariant T cell subset that are located in blood, liver and mucosal tissues. When activated MAIT cells display potent effector functions including secretion of pro-inflammatory cytokines such as interferon- γ (IFN γ) and tumor-necrosis factor alpha (TNF α) and lysis of bacterially-infected cells through granzyme B. MAIT cells recognize bacterial-derived metabolites, from commensal and pathogenic bacteria, via their semi-invariant T cell receptor (TCR) in the context of the MHC class I related molecule MR1. How MAIT cells distinguish between commensal and pathogenic bacteria is unknown.

MAIT cell frequency and function is decreased in the blood in the chronic phase of HIV, HCV, and tuberculosis infection and during these infections MAIT cells express inhibitory receptors. Whether MAIT cells are activated during the acute stage of infection and how MAIT cell responses are turned off is unclear.

Here, we address basic questions regarding MAIT cell activation and function during inflammatory disease states: 1) how MAIT cells distinguish between pathogenic and commensal antigen, 2) MAIT functional responses during acute viral infection and 3) how the MAIT cell response is turned off. We demonstrate that a TCR signal alone is not sufficient for sustained MAIT cell effector function, but rather a TCR + cytokine signal is necessary for MAIT cell activation. We propose that this unique control of effector function allows MAIT cells to respond to the same TCR signal (commensal or pathogen derived) in a dichotomous and situation-specific manner. Using an SIV model of infection, we demonstrate that MAIT cells at mucosal sites undergo rapid activation, and acquire effector functions such as expression of tumor-necrosis factor alpha ($TNF\alpha$) and granzyme B. In contrast, MAIT cells in the periphery display an early loss of cytokines important for barrier immunity, such as IL-17 and IL-22. These data suggest that MAIT cells become activated early in the mucosa after infection and may play an important role in mucosal homeostasis. Lastly, we interrogate MAIT cell function and transcriptional profiles in inflamed mucosal tissue to address how the MAIT cell response is turned off. We find distinct patterns of inhibitory transcripts by MAIT cells within the inflamed mucosa, including high expression of the inhibitory receptor cytotoxic T-lymphocyte associated protein 4 (CTLA-4), and that cytokine signals alone are sufficient for expression of CTLA-4. Together, our data highlight MAIT cells as important effector cells located at the site of pathogen entry, and begin to elucidate the mechanisms of MAIT cell regulation during inflammatory disease states.

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Dedication

To my Papa, who passed along his dedication to education and the pursuit of knowledge to the next generation.

Go Bears.

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Chapter 1:

Introduction

Conventional and unconventional T cells

The immune system of vertebrate animals has evolved to detect and appropriately respond to an incredible breadth of microbial diversity. Many of these microbial organisms living within the host provide benefits including important nutrition and growth factors as well as function to prevent invasion by pathogenic microbes. Often, these microbes are described as “commensal” due to their symbiotic relationship with the host. In contrast to commensal organisms, pathogenic organisms typically evoke a strong immune response that is initiated upon invasion. These organisms could either be external invading microbes or commensals that develop into pathogenic invaders¹. Protective immunity against these organisms is required to prevent life-threatening infections caused by viruses, bacteria, and parasites. Therefore, an intact immune system must be able to clearly identify pathogenic organisms, eliminate or contain them in such a way that is protective to the host, and remember the pathogen such that upon re-infection there is an immediate and efficient response resulting in clearance of the disease-causing organism. The specificity of these systems is incredibly important; recognition of a harmless microbe as a threat to the host can result in damage to tissues and an overt immune response, while a lack of an appropriate response to pathogenic organisms results in chronic infections or death of the host²⁻⁶. Thus, one of the fundamental purposes of the immune system is to appropriately

discern benign from deleterious.

The immune response to infection has been classically defined by two phases: the rapid, non-specific innate phase that is initiated within hours after pathogen invasion, followed by the antigen-specific adaptive response that takes time to develop and results in memory formation. Microorganisms that invade a vertebrate host are initially recognized by the innate immune system through germline-encoded pattern-recognition receptors (PRRs)⁷. Several classes of PRRs exist and include toll-like receptors (TLRs) expressed on the surface of cells or in endocytic vacuoles as well as cytoplasmic pathogen recognition systems. Engagement of TLRs or cytoplasmic receptors triggers the activation of signaling cascades and leads to the induction of genes involved in antimicrobial host defense^{8,9}. Genes encoding inflammatory cytokines are included in this response to pathogen invasion, and facilitate the development of adaptive immune responses¹⁰⁻¹⁴.

T cells have been typically categorized as adaptive immune cells due to their unique ability to recognize a large breadth of foreign and self-derived epitopes, clonally expand, and form memory against subsequent infections that often lasts the lifetime of the host^{15,16}. Naive T cells require three signals for activation: 1) Recognition of cognate antigen through TCR-MHC interactions, 2) co-stimulation through CD28/B7 ligation and 3) cytokine activation¹⁷. Upon recognition of cognate antigen in the context of an inflammatory response, naïve T cells begin to proliferate and acquire effector functions. Once pathogen has been cleared, the majority of effector cells undergo apoptosis (>90%)¹⁸. The remaining fraction of antigen-specific T cells is termed “memory” due to their ability to respond rapidly upon subsequent infections^{15,19}. More recently, it has

been demonstrated that memory CD8 T cell populations can be activated early after infection in an antigen-independent manner²⁰⁻²⁸. These cells are thought to behave in an “innate-like” manner during initial stages of infection, and are termed “bystander-activated” cytolytic T cells (BA-CTL).

In contrast to conventional T cells, a wide variety of unconventional, or innate-like T cells, participate in the initial immune response²⁹. These cell subsets include natural-killer T (NKT) cells³⁰, germline-encoded mycolyl-lipid reactive (GEM) T cells³¹, and mucosal-associated invariant T (MAIT) cells³². Similar to conventional T cells, innate-like T cells can develop antigen-specific responses²⁹. However, innate-like subsets display restricted TCR usage and recognize antigen that is presented via non-classical MHC-like molecules such as CD1 and MR1^{33,34}. In addition to antigen-recognition, it has been demonstrated that both NKT and MAIT cells can become activated through inflammatory cytokine stimulation³⁵⁻³⁹.

Mucosal-associated invariant T (MAIT) cells

MAIT cells are a recently described subset of T cells that bridge the gap between innate and adaptive immune responses. Expression of a semi-invariant T cell receptor confers antigen-specificity, while high expression of cytokine and activating receptors allows for a rapid, antigen-independent response by the MAIT population^{40,41}. Balk and colleagues first discovered MAIT cells in 1993 when randomly cloning TCR alpha chains from double-negative T cells isolated from intraepithelial lymphocytes. They had identified a new subset of cells that preferentially used an invariant TCR alpha chain, which paired with a limited number of beta chains^{32,42}. However, it wasn't until more than 10 years after their discovery that the non-classical MHC-like molecule, MR1, was

found to restrict the MAIT TCR⁴³. This unique cell population constitutes 1-8% of circulating lymphocytes in human blood and various mucosal tissues, and is easily identified in the T cell compartment in human blood and throughout various mucosal tissues by co-expression of the NK surface receptor CD161 and their invariant TCR alpha chain, V α 7.2⁴¹. Following their initial discovery, MAIT phenotype and functions were characterized, and it was later shown that the MAIT TCR recognizes metabolites presented by the major histocompatibility complex related protein 1 (MR1) derived from the riboflavin synthesis pathway of some bacteria and yeast^{40,44}. These metabolites are present in a subset of commensal and intracellular pathogenic bacteria located at the site of the mucosa^{44,45}. When activated, MAIT cells rapidly acquire effector function and secrete pro-inflammatory molecules such as interferon- γ and kill bacterially infected target cells by secretion of the cytolytic molecule granzyme B^{46,47}.

Activation and effector functions of MAIT cells

MR1 is ubiquitously expressed throughout various tissues at the transcript level, but not constitutively expressed on the cell surface^{48,49}. The mechanisms that drive MR1 surface expression remain unclear, although a role for TLR signaling has been demonstrated^{50,51}. B cells and other professional antigen presenting cells (APCs) express MR1 in mucosal tissues, and more recently MR1+ cells in the female genital tract have been identified⁵².

Early studies of MAIT cell activation began with investigations into the functional properties of MAIT cells through the use of whole peripheral blood mononuclear cell

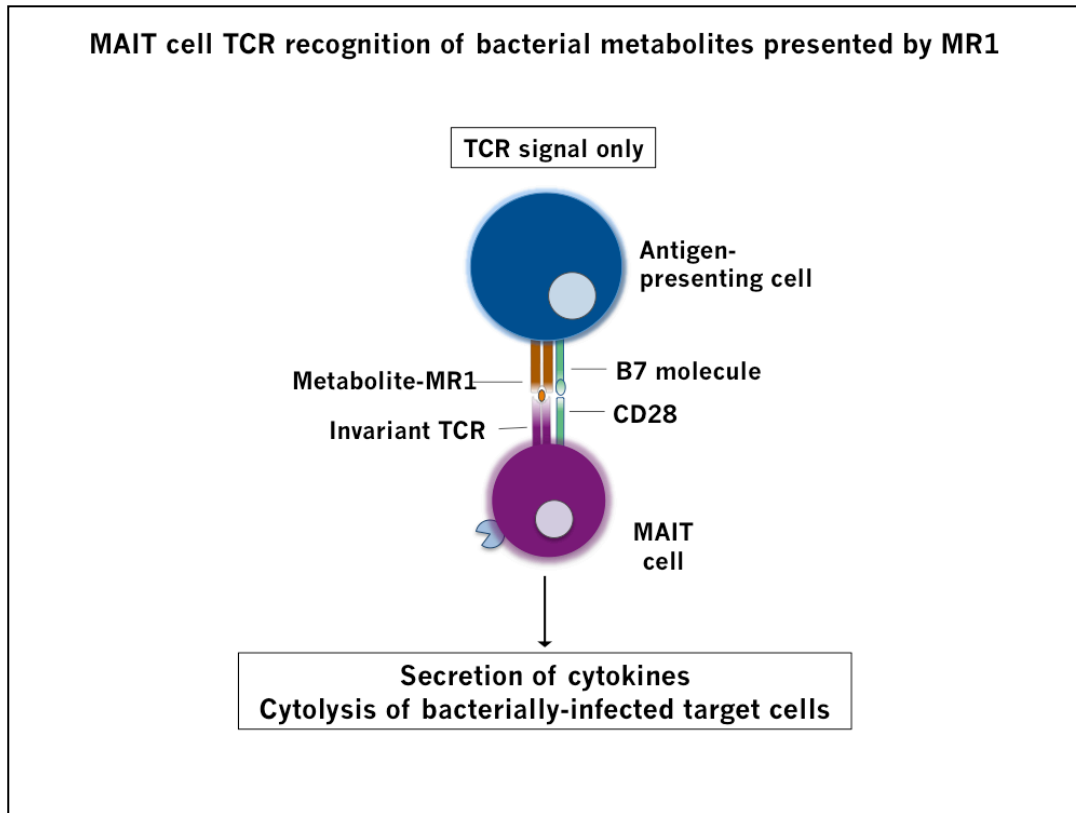


Figure 1.1. Schematic representation of TCR-dependent MAIT cell activation.

Upon recognition of bacterial-derived metabolites by the MAIT invariant TCR, MAIT cells acquire effector function and secrete cytokines such as $\text{IFN}\gamma$ and $\text{TNF}\alpha$, and kill bacterially-infected target cells through granzyme exchange. This model does not distinguish between MAIT cell responses to commensal or pathogen-derived antigen.

(PBMC) stimulation^{41,47}. These experiments focused on TCR-mediated mechanisms of activation and included activation of MAIT cells via stimulation of *E. coli*-fed antigen presenting cells (APCs) or cell lines⁴⁷. Upon stimulation with *E. coli*-fed APCs, MAIT cells upregulate the activation marker CD69 and begin to secrete inflammatory cytokines such as IFN γ and TNF α , and this activation can be partially blocked through the use of high concentrations of anti-MR1 blocking antibody^{41,47,53,54}. In addition to expression of activation markers and secretion of inflammatory cytokines, early studies of MAIT cell function also focused on MAIT cell lysis of bacterially infected cell lines⁴⁶. Thus, initial studies of MAIT cells centered on TCR-mediated activation and concluded that TCR signals alone were sufficient for MAIT cell activation (Figure 1). However, what these preliminary studies failed to address is the mechanism by which MAIT cells are able to distinguish commensal bacterial species from pathogenic, and mount an appropriate effector response. The distinction cannot be simply made due to differences in antigen, as the metabolite derived from both commensal and pathogenic bacteria is identical. Thus, there must be other means by which MAIT cells are able to discern between harmless and deleterious pathogens.

The first reports of TCR-independent activation of MAIT cells first came from studies of the CD161^{hi} CD8⁺ and MAIT cell subsets^{39,55,56}. Through whole PBMC stimulation with IL-12/18, or TLR agonists, it was determined that MAIT cells were responsive to inflammatory cytokine signaling in the absence of a TCR signal^{39,55}. These studies provided an additional activation mechanism of MAIT cells, but did not differentiate between the direct vs. indirect effects of cytokine or TLR-stimulated PBMCs

on the MAIT cell subset, or the downstream consequences of cytokine-induced activation.

MAIT cells and infectious disease

Soon after the initial characterization of MAIT cells in healthy human blood and tissues, the field began investigations into the MAIT cell response during HIV and tuberculosis infection. *Mycobacterium tuberculosis* (Mtb), the causative agent of tuberculosis (TB), infects alveolar macrophages and utilizes the riboflavin synthesis pathway that leads to the generation of the MAIT cell metabolite, 6 formyl-pterin (6-FP)^{44,57}. In early studies of MAIT cell activation, cell lines infected with Mtb, or dendritic cells fed cell wall isolates from Mtb, were used to demonstrate TB-specific activation of MAIT cells⁵³. During this time it was also demonstrated that MAIT cells were depleted in the blood of patients with active TB or HIV/TB co-infection, but not in those patients with latent TB infection^{47,53,58,59}.

At the same time of these TB studies, a few groups began investigations into MAIT cell alterations during chronic HIV infection. Since bacteria and yeast are the only microbes that have the potential to generate the MAIT cell metabolite, the question as to how a viral infection impacts MAIT cell function was addressed. It was initially demonstrated that individuals with chronic HIV infection had lower frequencies of circulating MAIT cells compared to healthy individuals, and that these MAIT cells displayed limited responses to *E.coli*-fed PBMC^{60,61}. However, a more recent conflicting report using the MR1 tetramer has noted that although MAIT cells are depleted early (within 6 months after transmission), the cells remain functional up to 2 years following infection⁶². It is known that the observed depletion in the blood is not due to direct

infection by HIV, as the most abundant CD8⁺ MAIT cell population does not express the CD4 co-receptor required for HIV entry⁶¹. Interestingly, MAIT cell frequencies do not rebound upon initiation of anti-retroviral therapy, indicating there is a significant and profound alteration to the population during the course of infection⁶¹. These initial studies provided some insight into MAIT cell alterations during chronic HIV infection, but failed to determine at what point MAIT cell population alterations occur, whether MAIT cells are trafficking to mucosal sites or undergoing cellular apoptosis, or if the lack of response to *E.coli*-fed PBMC is a MAIT intrinsic defect or due to a defect in antigen presentation or cytokine generation from the APCs in the PBMCs from these participants.

More recent work has begun to explore trafficking of MAIT cells to mucosal sites during HIV infection, although there have been conflicting reports as to whether there is a preservation, enrichment, or depletion of MAIT cells at mucosal sites⁶⁰⁻⁶². In addition to the difficulty in obtaining human mucosal biopsies, the methods to identify MAIT cells in mucosal tissue sections remains challenging due to the markers used to identify MAIT cells using flow cytometry. In the blood, MAIT cells are defined as CD161^{hi}V α 7.2⁺ cells in the T cell subset, however distinguishing between CD161 positive versus highly positive cells in tissue is not currently feasible. To get around this obstacle, other markers such as IL-18R α and MDR1 are used, however these markers alone do not distinguish MAIT cells from conventional T cells^{60,61,63}. This may partially explain conflicting reports on MAIT cell numbers in mucosal tissue.

These studies provided a basic understanding of alterations to the MAIT cell populations during chronic infections, however the kinetics of these responses and the

signals driving MAIT cell activation or migration have yet to be discovered. Recent work has demonstrated that MAIT cells are activated in an IL-12/18 dependent manner in a variety of viral infections, and provides some insight into the activation that may be occurring during HIV infection^{64,65}. In addition to these studies, investigations into MAIT cell functional responses in macaque models of infection have begun to explore basic questions regarding MAIT cell activation during chronic SIV infection and report similar kinetics to what has been observed in human cohorts⁶⁶.

Mucosal-specific immune responses by MAIT cells

The mucosa is often the first site of pathogen invasion and infection; therefore it is extremely important to understand the initial immune response as it functions to control early infection while shaping the subsequent adaptive responses. In addition to fighting infectious pathogens, the mucosal immune system must also protect the host from the commensal microflora that reside along mucosal sites. To achieve this, the mucosal immune system has developed a variety of mechanisms to maintain a homeostatic balance with commensal organisms while protecting the host from invading pathogens at the site of the mucosa^{67,68}. These mechanisms include secretion of mucus by goblet cells to create a physical mucosal barrier, generation of antimicrobial peptides secreted by epithelial cells, gut-associated lymphoid tissues and immune cells, and secretion of IgA into the gastrointestinal lumen⁶⁹⁻⁷⁴. In addition to these mucosal-specific responses, immune surveillance and cytokine production by innate and adaptive immune cells creates another layer of protection at barrier sites^{75,76}, and includes secretion of cytokines important for maintenance of barrier functions, such as IL-17 and IL-22⁷⁷⁻⁷⁹.

MAIT cells constitute 1-8% of all T cells throughout mucosal sites such as the gastrointestinal, lung, oral, and female genital mucosa^{41,52,53}. What is currently known about MAIT cell function is primarily based on activation studies of MAIT cells within the peripheral blood^{38-41,45-47,54,80,81}. Very few studies have investigated the effector response of MAIT cells at the site of the mucosa or within tissues in response to infection or inflammation^{56,59,63,82,83}. A handful of studies have shown that MAIT cells are depleted in the blood of individuals with chronic infections or autoimmune disorders such as HIV, tuberculosis, and multiple sclerosis^{58,60,61,84,85}. In these disease settings, it is unclear whether MAIT cells are recruited to the tissue from the blood resulting in the observed decreased frequencies, or if MAIT cells in the blood are specifically depleted during infection or autoimmunity. Recent work has begun characterizing MAIT cells within mucosal sites during inflammatory bowel diseases (IBD) or colorectal cancers^{82,83,86} and has noted increased frequencies of MAIT cells at these tissue sites, however the mechanisms driving this increase in the tissue is unknown.

We have examined MAIT cell frequencies in healthy and diseased mucosal tissues and found a wide range of MAIT cell frequencies (from 1-20%) among CD3⁺CD8⁺ cells suggesting that MAIT cell frequency in mucosal tissue and can vary greatly depending on the donor (data not shown). Their presence in mucosal tissues⁴¹ positions them as a putative early responder to any kind of infection. With the potential to secrete pro-inflammatory mediators to contribute to disease progression, it is important to understand the signals that drive MAIT cell activation at the site of the mucosa, and elucidate their role during infection. Furthermore, due to their location at various mucosal sites as well as expression of chemokine receptors specific for the

mucosa and sites of inflammation¹, MAIT cells may greatly influence not only the primary response to a natural infection, but also those generated during vaccination.

Regulation of MAIT cell responses

An important facet of the immune response to infection is the ability to regulate the immune response and temper highly potent effector responses that can lead to tissue damage and destruction of host processes⁸⁷⁻⁹⁰. Regulation of the cellular immune response can be achieved through cell-intrinsic mechanisms including expression of inhibitory markers such as cytotoxic leukocyte antigen-4 (CTLA4) or programmed cell death-1 (PD-1) on the surface of activated cells⁹¹, as well as expression of pro-apoptotic molecules on the cell surface such as Fas receptor⁹². Additionally, cell-extrinsic suppression via regulatory T cells (Treg)⁹³⁻⁹⁶ prevents overt immune activation. Tregs suppress immune responses through a variety of methods including secretion of inhibitory cytokines such as IL-10, sequestration of IL-2, engagement of B7 ligands through CTLA-4 on antigen presenting cells, or cytolysis of immune cells⁹⁷⁻¹⁰¹. To date, studies of MAIT cells have focused solely on elucidating the signals necessary for MAIT activation and function. How the MAIT cell response is shut off has yet to be discovered.

In the periphery, MAIT cells constitutively express the Fas receptor, suggesting that they are susceptible to Fas-mediated apoptosis⁴¹. Within certain tissues MAIT cells express PD-1, and during chronic inflammatory diseases such as HIV, HCV, and tuberculosis, an increase in PD-1 expression by MAIT cells in the periphery has been reported^{52,58,102-104}. During chronic HCV infection, MAIT cells display increased expression of inhibitory receptors such as PD-1 and CLTA-4¹⁰⁵. How these inhibitory receptors are induced on MAIT cells and if expression of these receptors confers

suppression of effector function is unknown. Additionally, whether Tregs function to suppress MAIT cell responses, either through direct suppression via secretion of anti-inflammatory cytokines such as IL-10 or indirect suppression by modulating inflammatory cytokine secretion by antigen-presenting cells^{106,107}, remains to be determined.

Questions to address

Altogether, our knowledge of basic MAIT cell functions and contributions to disease remain limited. MAIT cells have been characterized in a variety of chronic infections, autoimmune diseases, and cancers, yet basic questions about their activation requirements and subsequent effector functions remain. One of the major difficulties within the MAIT cell field is the lack of an appropriate mouse model, thereby leaving researchers to address these questions using human or non-human primate samples. This hurdle provides a substantial challenge to the MAIT cell field, while simultaneously allowing research to provide a directly translational link to the functions of this unique subset in a variety of important global health related diseases. Due to their location in the mucosa, it is of interest to study MAIT cell functional requirements not only in the periphery but also at the site of infection and pathogen invasion. Furthermore, the incredible effector potential of MAIT cells may act as a double-edged sword in certain scenarios. For instance, during chronic infections such as HIV or active TB, activated MAIT cells may initially contribute to the immune system's efforts to clear infection. However, over the course of infection this overt effector response may result in damage to tissues, chronic inflammation, and, in the case of HIV, generation of new

target cells¹⁰⁸. It is important, therefore, to not only determine the tissue-specific responses of MAIT cells during inflammation or infection, but also the mechanisms of MAIT cell control.

In Chapter 3, we investigate the basics of MAIT cell activation and provide an explanation as to how MAIT cells are able to distinguish between commensal and pathogenic bacteria and mount the correct effector response. Using blood and mucosal tissue from healthy human donors we begin to dissect the differences between MAIT cells isolated from the blood and mucosa, and gain insight into their functional potential at barrier sites. In Chapter 4, we investigate the kinetics of the MAIT cells response to acute viral infection using an SIV model of infection, and provide evidence for MAIT cell activation in both the mucosa and the blood within days of viral transmission. Using this acute infection model we show significant and sustained loss of IL-17 and IL-22 producing MAIT cells, and discuss how these effects may contribute to the breakdown in mucosal barrier function observed in HIV infection. Lastly, in Chapter 5, we address how MAIT cell responses may be regulated within inflamed tissue sites by executing a series of *in vitro* experiments using inflamed mucosal tissues and blood samples. We demonstrate that MAIT cells retain functional capacity in inflamed mucosal tissue, while expressing inhibitory markers that may regulate their effector responses at the site of the mucosa.

Together, the data presented address basic questions regarding MAIT cell activation and response to infection, elucidate signals that are sufficient for MAIT cell trafficking to mucosal tissues, and explore how the MAIT cell response is regulated during inflammatory states.

Chapter 2:

Materials and Methods

MAIT cell activation and gene expression

Flow cytometry and reagents

For the phenotypic identification or functional assays, bulk PBMC or sorted MAIT cells were stained with Aqua Live/Dead Fixable Dead Cell Stain and a combination of the following antibodies (from BD except as noted): CD3 Pacific Blue (OKT3, Biolegend), CD8 PerCPCy5.5 (SK1), CD4 ECD (SFC112T4D11, Beckman Coulter), CD161 PECy5 (DX12), V α 7.2 PE (3C10, Biolegend), CD56 PECy7 (NCAM16.2), CD16 APCCy7 (3G8), granzyme B (GB11), interferon- γ (4S.b3, Ebioscience), TNF α (MAb11), IL-17A (SHLR17, Ebioscience) CD45RO (UCHL1), CCR6 (Ebioscience, R6H1), CD62L (DREG-56). MAIT cells that were enriched for CD8⁺ CD161^{hi} expressing cells were sorted as live CD4⁻ CD8⁺ CD56⁺ CD16⁻ V α 24⁻ CD161^{hi} cells. MAIT cells that were enriched for high purity based on CCR6 expression were sorted as live CD4⁻ CD8⁺ CD56⁺ CD16⁻ CD161^{hi} CCR6^{hi} CD62L^{lo} cells. MAIT cells that were enriched for purity based on V α 7.2 expression were sorted as live CD4⁻ CD8⁺ CD56⁺ CD16⁻ CD161^{hi} V α 7.2⁺ cells. Sorted purities were typically >90-95% and performed on a FACS Aria II. For the phenotypic identification of conventional CD8⁺ T (CD8⁺ T_{mem}) cells, bulk PBMC or sorted CD8⁺ T_{mem} cells were stained with Aqua Live/Dead Fixable Dead Cell Stain and a combination of antibodies. CD8⁺ T_{mem} cells that were enriched for purity were sorted as live CD4⁻ CD8⁺ CD45RO⁺ V α 7.2⁻ cells.

Activation of cells

Cells were left untreated or were treated with one dose of IL-12 (eBioscience), IL-15 (eBioscience), and IL-18 (MBL) at 100ng/mL or a combination of the cytokines and anti-CD3/CD28-coupled beads per manufacturers instructions for whole PBMC; and increased to a 10:1 bead to cell ratio to ensure activation of sorted MAIT or CD8⁺ T_{mem} cultures (Invitrogen) and cultured for 6, 12, or 24 hours. Whole PBMC were stimulated at a concentration of 1x10⁶ cells per well and sorted cells were stimulated at a concentration of 5,000 cells per well. Golgi plug (BD) was added 4 hours before supernatant harvest and intracellular staining at each time point. Supernatant was collected from cultures where indicated 24 hours after stimulation. Cells were analyzed by FACS and Luminex according to the manufacturer's instructions (Luminex Corp.).

Monocyte isolation and stimulation

Cryopreserved PBMC were thawed and immediately prepared for CD14⁺ monocyte isolation using a CD14 positive isolation kit (Miltenyi). CD14⁺ monocytes were left untreated or treated with either LPS at 1ng/mL or ssRNA at 1µg/mL (Invivogen) for 24 hours in a 37°C incubator. Cytokine concentrations within supernatants were assessed by Luminex. Following culture MAIT cells were analyzed for activation by flow cytometry.

Culture of MAIT cells with TLR-activated monocytes

After 24 hours of monocyte activation, MAIT cells were sorted from a separate vial of cryopreserved PBMC from the same donor, and cultured with monocytes at a 1:10 ratio or treated with 100-150µl of supernatant from monocyte cultures for an additional 24 h, and stained for MAIT cell markers and cytokine expression.

Isolation of cells from mucosal tissue

Five 3mm² punch rectal biopsy specimens, obtained 10cm proximal to the anal verge, were collected during the same visit as blood was collected, transported on wet ice, and placed immediately into RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin sulfate (100 µg/ml), and Fungizone (2.5 µg/ml) (all from GibcoBRL); repeatedly washed; and mucosal mononuclear cells (MMC) were isolated by two rounds of digestion with collagenase II (Sigma-Aldrich). Cells were immediately stained for single cell sorting described below.

Ex-vivo cytokine staining of blood and mucosal MAIT cells

Golgi plug was added for four hours to mucosal mononuclear cells (MMC) before staining MAIT cells for IFN γ , granzyme B, TNF α , and IL-17A expression. MAIT cells are identified by gating on live, CD45⁺CD3⁺CD8⁺CD161^{hi}V α 7.2⁺ cells.

Single-cell gene expression

For Nanostring experiments, CD8⁺ MAIT cells were sorted as live CD3⁺CD8⁺ CD4⁻ CD161^{hi}V α 7.2⁺ cells, CD8⁻ MAIT cells were sorted as live CD3⁺CD8⁻CD4⁻CD161^{hi} V α 7.2⁺ cells, CD8⁺ conventional T cells were sorted as live CD3⁺CD8⁺CD4⁻CD161^{neg} V α 7.2⁻ cells, and NK cells were sorted as live CD3⁻CD56⁺CD16⁺ cells. Single cells were sorted into 96-well polypropylene PCR plates (Eppendorf) containing lysis buffer (NanoString Technologies) and immediately frozen. After lysis, RNA was converted to cDNA with SuperScript VILO (Life Technologies). Primers for 145 genes were pooled and cDNA was enriched in a multiplexed amplification (MTE) reaction according to the nCounter Single Cell Expression protocol (NanoString). The MTE samples were

hybridized overnight at 65°C with an nCounter CodeSet containing probes for all enriched targets (inflammation, activation, migration, unrelated genes and controls), and internal controls as recommended by the manufacturer. Gene expression was analyzed using the nCounter system (NanoString Technologies).

Statistical analysis

Nonparametric tests were performed using Mann-Whitney or two-way ANOVA tests, as appropriate and corrected for multiple comparisons using Dunnett's test. P-values >0.05 were considered not significant (ns), and values denoted with (*) symbols reflect significance levels as follows: $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***), and $P \leq .0001$ (****). Stimulated samples were compared to the negative control using a one-tailed test and to each other using a two-tailed test. Analyses were performed using GraphPad software (GraphPad Prism) version 6.0e.

Statistical and computational methods for Nanostring analysis

A total 756 wells containing single cells were sampled. Single-cell gene expression data were quality controlled and preprocessed as previously described^{109,110}. After filtering, 701 cells remained. 40% of filtered cells corresponded to wells without detectable expression. We did not consider any explicit normalization, as single cells were sampled, providing natural, atomic units of input RNA normalization.

Multivariate classification and dimension reduction

We considered to what degree gene expression patterns could discriminate cells into their (a) cell subset within blood and tissue lymphocytes and (b) tissue type, within MAITs. To do this, we used Fisher's Linear Discriminant Analysis (LDA). LDA performed

on cells belonging to K distinct phenotypic groups results in the gene expression vector profile of each cell being reduced to a set of $K-1$ canonical coordinates via a linear transformation of the gene expression vector. This is akin to doing Principal Components Analysis in a manner that maximizes the spread between the K groups, i.e. between cell-subsets and tissue types.

We considered LDA of lymphocytes in blood (Figure 6A), and within MAITs across tissue type (Figure 6B and 6C) and plot the location of each blood and mucosa sample according to the LDA ordination. Out of the three canonical coordinates to separate the four cell types collected from blood, the coordinates that discriminate MAITs and NK from all other cell types are shown in Figure 6A. In Figures 6B and 6C, the coordinate that discriminates between tissues in MAITs and the two coordinates that discriminate $CD8^{+/-}$ in each tissue type are shown. Ellipses are 75% confidence regions of the location (centroid) of the points for each donor and cell type assuming bivariate normality.

Differential gene expression

Using the Hurdle linear model^{109,110}, developed to accommodate bi-modality in single cell gene expression, we tested for changes between tissues by $CD8^{+/-}$ status. A total of 28 genes had any difference between subsets (6 degree of freedom chi-square test, 5% Bonferroni significant across inflammation, activation and migration genes). The differences were further decomposed into an additive effect due to tissue differences, an additive effect due to $CD8^{+/-}$ status, and an interaction between tissue and $CD8^{+/-}$ status. Genes with consistent tissue differences were found, by screening for genes with stable $CD8^{+/-}$ patterns and no evidence of an interaction between $CD8$ status and

tissue (FDR q-values > 10%). The genes with the 6 most significant tissue differences (all Bonferroni significant <1%) are shown in plots 6D and 6E. We calculate the signed signal to noise ratio by taking the average log expression for each of the four subtypes, centering 4 subtype averages about zero, and dividing by the pooled standard deviation of single cell expression. Genes with larger differences between subgroups with respect to the residual single cell variability in the gene have more extreme heatmap values.

Study approval

Twelve healthy, HIV-uninfected adults (7 male, 5 female; age range of 31-60 years) were recruited at the Seattle HIV Vaccine Trials Unit, Seattle, WA, as part of the study “Establishing Immunologic Assays for Determining HIV-1 Prevention and Control”, also referred to as Seattle Assay Control or SAC. All participants signed informed consent and the following institutional human subjects review committee approved the protocol prior to study initiation: Fred Hutchinson Cancer Research Center IRB, Seattle, WA.

MAIT cell response to SIV infection

Study animals

Animals were housed and cared for in Association for the Assessment and Accreditation of Laboratory Animal Care international (AAALACi) accredited facilities, and all animal procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of University of Washington. Six male rhesus macaques (*Macaca mulatta*) were infected intrarectally with 100,000 TCID₅₀ SIV_{MAC239x}. Baseline samples for blood and jejunum biopsies were taken 63, 49 and 21 days pre-infection. Data from the three baseline samples were averaged and graphed

as day -21. Blood samples were drawn prior to infection on day 0 and 1, 3, 7, 14, 21, and 28 days post-infection. Additionally, jejunum biopsies were sampled 3, 14, and 28 days post-infection.

Tissue processing

Gut biopsies were processed immediately as follows: 1-2 biopsies/tissue were frozen in RNAlater (QIAGEN, Valencia, CA) for subsequent DNA extraction and 16S sequencing. The remaining biopsies were enzymatically digested with R10 media supplemented with Liberase (40 μ g/ml, Sigma-Aldrich, St. Louis, MO) and DNase (4 μ g/ml, Sigma-Aldrich) for 1 hour at 37°C with vigorous stirring, then ground through a 70- μ m cell strainer into a single cell suspension, then analyzed by flow cytometry.

Flow cytometry and reagents

Whole blood and single cell isolations from biopsies were stained immediately using the following surface antigen antibodies with clone denoted in (), from BD biosciences unless otherwise stated: CD45 PerCP (D058-1283), CD3 PE-CF594 (SP34-2), CD8 APC-H7 (SK1), CD25 BV786 (M-A251), CD69 BV711 (FN50), CD4 Pe/Cy7 (Biolegend, OKT4), IL-18R (Biolegend, H44), and CD161 Brilliant Violet 605 (Biolegend, HP-3G10). Following surface staining, cells were permeabilized using Cytotfix/Cytoperm (BD Biosciences), and stained using the following intracellular antigen antibodies from eBiosciences with clone denoted in (): IL-17 PE (ebio64CAP17), IL-22 PerCP-eFluor710 (IL22JOP), and TNF α alexafluor 700 (Mab11). Cells were also stained intracellularly with granzyme B v450 (BD Biosciences, GB11). Stained samples were fixed in 1%

paraformaldehyde and collected on an LSR II (BD Biosciences, La Jolla, California). Analysis was performed in FlowJo (version 9.9, Treestar Inc., Ashland, Oregon).

Assessment of plasma LPS binding protein

Levels of LPS binding protein were assessed via ELISA using a pre-prepared kit (Biometric, Greifswalkd, Germany). ELISAs were read using an iMark Microplate Reader (Biorad, Hercules, CA).

TCR sequencing

TCR α genes were cloned from sorted CD8⁺ CD161^{hi} IL-18R α ⁺ T cells using the SMARTer RACE PCR kit (Takara Bio USA), with the TCR α gene specific reverse primer 5'-CAGCCGCAGCGTCATGAGCAGATTA-3'. RACE PCR products were topocloned into pENTR-D-Topo (Thermofisher Scientific), individual colonies were sequenced, and TCR α gene sequences were analyzed using IMGT/V-QUEST^{111,112}.

Statistical analyses

Statistical analysis was performed using GraphPad Prism statistical software (Version 6, GraphPad Software, San Diego, CA). Averaged baseline values (week -21) were compared to post-SIV-infection time points (days 1, 3, 7, 14, 21, 28), and significance was evaluated using a paired *t*-test. Correlations were assessed using the Spearman's rank correlation analysis.

MAIT cell activation and gene expression at mucosal sites

Isolation of cells from blood and mucosal tissue

Gingival mucosal tissues were collected into complete media transported on wet ice, and placed immediately into RPMI 1640 supplemented with penicillin (100 U/ml), streptomycin sulfate (100 µg/ml), and Fungizone (2.5 µg/ml) (all from GibcoBRL); repeatedly washed; and mucosal mononuclear cells (MMC) were isolated by two rounds of digestion with collagenase II (Sigma-Aldrich). MMC and peripheral blood used for direct *ex vivo* cytokine analysis were digested and processed in the presence of Golgi inhibitor (1:1000 dilution, BD). During the same visit blood was collected into 1X PBS (Gibco), transported on wet ice, and red blood cells were lysed using ACK Lysis Buffer (Gibco). Live, CD3⁺ cells were counted using the Guava cell counter (EMD Millipore) immediately after processing.

Flow cytometry and reagents

For the phenotypic identification or functional assays, bulk PBMC or sorted MAIT cells were stained with Aqua Live/Dead Fixable Dead Cell Stain and a combination of the following antibodies (from BD except as noted): CD3 Pacific Blue (OKT3, Biolegend), CD8 PerCPCy5.5 (SK1), CD4 ECD (SFC112T4D11, Beckman Coulter), CD161 PECy5 (DX12), V α 7.2 PE (3C10, Biolegend), granzyme B (GB11), interferon- γ (4S.b3, Ebioscience), TNF α (MAb11), CD45RO (UCHL1), CCR6 (Ebioscience, R6H1).

MAIT cells that were enriched for purity based on V α 7.2 expression were sorted as live CD4⁻CD8⁺CD161^{hi}V α 7.2⁺ cells. CD8⁺ T_{mem} cells that were enriched for purity were sorted as live CD4⁻CD8⁺CD45RO⁺V α 7.2⁻ cells. Sorted purities were typically >90-95% and performed on a FACS Aria II.

Activation of cells

Following processing, PBMC or MMC were left untreated or were treated with one dose of IL-12 (eBioscience), IL-15 (eBioscience), and IL-18 (MBL) at 100ng/mL or anti-CD3/CD28-coupled beads per manufacturers instructions for whole PBMC (Invitrogen) and cultured for 6 hours in the presence of Golgi inhibitor (BD) at a concentration of 1:1000.

CCL20 chemotaxis assay

PBMC were enriched for CD8⁺ T cells using a positive selection kit (Miltenyi) and then re-suspended at 1.9×10^6 cells per stimulation condition. CD8⁺ T cells were left at rest or incubated with plate-bound anti-CD3 (1ug/mL, Mabtech) and soluble CD28 (1:500 dilution, BD), IL-12/15/18 (100ng/mL per cytokine) at 37°C and 5% CO₂ in complete medium. Cells were washed and added to the top chamber of a Transwell with a pore size of 5µm (Corning). CCL20 diluted in complete medium was added to the bottom chamber to a final concentration of 500ng/mL. Complete medium alone was added to the bottom of control chambers. Pre-migrated controls from each stimulation condition were kept aside to calculate the number of cells after migration. After 4 hours of incubation at 37°C and 5% CO₂, 150ul was collected from the bottom of each well and stained for MAIT cells identified as CD3⁺CD8⁺CD161^{hi}Vα7.2⁺ cells and run on an LSRII with counting beads (Invitrogen) to calculate total numbers of migrated cells. Percent specific migration was calculated by normalization of the frequency of migrated CD3⁺CD8⁺CD161^{hi}Vα7.2⁺ cells to the input population.

Low input RNA sequencing

Up to 1000 MAIT cells were sorted directly into SMARTer v3 or SMARTseq v4 lysis reagent (Clontech), lysed and converted to cDNA. After amplification, sequencing

libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina) according to C1 protocols (Fluidigm). Barcoded libraries were pooled and quantified using a Qubit® Fluorometer (Life Technologies). Single-read sequencing of the pooled libraries was carried out on a HiSeq2500 sequencer (Illumina) with 58-base reads, either using TruSeq v4 or Rapid Run v1 Cluster and SBS kits (Illumina).

Statistical analysis

Nonparametric tests were performed using Mann-Whitney or two-way ANOVA tests, as appropriate and corrected for multiple comparisons using Dunnett's test. P-values >0.05 were considered not significant (ns), and values denoted with (*) symbols reflect significance levels as follows: $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***), and $P \leq .0001$ (****). Stimulated samples were compared to the negative control using a one-tailed Students t-test. Analyses were performed using GraphPad software (GraphPad Prism) version 7.0e.

Bulk RNAseq read processing

General quality control of the raw reads was performed using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Reads were then mapped against a human reference genome (hg38, from the UCSC genome browser <http://genome.ucsc.edu>) using STAR (v2.4.0h1)¹¹³. Quantitative gene counts were produced from this alignment using RSEM utilizing the human annotation associated with the genome.

Differential expression analysis

Gene counts for each sample were loaded into R (<http://www.r-project.org/>). Genes with no counts were removed and counts across samples were normalized with edgeR (version 3.10.2) using the weighted trimmed mean of M-values¹¹⁴.

Differentially expressed genes were identified using Limma between samples and defined by using an absolute fold change cutoff of 2x and a p-value of ≤ 0.05 after adjustment using the Benjamini-Hochberg multiple testing correction. Additional clustering, creation of heatmaps, and other statistical analyses were performed using R.

Functional enrichment analysis

Functional analysis of the differential gene expression data was performed with QIAGEN's Ingenuity® Pathway Analysis (IPA®, QIAGEN Redwood City, www.qiagen.com/ingenuity). Differentially expressed genes were analyzed in the context of all known biological functions and pathways in the Ingenuity Pathways Knowledge Base. IPA-generated P values for enrichment of functions and pathways were calculated using the right-tailed Fisher exact test and adjusted using the Benjamini-Hochberg multiple testing corrections. Biological diseases and functions enrichment were considered to be significant based on a Z score calculation. Z score is calculated in IPA to determine whether gene expression changes for known targets are consistent with their information in literature. Biofunctions with Z score >2 were examined, and cell migration, inhibition and cell death were selected. Top scoring networks for these biological functions were merged together to create networks.

Chapter 3:

Distinct activation thresholds of human conventional and mucosal-associated invariant T (MAIT) cells

Introduction

Three major categories of antigen have been identified so far that can be recognized by different T cell subsets: conventional T cells recognize peptides in context of MHC class I or II, natural killer T (NKT) cells recognize lipids/glycolipids in context of CD1, and mucosal-associated invariant T (MAIT) cells recognize bacterially-derived metabolites in context of MHC class I-related protein (MR1)¹¹⁵. The respective roles of TCR signals and pro-inflammatory cytokines in regulating activation of primary human MAIT cells have not been well characterized to date.

Mouse model studies introduced the concept of inflammation-driven, T cell receptor (TCR)-independent activation of memory CD8 T cells^{20-22,116}. More recent studies further defined the mechanisms and inflammatory cues leading to this “bystander-activation” of memory T cells^{27,117} and demonstrate that human memory T cells also become bystander-activated in many inflammation-inducing scenarios including infection and cancer immunotherapy^{23,25,26,28,118-120}. Bystander-activation of memory T cells leads to rapid secretion of IFN γ and enhanced early pathogen clearance^{21,22,27,121}, which suggests that memory T cells contribute to host immunity in a TCR-independent fashion. Bystander-activated memory T cells also express granzyme B and can kill NKG2D-ligand expressing target cells in an NKG2D-dependent, innate-like manner²⁷. This mechanism of target cell elimination can help curtail pathogen

spread following an infection²⁷, but can also exacerbate pathology in context of an infection^{122,123} and autoimmunity²⁴.

These cytokine-driven, innate-like responses by conventional memory T cells are similar to the inflammation-driven activation of NKT cells^{35,36,124} and MAIT cells³⁹. While NKT cells are fairly rare in human blood and tissue, MAIT cells are quite abundant and make up 1-8% of T cells in blood and mucosal tissues, and 20-45% of T cells in the liver^{41,55}. Importantly, MAIT cells have a memory-like phenotype⁴¹ and effector functions, including cytotoxicity, that are comparable with conventional memory CD8 T cells. MAIT cells can be activated by the same inflammatory signals as conventional memory T cells to express IFN γ and granzyme B^{39,125}.

Changes in MAIT cell abundance or location have been described in a series of studies including chronic infections, cancer, and autoimmune disorders, indicating that MAIT cells respond and are thus relevant in a wide array of conditions^{81,126}. There is substantial overlap in activation requirements, functional program and location between conventional memory CD8 T cells and MAIT cells, thus we sought to stringently define unique activation and functional characteristics to better understand each subset's potentially unique role and contribution to immunity and pathology.

While conventional memory CD8 T cells and MAIT cells appear to have similar inflammation-driven responses, it is unclear if MAIT cells respond like conventional memory CD8 T cells when stimulated via their TCR. We hypothesized that the role of TCR-mediated signals for primary human MAIT cell and conventional memory CD8 T cell activation are different due to the following consideration: since MAIT cells recognize bacterial metabolites that are produced by commensal as well as pathogenic

bacteria^{44,45}, specific activation requirements must exist to allow for dichotomous responses. Moreover, assuming that a conventional memory CD8 T cell has a more focused antigenic specificity than a MAIT cell (specific peptide sequence vs. a metabolite), we reasoned that TCR-mediated memory CD8 T cell responses are inherently more restricted and conventional memory T cells may have thus a different TCR-activation threshold compared to MAIT cells.

We report here that in contrast to conventional memory CD8 T cells, even a strong TCR + costimulatory signal is not sufficient to induce robust and sustained MAIT cell effector function. Importantly, we demonstrate that inflammatory signals and TCR signals synergize to induce MAIT cell effector function.

To better understand MAIT cell characteristics and contributions to immunity at the site of the mucosa, we used single-cell gene expression analysis of MAIT cells isolated from blood and mucosal tissue biopsies. We did not observe direct ex vivo effector function in MAIT cells isolated from healthy mucosal tissues; however, we found that MAIT cells in mucosal tissue have pronounced pro-inflammatory/activating potential. This suggests that mucosal MAIT cells are poised to respond rapidly and further underlines the need for tight functional control to prevent unwanted effector responses. We propose that the previously unappreciated requirement for inflammatory cues to acquire MAIT cell effector function provides an explanation for the co-existence of MAIT cells and commensals in mucosal tissues without eliciting pathology (TCR signal without inflammation), while maintaining responsiveness to pathogens (TCR signal plus inflammation). Our findings also have important implications for understanding changes in MAIT cell function following bone marrow transplant

conditioning¹²⁷ or HIV infection^{60,61,128,129}, which damage the gastrointestinal luminal integrity and elicit inflammatory responses.

Results

TCR signals are not sufficient to directly induce MAIT cell effector function

The functional properties of MAIT cells have typically been examined following stimulation of whole peripheral blood mononuclear cells (PBMC) or enrichment of CD161^{hi} CD8⁺ cells. We wanted to stringently define the ability of purified MAIT cells and conventional memory T cells (CD8 T_{mem}) to directly respond to cytokine- and TCR-mediated stimuli. We tested two different approaches to purify MAIT cells. We first developed a sorting strategy that relies on the CCR6^{hi} expression levels of MAIT cells⁴¹ (Figure 1A). Using this strategy we typically increased MAIT cell purity to >90% (Figure 3.1A, right panel). To further increase the sort purity, we next included an antibody against V α 7.2 (the invariant MAIT TCR α chain) in the sorting panel and further increased the cell purity to >95% (data not shown). Importantly, comparing these two methods we found no evidence that the anti-V α 7.2 antibody significantly altered MAIT cell responses (Supplemental Figure 3.1) and we thus continued to use the V α 7.2 sorting strategy for greatest MAIT cell purity. Conventional memory CD8 T cells were sorted as CD8⁺ CD45RO⁺ V α 7.2⁻ cells (see Methods for full sort panel).

Sorted MAIT and conventional memory CD8 T cells were incubated for 6, 12, or 24 hours with a combination of the cytokines IL-12, IL-15 and IL-18 (IL-12/15/18) or with anti-CD3/CD28 beads (TCR). Anti-CD3/CD28 beads allowed us to compare the MAIT cell response to the memory CD8 T cell population and deliver a well-defined and

standardized TCR + costimulatory signal across stimulation conditions and cell types. We first analyzed both T cell subsets by intracellular cytokine staining. MAIT cells responded robustly and expressed IFN γ , but not TNF α , as early as 12 hours and at 24 hours after stimulation with recombinant IL-12/15/18 (Figure 3.1B). In contrast, after TCR stimulation we observed a short burst of IFN γ and TNF α expression by MAIT cells at 6 hours, but this effect was lost by 12 hours, indicating that TCR signaling was not sufficient to maintain expression of IFN γ and TNF α . The population of conventional memory CD8 T cells was overall less responsive to IL-12/15/18 compared to the MAIT cell population, but responded to TCR signaling and produced TNF α at all time points, while IFN γ production peaked at 6 hours and was still significantly produced at 12 hours before declining by 24 hours post-TCR stimulation (Figure 3.1C).

We were initially surprised to find only limited IFN γ production by MAIT cells in response to TCR signaling (Figure 1B, left panel) and considered that MAIT cells may be hyporesponsive to a direct TCR signal. Thus, we next screened the culture supernatant for secreted cytokines and chemokines by Luminex analysis (Figure 3.2A and 3.2B) to define which cytokines are secreted within the first 24 hours of activation. IL-12/15/18-stimulated MAIT and conventional memory CD8 T cells secreted IFN γ , but not TNF α . We only detected IFN γ and TNF α in the supernatant of TCR-stimulated conventional memory CD8 T cells, but not TCR-stimulated MAIT cells. This further supports the notion that conventional memory CD8 T cells, but not MAIT cells secrete significant amounts of effector cytokines following TCR stimulation.

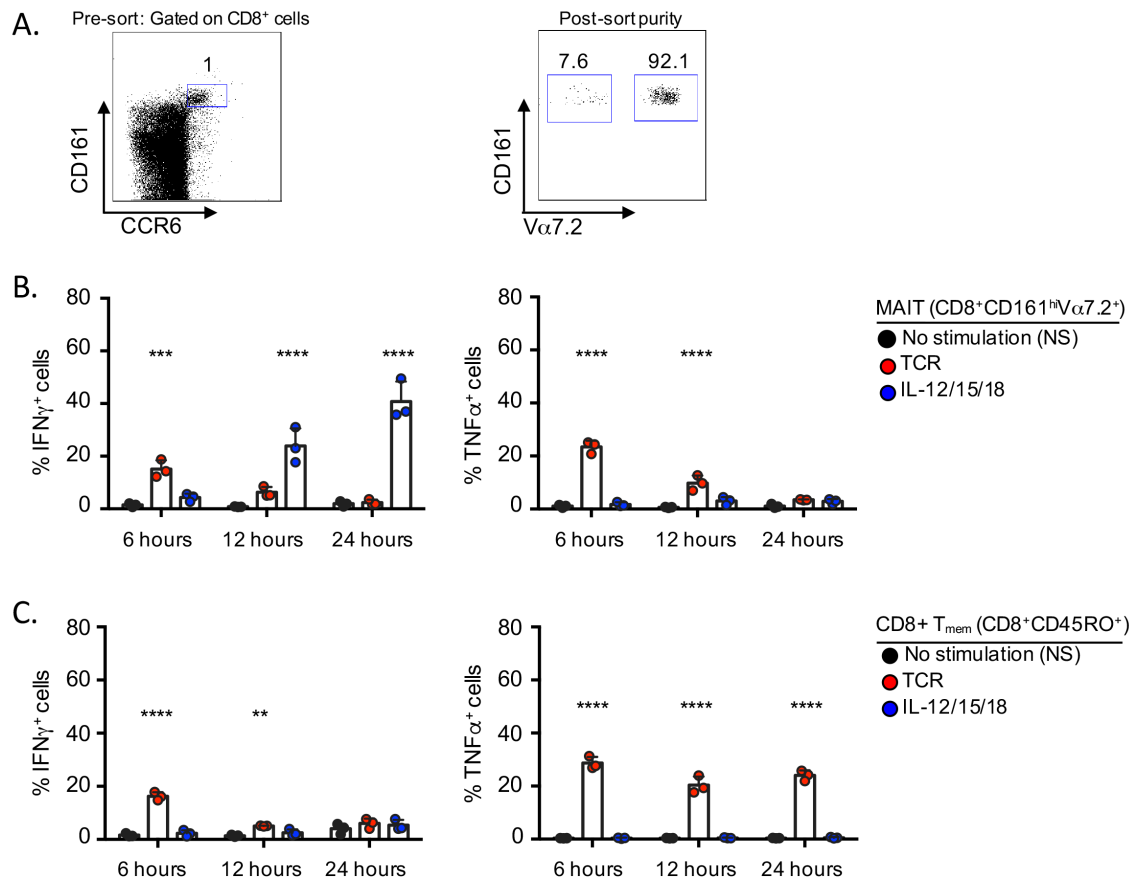


Figure 3.1. A TCR signal alone is not sufficient for sustained effector function in MAIT cells.

(A) A representative flow plot of the CD161^{hi}CCR6^{hi} MAIT cell population in peripheral blood mononuclear cells (PBMC) after gating on CD8⁺ cells is shown (left panel). MAIT cell purity was confirmed after sorting CD8⁺CD161^{hi}CCR6^{hi} cells by determining Vα7.2 expression in a small aliquot (right panel). (B) 5,000 sorted CD8⁺CD161^{hi}Vα7.2⁺ MAIT cells were rested (No Stimulation, black circles), stimulated with anti-CD3/CD28 beads (TCR, red circles) or 100ng/mL of IL-12/15/18 (blue circles) for 6, 12, or 24 hours and analyzed for expression of IFN γ and TNF α (n=3). (C) 5,000 sorted CD8⁺CD45RO⁺Vα7.2⁻ memory T cells (CD8⁺ T_{mem}) were rested (No Stimulation, black circles), stimulated with anti-CD3/CD28 beads (TCR, red circles) or 100ng/mL of IL-12/15/18 (blue circles) for 6, 12, or 24 hours and analyzed for expression of IFN γ and TNF α (n=3). Data displayed are the average of two to three technical replicates for each donor, with each data point representing a single donor (n=3, B and C) and data are displayed as mean \pm SEM (where applicable). P values were determined by comparing treatment conditions to no stimulation conditions for each time point. Two-way ANOVA, with Dunnett's test for multiple comparisons (B and C).

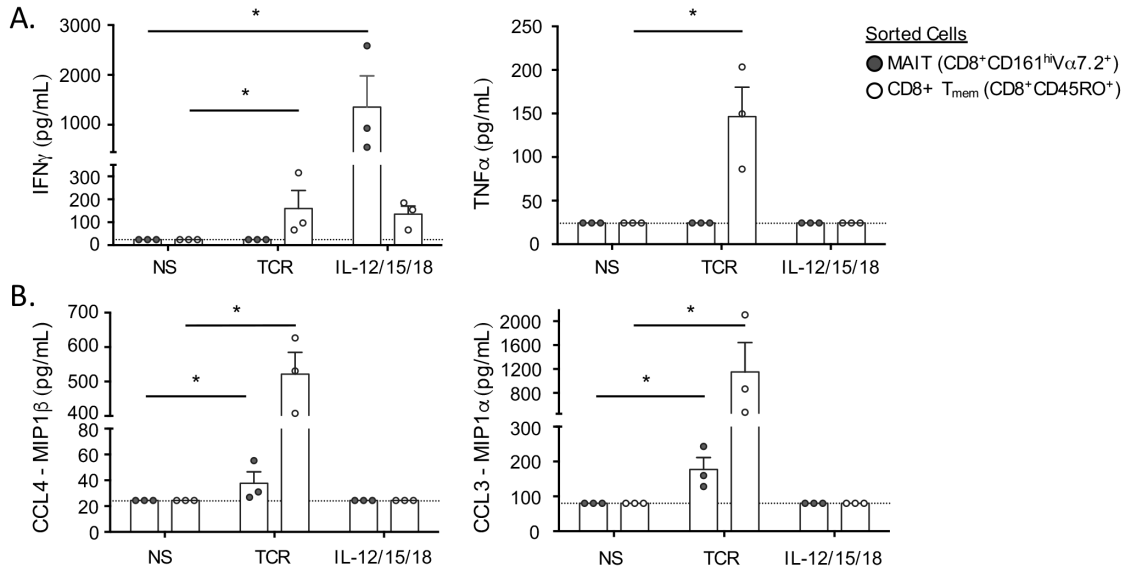


Figure 3.2. A TCR signal is sufficient to induce effector function in conventional memory CD8 T cells, but not in MAIT cells.

(A and B) Using the supernatant from the same experiments as in (Figure 1) MAIT (dark gray circles) or Tmem (white circles) supernatants were collected after 24 hours of culture and analyzed using Luminex (n=3). Dashed lines denote limit of detection for each analyte. Three different donors were used for each experiment shown. MAIT cell data shown are representative of at least two independent, technical replicates. Data are displayed as mean +/- SEM (where applicable). P values were determined by comparing treatment conditions to unstimulated conditions using Mann Whitney one-tailed U-test (A and B).

MAIT cells have been reported to express IL-17 upon activation, particularly after prolonged in vitro culture and stimulation⁴¹, however we could not detect IL-17 in the supernatant of our ex vivo stimulated MAIT cells regardless of the experimental stimulation condition (data not shown). Importantly, TCR-mediated signals induced secretion of CCL3 and CCL4 from both MAIT cells and CD8 T_{mem} (Figure 3.2B), suggesting MAIT cells are not simply hyporesponsive to TCR stimulation. Finally, we considered the possibility that anti-CD3/CD28 stimulation in the absence of inflammatory stimuli could also lead to induction of immune-suppressive mechanisms as an additional control mechanism. We examined IL-10 secretion, but could not detect IL-10 in any of the experimental conditions (data not shown). Together, these data show that inflammation- but not TCR-mediated signals are sufficient to induce MAIT cell effector function. Our approach allowed us to define the signals that act directly on a pure MAIT cell population and compare these results to previously published experiments analyzing MAIT cell responses in the context of other cell types^{39,55}. When not purified, MAIT cells may be indirectly activated by other (non-MAIT) T cells responding to TCR signals and in turn secrete cytokines that then act on MAIT cells. Since the current model of MAIT cell activation suggests that TCR signals are sufficient for MAIT cell activation based on experiments that examined MAIT cell function in the context of other cell types, we next wanted to explain this seemingly contradictory outcome of our data and these previous studies.

Dissecting direct and indirect MAIT cell activation mechanisms

To distinguish direct from indirect activation mechanisms we examined how memory CD8 T and MAIT cells respond to inflammatory cues and TCR stimulation in context of

other PBMCs. PBMCs were incubated for 24 hours with a combination of the induced strong granzyme B expression in MAIT cells and CD8 T_{mem} (Figure 3.3B), but IFN γ expression (Figure 3.3A) was more limited following CD3/CD28-mediated stimulation compared to cytokine stimulation. We examined MAIT cells again for expression of IL-17 following ex vivo stimulation (with anti-CD3/CD28 beads for 24hrs or PMA/ionomycin for 4hrs, data not shown), but found only minimal to no IL-17 production (data not shown). Together, these data show that MAIT cell activation occurs when bulk PBMCs are activated with anti-CD3/CD28 as previously demonstrated, but – in context of Figure 1 – this acquisition of effector function is the result of indirect activation and not due to an intrinsic TCR-mediated signal in MAIT cells. Finally, we wanted to define the contribution of each cytokine alone and in combination to the pronounced MAIT cell effector phenotype (Figure 3.3C). IL-18 was not sufficient to elicit strong effector function, while IL-12 and IL-15 alone could induce granzyme B expression. IL-12 appeared particularly important for IFN γ expression, since IL-12/15 and IL-12/18 stimulation conditions were sufficient for IFN γ production, while IL-15/18 stimulation resulted primarily in granzyme B expression. Interestingly, the combination of IL-12/15/18 resulted in such strong MAIT cell activation that adding additional TCR signaling had seemingly little impact on further increasing the frequency of responding cells. Together, the experiments so far demonstrate that a TCR signal is not sufficient to directly activate MAIT cells and explain why previous studies missed this characteristic when stimulating bulk PBMCs.

We next sought a better understanding of the role of inflammatory and TCR signals on MAIT cell activation by more closely mimicking physiologically relevant conditions.

Activated monocytes are sufficient to activate MAIT cells in a cell-contact dependent and –independent manner

We set up an experimental system to interrogate the ability of activated professional antigen-presenting cells (APC) to stimulate MAIT cells in a cell contact–dependent vs. –independent manner. We used monocytes for this purpose, since monocytes are the most readily available human APC and recent data suggest that monocytes can enter non-lymphoid tissue and recirculate, indicating that they can interact with MAIT cells in blood and tissues¹³⁰.

We asked if TLR-stimulated monocytes are sufficient to activate MAIT cells and first wanted to distinguish if this happened in a cell contact-dependent or –independent manner. We isolated primary human CD14⁺ monocytes from PBMC and treated the monocytes with a TLR8 agonist (ssRNA). After 24 hours, we co-cultured them with CD8⁺CD161^{hi} sorted MAIT cells from the same donor for an additional 24 hours, then measured cytokine expression by MAIT cells. MAIT cells that were co-cultured with unstimulated monocytes for 24 hours served as the negative control. MAIT cells expressed granzyme B and low levels of IFN γ when co-cultured with TLR8-activated monocytes (Figure 3.4A), in line with previous studies^{39,55}. To determine if cytokines secreted by monocytes are sufficient for activating MAIT cells or if MAIT cell activation is cell contact-dependent, we treated monocytes with a TLR8 agonist for 24 hours, removed the supernatant and cultured MAIT cells in the monocyte culture supernatant

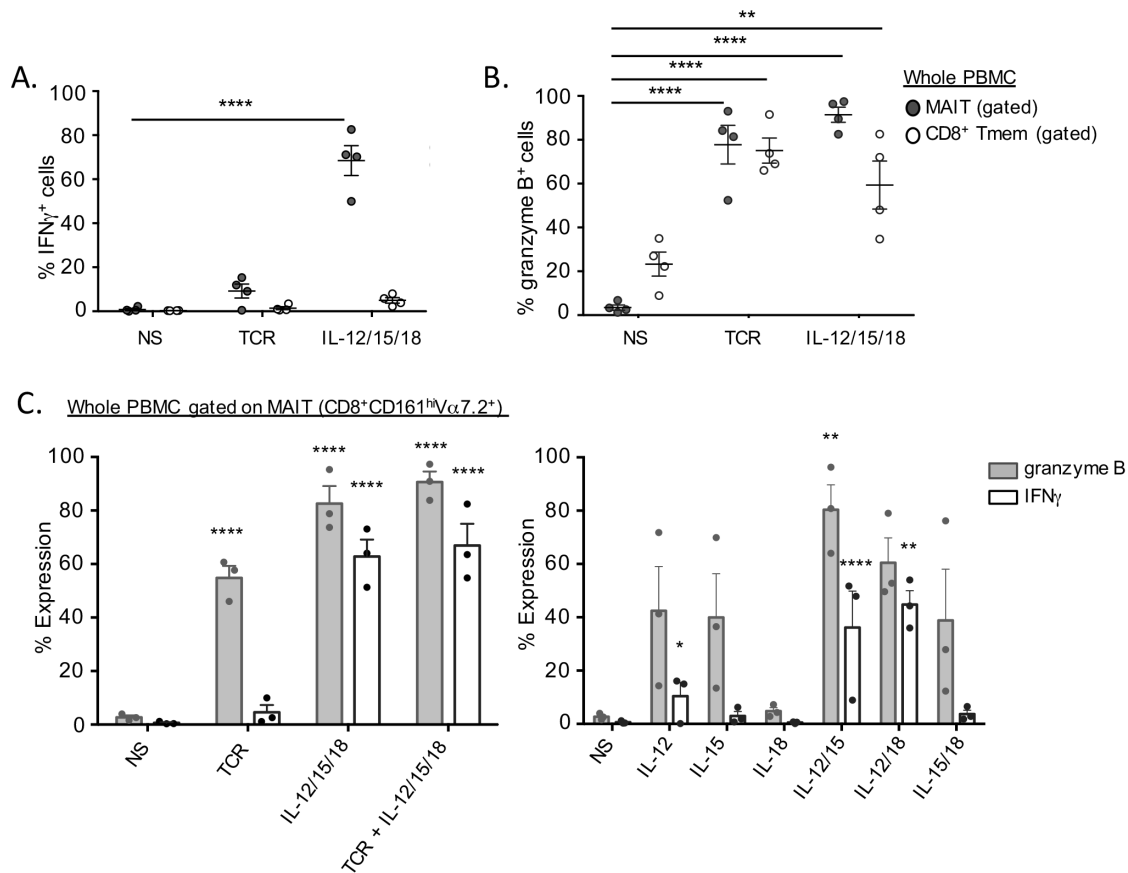


Figure 3.3. Indirect effects lead to MAIT cell activation when PBMCs are stimulated with anti-CD3/CD28 beads.

Whole peripheral blood mononuclear cells (PBMC) stimulated for 24 hours with anti-CD3/CD28 beads (TCR) or 100ng/ml IL-12/15/18 or left unstimulated (NS) and analyzed for (A) IFN γ and (B) granzyme B expression by MAIT and CD8 Tmem cells (n=4). MAIT cells (dark gray circles) were identified by gating on CD3⁺CD8⁺CD161^{hi}Va7.2⁺ cells. CD8 memory T cells (CD8⁺ Tmem, white circles) were identified as CD3⁺CD8⁺CD45RO⁺Va7.2⁻ cells. (C) Expression of IFN γ (white bars) and granzyme B (gray bars) by MAIT cells after stimulating PBMC (n=3) for 24 hours with individual cytokines, a combination of cytokines, anti-CD3/CD28 beads (TCR), a combination of cytokines and anti-CD3/CD8 beads, or left unstimulated (NS). Data in A and B were generated in separate experiments from data shown in C. Data are displayed as mean \pm SEM (where applicable). P values were determined by comparing treatment conditions to unstimulated conditions using Mann Whitney U-test (A and B) or two-way ANOVA with Dunnett's test for multiple comparisons (C).

for an additional 24 hours. We found that stimulation with supernatant from TLR-activated monocytes led to minimal, but detectable granzyme B and IFN γ production by MAIT cells, suggesting that cytokine secretion alone, though not optimal, is sufficient for activation (Figure 3.4A and 3.4B).

Synergy of T cell receptor signaling and inflammatory cytokines

To understand which inflammatory cytokines were secreted by these monocytes we analyzed the cytokines in the culture supernatant. We determined that IL-1 α and β (Figure 3.4C) and (in some donors) IL-12 were present, but neither IL-18 nor IL-15 could be detected consistently above background levels (Figure 3.4C, right panel). These data suggest that the granzyme expression of MAIT cells could be primarily driven by IL-12 as well as by cytokines other than IL-12, IL-15 and IL-18.

Together these data show that an activated APC is sufficient to induce MAIT cell effector function in a cell contact-independent manner. Since activation occurred in a cell contact-independent manner, this also suggests that it occurred in the absence of a TCR signal. While a TCR signal had minimal impact on enhancing effector function in IL-12/15/18 stimulated MAIT cells (Figure 3.3C), we next wanted to define the role of the TCR signal in the context of activated APCs secreting inflammatory cytokines. To define how MAIT cells respond to TCR signals in the presence of pro-inflammatory signals provided by activated APCs, we used a similar experimental setup as in Figure 4A. We isolated primary human CD14⁺ monocytes from PBMC and treated the monocytes with a TLR8 (ssRNA) or TLR4 (LPS) agonist. Supernatant from unstimulated monocytes was used as a negative control. After 24 hours, we removed the supernatant and added it to CD8⁺CD161^{hi}CCR6⁺ sorted MAIT cells from the same donor and

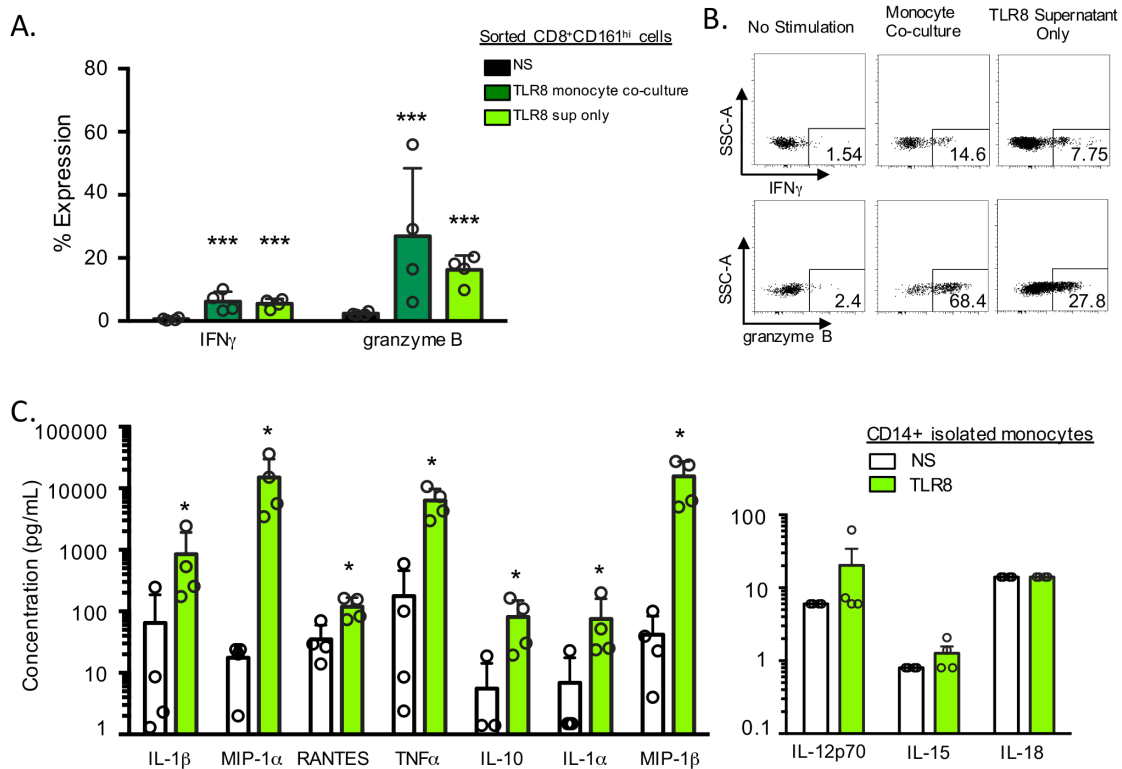


Figure 3.4. Cell contact dependent- and independent-activation of MAIT cells by TLR-stimulated monocytes.

(A) Sorted CD8⁺CD161^{hi} MAIT cells were co-cultured with unstimulated monocytes (NS, black), ssRNA (a TLR8 agonist)-activated monocytes (TLR8 monocyte co-culture, dark green) or supernatant from ssRNA activated monocytes (TLR8 sup only, light green) for 24 hours. After 24 hours of co-culture directly with monocytes or with supernatant, MAIT cells (CD8⁺CD161^{hi}V α 7.2⁺) were analyzed for IFN γ and granzyme B expression (n=4). (B) A representative FACS plot is shown illustrating IFN γ and granzyme B expression by MAIT cells. (C) Luminex analysis of statistically significant analytes (left panel) or from the cytokines IL-12, IL-15, and IL-18 (right panel) from supernatants collected from CD14⁺ monocytes that were rested (NS, black) or activated with ssRNA (TLR8, light green) reveal differences in cytokine expression dependent on stimulation (n=4). A lack of a visible error bar in C (right panel) is due to identical data points. Data shown are displayed as mean +/- SEM (where applicable). P values were determined by comparing treatment conditions to unstimulated conditions using Mann Whitney one-tailed or two-tailed U-test (A-C).

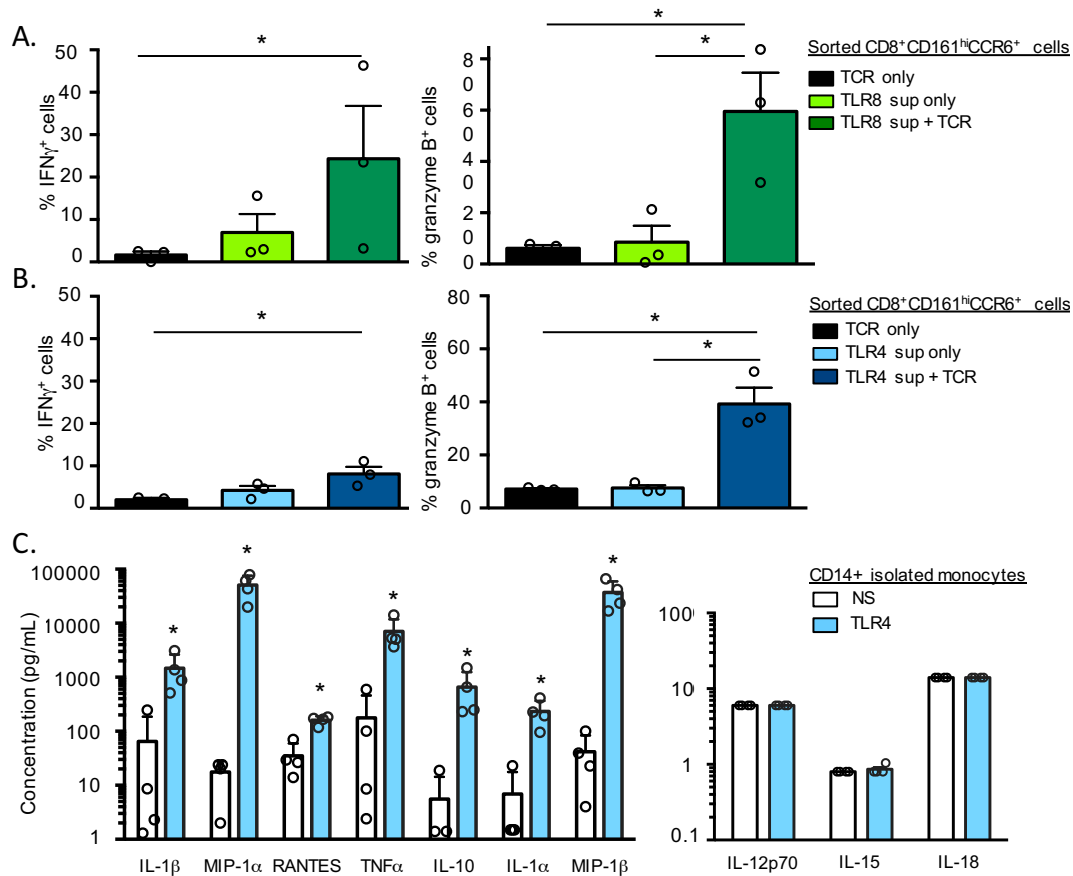


Figure 3.5. Inflammatory cytokines and TCR-mediated signals synergize to induce MAIT cell effector function.

(A) Monocytes were stimulated with ssRNA (a TLR8 agonist); after 24 hours, supernatant was removed and CD8⁺CD161^{hi}CCR6^{hi} MAIT cells were co-cultured with TLR8 supernatant in the presence (TLR8 sup + TCR, dark green) or absence (TLR8 sup only, light green) of anti-CD3/CD28 beads for 24 hours followed by analysis of IFN γ and granzyme B expression (n=3). MAIT cells stimulated only with anti-CD3/CD28 (TCR only) beads for 24 hours are shown in black. (B) Using the same donors and experimental setup as in (A), but monocytes were stimulated with LPS (a TLR4 agonist) in the presence (TLR4 sup + TCR, dark blue) or absence (TLR4 sup only, light blue) of anti-CD3/CD28 beads for 24 hours followed by analysis of IFN γ and granzyme B expression (n=3). MAIT cells stimulated only with anti-CD3/CD28 (TCR only) beads for 24 hours are shown in black. (C) Luminex analysis of statistically significant analytes (left panel) or from the cytokines IL-12, IL-15, and IL-18 (right panel) from supernatant collected from CD14⁺ monocytes that were rested (white bars) or activated with LPS (TLR4, light blue bars), reveal differences in cytokine expression dependent on stimulation (n=3). A lack of a visible error bar in C (right panel) is due to identical data points. Data are displayed as mean +/- SEM (where applicable). P values were determined by comparing treatment conditions to unstimulated or TCR only conditions using Mann Whitney one-tailed or two-tailed U-test (A-C).

examined MAIT cell effector function 24 hours later. There were three experimental groups: (1) MAIT cells + TCR signals + negative control supernatant, (2) MAIT cells + TLR-stimulated supernatant and (3) MAIT cells + TCR signals + TLR-stimulated supernatant. We observed minimal expression of granzyme B and IFN γ by MAIT cells when stimulated via their TCR in the presence of supernatant from unstimulated monocytes (Figure. 3.5A and 3.5B). Addition of supernatant from TLR4- or TLR8-stimulated monocytes was sufficient to activate MAIT cells and induce detectable amounts of IFN γ and granzyme B (Figure 3.5A and 3.5B). When both signals were combined (supernatant + TCR signal) the effector response was significantly increased. Given the size of the effector response when both signals are combined, it indicates that TCR signals and inflammatory signals synergize to elicit a robust effector response. This is particularly apparent for granzyme B expression regardless of the TLR stimulus and for IFN γ when MAIT cells receive TLR8 supernatant and a TCR signal. To understand how the TLR4 supernatant differs from the TLR8 supernatant, we determined the cytokine profile using the same approach as described in Figure 3.4C. Overall, many cytokines were secreted at comparable levels (Figure 3.4C and 3.5C), including IL-1 α and β . Neither IL-18 nor IL-15 could be detected above background levels (Figure 3.4C and 3.5C, right panels) and, in contrast to TLR8 supernatant, we could not detect IL-12 in supernatant from TLR4 stimulated monocytes. These data show that the inflammatory environment controls the extent of effector function, but regardless of the specific inflammatory milieu, there is strong evidence for synergy between inflammatory signals and TCR signals in inducing MAIT cell effector function.

Thus far, all experiments were done with MAIT cells isolated from PBMCs. We considered that MAIT cells located in mucosal tissues may have decreased functional potential that could serve as a cell intrinsic mechanism to avoid unwanted effector responses within tissues. Thus, we wanted to determine functional commonalities and potential differences of MAIT cell populations in blood and mucosal tissue.

MAIT cells in the tissue express higher levels of pro-inflammatory gene transcript compared to those in the blood

We considered the possibility that a decrease in MAIT cell effector potential, concomitant with mucosal tissue residency, could serve as a potential additional or alternative safety mechanism to curtail unwanted effector responses against commensal antigen. Due to the limited availability of mucosal tissue from healthy donors restricting the ability to perform direct ex vivo stimulation assays, we chose a single-cell gene expression analysis approach to answer this question as comprehensively as possible. Although CD8a⁺ MAIT cells are typically the predominant population, MAIT cells are also found in the CD4⁻CD8⁻ (double negative; DN) CD3⁺ population⁴³. Aside from this phenotypic difference, the functional differences of MAIT cell populations are not well understood and it is furthermore unclear whether populations with distinct functional properties exist in mucosal tissues. We compared gene expression profiles from CD8⁺ and CD8⁻ MAIT cells, conventional CD8⁺ T cells (all CD8 T cells, i.e. naïve and memory) and NK cells isolated from peripheral blood and mucosal tissue (CD8⁺ MAIT, CD8⁻ MAIT and CD8⁺ T cells only) of two healthy donors (Table 3.1). For these experiments anti-CD3 and anti-Vα7.2 antibodies were included in the sorting panel to ensure optimal sort purity. Expression of 145 genes in each single-cell was determined using the nCounter NanoString platform. The NanoString technology relies on a capture probe and a

	TISSUE	CD8 ⁺ T cell	CD8 ⁻ MAIT	CD8 ⁺ MAIT	NK cell
Donor 1	Blood	48	47	48	48
	Mucosal	43	53	66	0
Donor 2	Blood	44	47	44	45
	Mucosal	46	57	65	0

Table 3.1. Single-cell gene expression analysis of MAIT cells isolated from blood and mucosal tissue.

The number of single-cells in each population that passed quality control and used for subsequent analysis is shown.

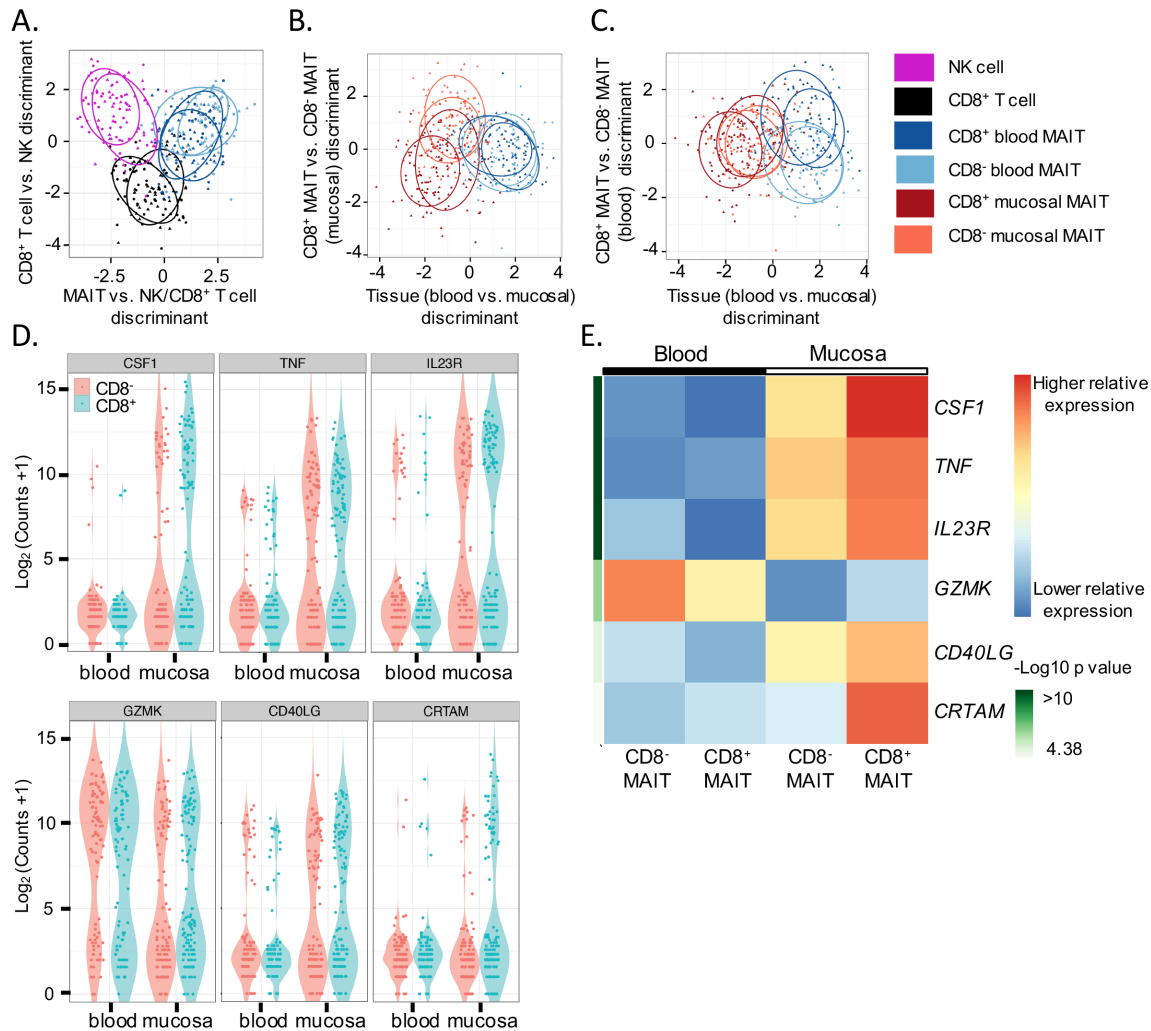


Figure 3.6. Mucosal MAIT cells are poised to respond more rapidly based on single-cell gene expression analysis of MAIT cells isolated from blood and mucosal tissue.

(A). Linear discriminant analysis of selected activation and inflammation genes expressed in CD8⁺ MAIT cells (dark blue), CD8⁻ MAIT cells (light blue), CD8⁺ T cells (black) and NK cells (purple) isolated from the blood (n=2). (B-C) CD8⁺ and CD8⁻ MAIT cells from blood are compared to CD8⁺ (dark orange) and CD8⁻ (light orange) MAIT cells isolated from rectal mucosal tissue. A confidence ellipse shows the expression profile of each donor (n=2). (D). Violin plots of the top 6 genes that are differentially expressed in CD8⁺ and CD8⁻ MAIT cells from blood and mucosa show gene expression on a single-cell level. Each single CD8⁺ (blue) and CD8⁻ (red) MAIT cell in the blood and tissue is represented as a single dot (n=2 donors). (E) Relative gene expression levels of the top 6 genes that are differentially expressed in CD8⁺ and CD8⁻ MAIT cells from blood (columns 1 and 2) and mucosa (columns 3 and 4) are indicated in red (high) and blue (low).

barcoded reporter probe to detect gene transcripts of interest. Using a two-probe detection system ensures specificity, and transcript abundance is measured by single molecule imaging of the reporter probe. We specifically interrogated the expression pattern of 119 genes controlling or indicating inflammation, activation and migration (IAM) status of a cell, as well as a set of 26 genes for quality control. CD8⁺ T and NK cells served as reference populations and were also included to facilitate interpretation of the data. Both MAIT cell populations had a distinct gene expression signature compared to conventional CD8⁺ T and NK cells in the blood, indicating their distinct functional properties (Figure 3.6A). We next compared the two MAIT cell populations from each tissue to each other to determine their relationship. We found that the two MAIT cell populations share extensive transcriptional overlap in mucosal tissue (Figure 3.6B, y-axis discriminant) and blood (Figure 3.6C, y-axis discriminant), but differences in the transcriptional profile became apparent when comparing MAIT cells from blood and mucosal tissue (Figure 3.6B and 3.6C – x-axis discriminant). We next interrogated which genes drive this tissue-based difference. Interestingly, we found that expression of genes associated with activating/pro-inflammatory functions (*TNF*, *IL23R*, *CSF1*, *CD40L*) was increased in mucosal MAIT cell populations, indicating that mucosal MAIT cells seem poised to respond more quickly compared to their counterparts in blood due to the increase in mRNA transcript (Figure 3.6D and 3.6E). The increase in *TNF* gene expression in the mucosal MAIT cell populations does not result in the display of actual effector function directly ex vivo (Figure 3.7A) and thus may reflect the potential to respond rapidly upon activation as has been shown for other cytokines^{131,132}. These data show that MAIT cells have an increased effector potential in healthy mucosal

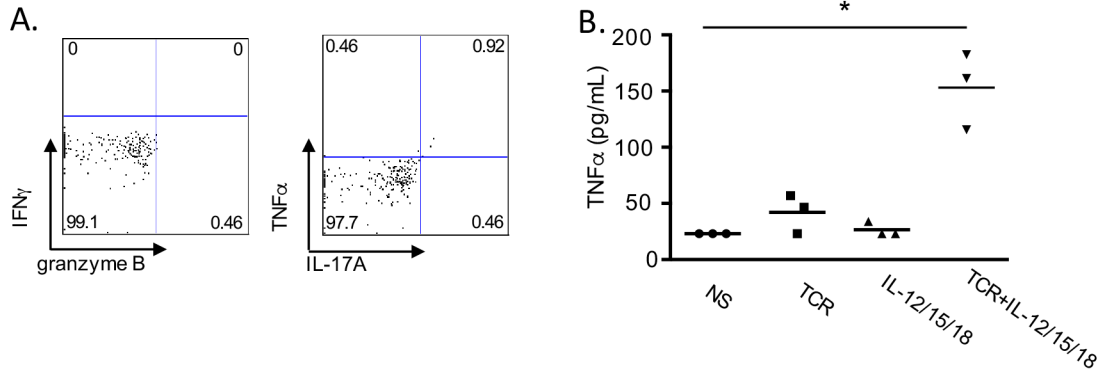


Figure 3.7. MAIT cells from healthy mucosal tissue do not secrete inflammatory cytokines ex vivo.

(A). Representative FACS plot from one donor of cytokines produced directly ex-vivo by CD8⁺ MAIT cells isolated from rectal mucosal biopsies. (B). 3,000 CD8⁺CD161^{hi}CCR6^{hi} cells were sorted from peripheral blood mononuclear cells (PBMC) and rested (NS, circles), stimulated with anti-CD3/CD28 beads (TCR, squares), 100ng/mL of IL-12/15/18 (triangles), or a combination of anti-CD3/28 beads plus IL-12/15/18 (TCR+IL-12/15/18, upside down triangles) for 24 hours followed by analysis of the culture supernatants by Luminex (n=3) for TNF α secretion. TNF α secretion that was below the limit of detection (LOD, 23 pg/mL) is plotted at the LOD. P values were determined using Mann Whitney one-tailed U-test (B).

tissue and further underline the necessity for stringent regulation of their effector function. To further support the notion of stringent activation requirements, we sorted MAIT cells from PBMC and asked how to most effectively elicit a $\text{TNF}\alpha$ response. Sorted MAIT cells were stimulated with IL-12/15/18 and/or via their TCR (Figure 3.7B). Robust $\text{TNF}\alpha$ production occurred only when TCR signals synergized with inflammatory signals (Figure 3.7B) further underlining the need for both signals for inducing comprehensive MAIT cell effector function.

Discussion

Conventional memory CD8 T cells and MAIT cells are important components of the immune system and have multiple overlapping characteristics. Both populations are fairly abundant in the blood, liver and mucosal tissues, where they exert their effector function upon activation, specifically secretion of $\text{TNF}\alpha$, $\text{IFN}\gamma$ and granzyme B. MAIT cells express effector molecules in response to inflammatory signals and, as proposed by recent reports, also following TCR stimulation^{39,55,115}. This would suggest that these two populations would respond in a very similar fashion and have overlapping function in response to inflammation and when encountering their specific antigen. However, we hypothesized that at least the sensitivity to TCR-mediated signals for primary human MAIT cell and conventional memory CD8 T cell activation is different due to the nature of the antigen they recognize. Conventional CD8 T cells have a highly diverse TCR repertoire and recognize their cognate antigen (peptides with a typical length of 8 – 10 amino acids) in context of MHC class I with great specificity. MAIT cells use an invariant $\text{TCR}\alpha$ chain and a limited repertoire of $\text{TCR}\beta$ chains to recognize a specific class of

antigen, bacterial metabolites and, potentially, metabolites of other origin as well. Since many metabolic pathways are essential and conserved across bacterial species, MAIT cells cannot use their TCR to distinguish between metabolites from commensal vs. pathogenic bacterial origin. Importantly, the current MAIT cell activation model suggests that a TCR signal is sufficient for inducing MAIT cell effector function. However, this model cannot explain how MAIT cells avoid responses against commensal-derived antigen or how MAIT cells could selectively respond to metabolites derived from commensal versus pathogenic bacteria.

To address these questions, we first wanted to clearly define which signals are sufficient to directly induce MAIT cell effector function *ex vivo*. We show here that an anti-CD3/CD28 signal is not sufficient to induce sustained robust effector function in FACS-purified MAIT cell populations (Figure 3.1B), while the same signal was sufficient to induce sustained effector function in memory CD8 T cells (Figure 3.1C). Importantly, stimulating both populations with anti-CD3/CD28 beads has two important advantages: it allows us to directly compare the responses of both populations and it avoids the pitfall of using antigen, specifically metabolites, with still undefined TCR affinity. It is also noteworthy that a TCR signal is sufficient for MAIT cells to elicit a brief $\text{TNF}\alpha$ and $\text{IFN}\gamma$ pulse 6 hours after TCR stimulation. This is akin to what has been described in naïve CD8 T cells, which also briefly make $\text{IFN}\gamma$ early after activation and prior to their first cell division¹³³. We could not detect significant production of $\text{IFN}\gamma$ in MAIT cells by ICS staining at later time-points (Figure 3.1B) or in MAIT cell culture supernatant by Luminex analysis (Figure 3.2A) 24 hours post-stimulation, further arguing that the production is very brief and limited. A previous study demonstrated that this brief $\text{IFN}\gamma$ pulse does not

result in anti-viral immunity and thus the purpose is still unclear¹³³. An IFN γ response following TCR stimulation was reported in MAIT cell clones and lines^{44,53}, which are useful tools to identify antigens, but not a suitable substitute for studying ex vivo activation requirements of primary human T cells.

Although a TCR signal is not sufficient to induce robust effector function in MAIT cells, primary human MAIT cells still respond to a TCR + costimulatory signal. However, instead of eliciting prolonged, robust effector function, a TCR + costimulatory signal elicits secretion of the chemokines CCL3 and CCL4 (Figure 3.2B). Similarly, conventional memory CD8 T cells secrete CCL3 and CCL4 in response to TCR stimulation (Figure 3.2B). The functions of CCL3 and CCL4 include recruitment of monocytes¹³⁴, which may serve as a surveillance population. Thus, our data demonstrate that MAIT cells do not secrete significant levels of effector molecules following TCR stimulation, but are not generally hyporesponsive to TCR signaling based on their ability to express CCL3 and CCL4 (Figure 3.2B). This phenotype resembles the altered TCR responsiveness of other innate-like T cells previously demonstrated in a mouse model system¹³⁵ and CD161^{hi}CD8 α^+ T cells³⁸. More work is needed to determine the downstream signaling cascade when MAIT cells are stimulated via their TCR. Similarly, the indirect signals that lead to activation of MAIT cells when bulk PBMCs are activated with anti-CD3/CD28 stimuli (Figure 3.3A and 3.3B) are still undefined. It is important to consider that additional mechanisms may be in place to further control MAIT cell function: for example, MAIT cell exposure to antigen could be further controlled via regulation of MR1 expression, which does not appear to be constitutively expressed on the cell surface^{136,137}. In summary, our data argue for a

fundamentally different role of a TCR signal for MAIT cells vs. memory CD8 T cells to elicit effector function (Figure 3.8).

We and others have previously shown that inflammatory cues (IL-12, IL-15, IL-18) are sufficient to directly activate MAIT cells^{39,55}. We demonstrate here that the MAIT cell population responds more vigorously compared to the memory CD8 T cell population (Figure 3.1B, 3.1C, 3.3A, 3.3B) suggesting that MAIT cells also play an important role in inflammatory processes in the absence of their cognate antigen. Following cytokine-induced activation, conventional memory CD8 T cells can kill target cells in an NKG2D-dependent and TCR-independent manner^{24,27,119,120,138}. Future studies will need to examine if MAIT cells can similarly exert direct cytotoxic effector function without cognate antigen recognition by the TCR. To more stringently test the ability of MAIT cells to respond in an inflammation-driven activation scenario under physiologically relevant conditions, we examined the ability of MAIT cells to respond to monocytes that were activated with ssRNA, a TLR8 agonist. We specifically asked whether TLR-stimulated monocytes could activate MAIT cells in a cell contact-dependent and –independent manner by culturing MAIT cells with monocytes or monocyte supernatant as outlined in Figure 3.4. MAIT cells acquired effector function following incubation with supernatant alone demonstrating that direct monocyte to MAIT cell contact is not necessary for activation (Figure 3.4A and 3.B).

Together, the experiments described so far allowed us to define the respective contributions of inflammatory vs. TCR signals to inducing MAIT cell effector function. To determine how TCR and cytokine signals integrate to activate MAIT cells, we either delivered a TCR signal, a cytokine signal (supernatant from monocytes that were

stimulated with ssRNA or LPS) or a combination of TCR + cytokine signals (Figure 3.5A and 5B). Importantly, the latter combination of signals led to a synergistic increase in effector function regardless of the nature of the TLR agonist. Only TLR8- but not TLR4-stimulated monocytes secreted detectable amounts of IL-12p70 (Figures 3.4C and 3.5C). However, both supernatants were sufficient to elicit MAIT cell effector function, which suggests that inflammatory cues other than IL-12 and IL-18 are sufficient to synergize with the TCR signal to induce effector function. Future studies will need to determine the nature of these cytokines.

Finally, we sought to compare MAIT cells from blood and mucosal tissue to determine if stringent control of effector functions is necessary for MAIT cells located in tissues. We considered the possibility that MAIT cells in the tissues may have inherently lower functional potential to prevent unwanted responses in healthy tissues. Importantly, our single-cell gene expression analysis showed enhanced immediate effector potential in a large portion of the mucosal MAIT cell population (Figure 3.6D and 3.6E). The single-cell analysis shows a biphasic distribution within the mucosal MAIT cell population for most genes (Figure 3.6D, violin plots). This distribution may be due to the specific location of MAIT cells in the tissue, due to the amount of time the cells have been in the tissue and there could also be a stochastic component. Future studies will need to address the underlying mechanisms. Interestingly, granzyme K (*GZMK*) was an exception in that its expression was decreased in mucosal tissue-derived MAIT cells (Figure 3.6D and 3.6E). Granzyme K can enhance LPS-induced cytokine secretion from human monocytes¹³⁹ The decrease in *GZMK* expression we observed could serve to prevent unwanted cytokine responses against commensal-derived LPS thus avoiding

eliciting an inflammatory response and induction of MAIT cell effector function. Finally, $\text{TNF}\alpha$ and other cytokines are not expressed on a protein level by MAIT cells in healthy mucosal tissue directly ex vivo (Figure 3.7A). This demonstrates that the increase in transcripts of effector molecules in mucosal MAIT cells does not indicate an ongoing effector response, but instead suggests that mucosal MAIT cells are poised to respond quickly.

In summary, our data provide a novel model of human MAIT cell activation requirements. Since inflammatory signals are necessary for acquisition of robust MAIT cell effector function and synergize with TCR signals, we propose that this previously unappreciated requirement for acquiring MAIT cell effector function provides an explanation for the co-existence of MAIT cells and commensals in mucosal tissues without eliciting pathology (TCR signal without inflammation¹⁴⁰), while maintaining responsiveness to pathogens (TCR signal plus inflammation). Future studies will address the consequences of (wanted and unwanted) MAIT cell activation in mucosal tissue and their role in inflammatory disorders of the mucosal tissue¹⁴¹, including bacterial translocation following HIV/SIV infection^{129,142} as well as intestinal inflammation following bone marrow transplantation¹²⁷. Importantly, MAIT cells express the drug efflux reporter ABCB1, which allows them to preferentially survive cytotoxic chemotherapy^{41,143}. In the latter context, it will be of particular interest to investigate the effect of MAIT cell activation on the diversity of the microbiome, which has been implicated in directly affecting patient outcome¹⁴⁴⁻¹⁴⁶. Importantly, our study outlines the mechanisms for inducing MAIT cell effector function and how they could be targeted for improving prevention and treatment strategies.

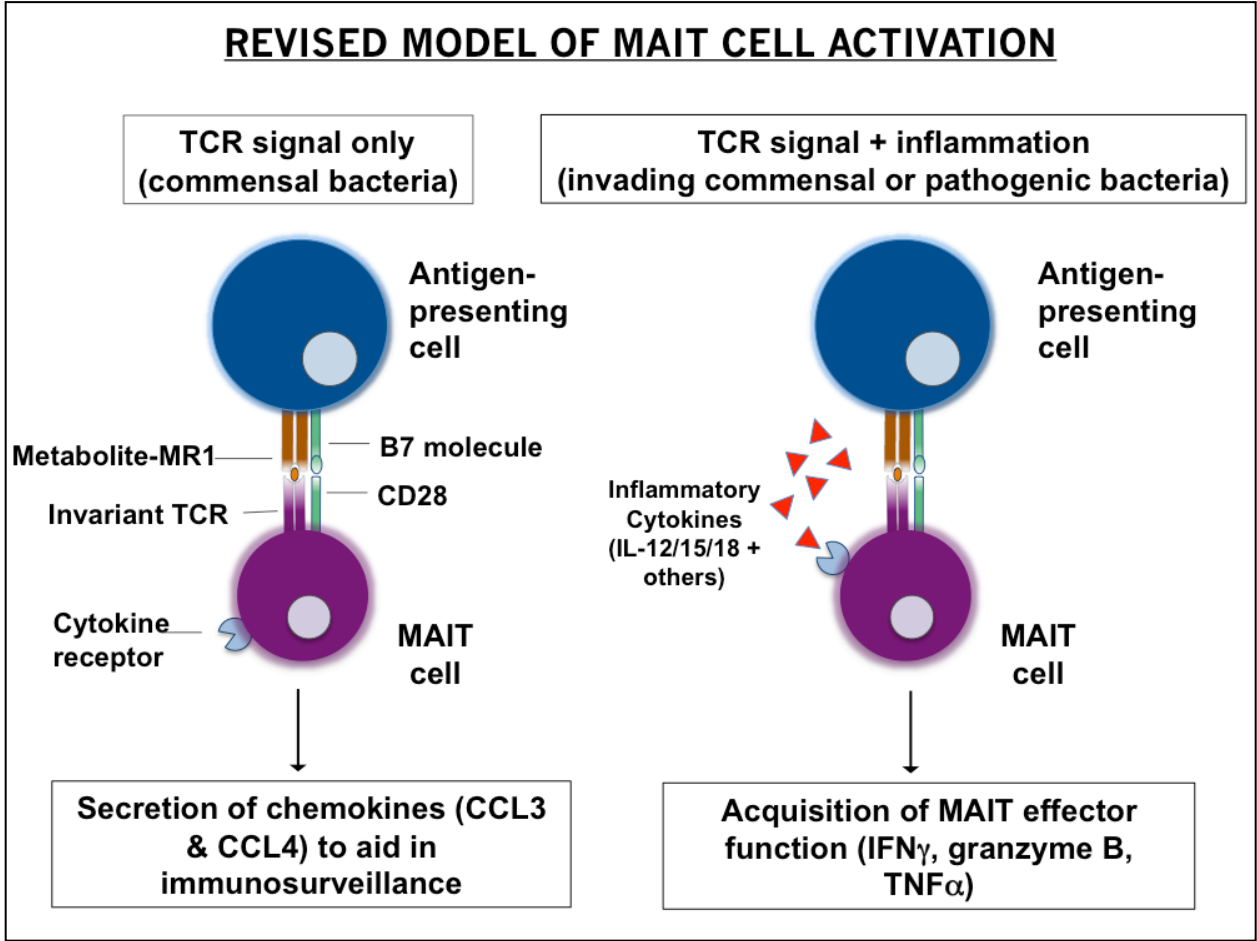


Figure 3.8. Revised model of MAIT cell activation.

In contrast to a TCR-dependent activation model of MAIT cells (Figure 1), we demonstrate that upon interaction with commensal-derived antigen in the absence of inflammation, MAIT cells function to secrete chemokines (CCL3 and CCL4) as a possible immune-surveillance mechanism. In contrast, when MAIT cells receive a TCR signal, derived either from pathogenic or invading commensal bacteria, in the context of inflammation (IL-12/15/18), MAIT cells acquire effector functions (IFN γ , granzyme B, TNF α) and function to clear the invading microbe. We propose that this model of MAIT cell activation allows argues for a dichotomous and situation-specific response by MAIT cells located in mucosal tissues.

Chapter 4:

MAIT cell activation and function during acute SIV infection

Introduction

Human immunodeficiency virus (HIV) infection in humans and simian immunodeficiency virus (SIV) infection in macaques leads to early and rapid depletion of CD4⁺ T cells in the gastrointestinal (GI) lamina propria¹⁴⁷⁻¹⁵⁰. During the acute phase of infection, a preferential loss of IL-17 (Th17) and IL-22 (Th22) producing CD4⁺ T cells within the lamina propria coincides with increased apoptosis of epithelial cells and areas of damage to the epithelial barrier of the GI tract¹⁵¹⁻¹⁵⁴. This damage disrupts the integrity of the mucosal barrier, allowing for translocation of microbial products into the lamina propria and increased systemic levels of inflammatory mediators, leading to chronic immune activation within the host^{142,155-157}.

More recently, the role of IL-17-producing CD8⁺ T cells (Tc17) has been investigated during SIV infection. In pathogenic SIV infection, Tc17 magnitude and functional responses are preserved during the first two weeks of infection. During chronic infection or at end-stage disease, Tc17 are depleted in the blood and throughout various tissues¹⁵⁸. In addition to conventional Tc17 subsets, unconventional semi-invariant T cells are also capable of producing IL-17³⁸. CD8⁺ CD161^{hi} T cells, including mucosal-associated invariant T (MAIT) cells, secrete IL-17 when stimulated with superantigen or after prolonged *in vitro* culture^{41,159}. MAIT cells are defined as V α 7.2⁺ CD161^{hi} IL-18R α ⁺ cells that recognize bacterial-derived metabolites via their semi-invariant TCR in the context of the MHC class I related molecule MR1^{43,44,160}.

Activated MAIT cells then function to lyse bacterially infected cells through the secretion of cytolytic molecules including granzyme B^{46,161}. MAIT cells have a memory-like phenotype and, similarly to conventional memory CD8⁺ T cells, become activated in response to stimulation with inflammatory cytokines¹⁶². Importantly, these cytokines act directly on MAIT cells and most MAIT cells respond to these signals as recently demonstrated in single-cell RNAseq experiments¹⁶³. We recently demonstrated that a TCR signal is not sufficient to induce a sustained MAIT cell effector response; rather, pro-inflammatory cytokine signals are necessary and synergize with TCR signals to induce sustained MAIT cell effector function¹⁶⁴.

Previous work on CD161^{hi} CD8⁺ T cells and MAIT cell subsets during chronic HIV infection has shown that these subsets are preferentially depleted in the blood of HIV-infected individuals even after initiation of highly active antiretroviral therapy (HAART)⁵⁹⁻⁶¹. Through *in vitro* stimulation experiments, it has been shown that MAIT cells isolated from chronically infected individuals have reduced effector function when stimulated with *E. coli*-fed antigen presenting cells⁶⁰. However, at what point during infection the depletion of these cells from the circulation occurs, and the mechanisms driving this depletion, remain unclear.

Due to an extended eclipse phase in patients infected with HIV, and limited number of blood and mucosal samples available, few studies have addressed the contributions of CD161^{hi} CD8⁺ semi-invariant T cells to HIV during acute infection^{115,165,166}. We sought to better understand the role of CD161^{hi} CD8⁺ semi-invariant T cells to immunity during the acute phase utilizing the non-human primate

model of acute SIV infection in order to identify how alterations in this subset may influence disease pathogenesis and progression.

Results

Functional characterization of CD8⁺ CD161^{hi} IL-18R α ⁺ T cells in rhesus macaque peripheral blood and jejunum

The C-type lectin receptor, CD161, is expressed highly on semi-invariant CD8⁺ T cells, including the MAIT cell subset, that are found in the blood and throughout various mucosal tissues^{41,80}. CD161^{hi} semi-invariant T cells also express cytokine receptors, including the IL-18 receptor α -chain⁴¹. Changes in frequency and function of CD161^{hi} semi-invariant T cells have been reported in a variety of disease states, including during chronic HIV and SIV infection, but have yet to be studied during acute infection^{59,61,63}. To investigate the changes that occur in the CD8⁺ CD161^{hi} IL-18R α ⁺ T cell subset during acute SIV infection, we developed a gating strategy to identify CD45⁺ CD3⁺ CD8⁺ CD161^{hi} IL-18R α ⁺ lymphocytes (Figure 4.1A). The majority of human CD161^{hi} CD8⁺ semi-invariant T cells are MAIT cells and express the TCR V α 7.2 (TRAV1-2) chain⁴¹. The anti-V α 7.2 antibody used for human MAIT studies lacks cross-reactivity for macaques. Therefore, to determine if macaque CD8⁺ CD161^{hi} IL-18R α ⁺ T cells also predominantly express the V α 7.2 chain, we sorted CD3⁺ CD8⁺CD161^{hi} IL-18R α ⁺ T cells at one baseline time point and used the cells for TCR sequencing analysis. Our results showed that CD3⁺ CD8⁺CD161^{hi} IL-18R α ⁺ cells typically express the canonical MAIT cell TRAV (TRAV1-2) and TRAJ genes (TRAJ33, TRAJ12, TRAJ20) (Supplemental

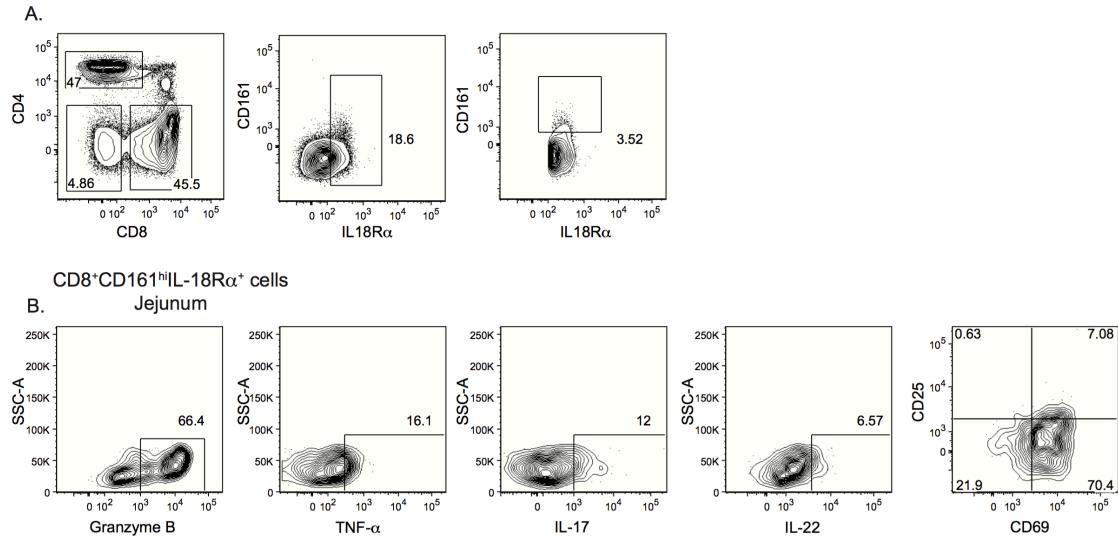


Figure 4.1. Identification of innate-like T cells in rhesus macaques.

(A) Innate-like T cells were characterized as viable CD3⁺ CD45⁺ CD8⁺ CD161^{hi} IL-18Rα⁺ lymphocytes. Representative flow plots from the blood of one animal at a pre-infection time point. (B) Representative flow plots from intracellular cytokine staining of innate-like T cells directly *ex vivo* from one animal at a pre-infection time point in the mucosa.

Figure 4.1A) similarly to what has been reported for human CD161^{hi} CD8⁺ semi-invariant T cells. These results indicate that a substantial fraction of the CD8⁺ CD161^{hi} IL-18R α ⁺ T cells analyzed in this study are MAIT cells.

In addition, we analyzed CD8⁺ CD161^{hi} IL-18R α ⁺ and total CD8 T cells directly *ex vivo* for expression of CD69, CD25, granzyme B, TNF α , IL-17, and IL-22 (Figure 4.1B) prior to infection.

Alterations in CD8⁺ CD161^{hi} IL-18R α ⁺ T cell frequency during acute SIV infection

Given multiple reports that CD8⁺ CD161^{hi} IL-18R α ⁺ T cells in the blood and mucosal tissue of chronically HIV-infected individuals or SIV-infected macaques are depleted^{59,61,62,66}, we assessed semi-invariant T cell frequencies in the blood and mucosal tissue during acute SIV infection to define when this cell population is affected by SIV infection. Six male rhesus macaques (*Macaca mulatta*) were infected intrarectally with 100,000 TCID SIVMAC239x. Frequencies of semi-invariant T cells were significantly increased in the blood 3 and 7 days post-infection, but frequencies decreased to baseline by day 14 in conjunction with peak viral load, before rebounding by day 28 once viral set point had been achieved (Figure 4.2A). Total CD8 T cell frequencies in the blood increased as early as day 3, and continued increasing throughout infection (Figure 4.2B). Absolute cell numbers reflected similar kinetics in both the CD8⁺ CD161^{hi} IL-18R α ⁺ T cell and total CD8 T cell populations (Figures 4.2C and 4.2D). In the jejunum, CD8⁺ CD161^{hi} IL-18R α ⁺ T cell frequencies decreased at day 3, followed by a rebound by day 14 before returning to baseline levels by day 28 (Figure 4.2E). This observation is specific to CD8⁺ CD161^{hi} IL-18R α ⁺ T cells as conventional CD8 T cell frequencies increased in the jejunum, similar to what was observed in the

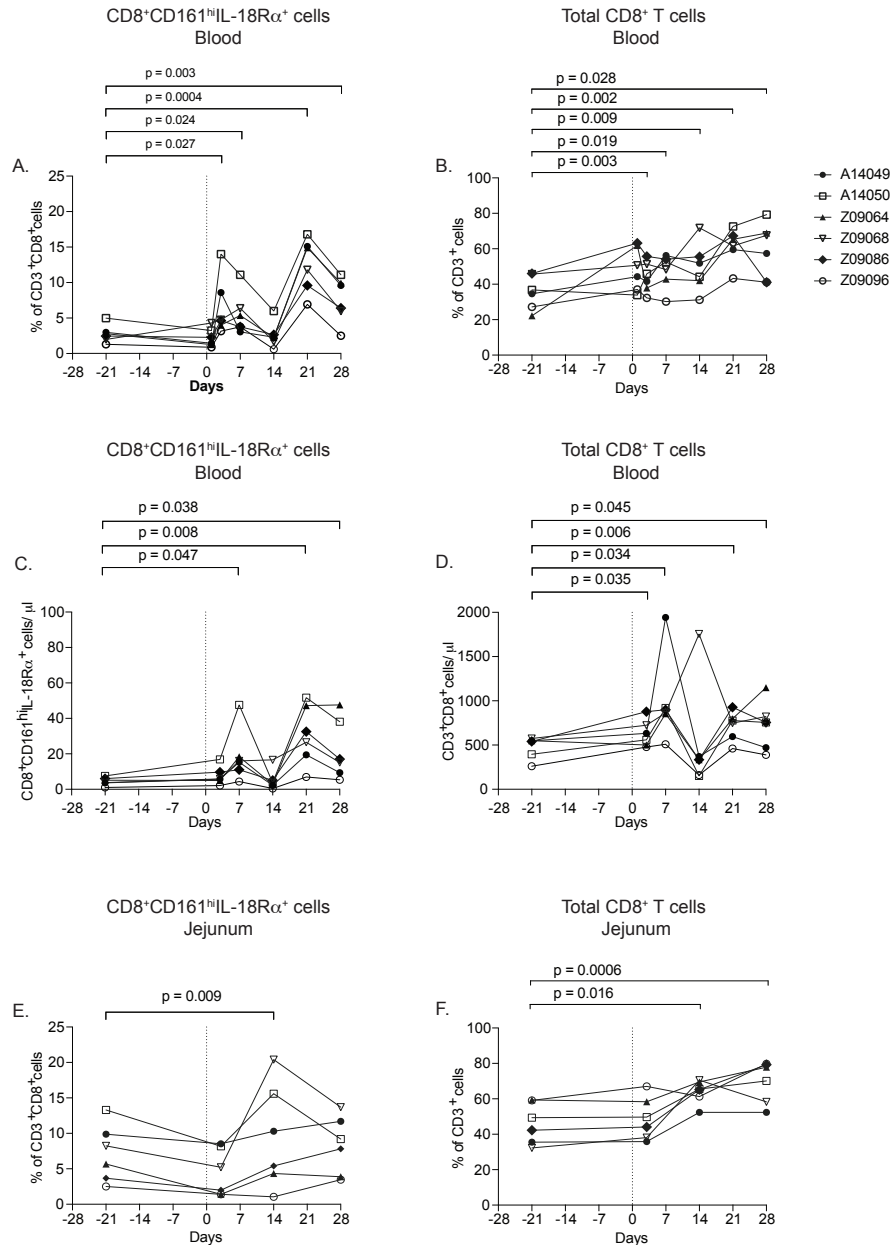


Figure 4.2. Kinetics of innate-like T and conventional T cells during early SIV infection time points.

(A). Frequency of CD8⁺CD161^{hi}IL-18R α ⁺ cells and (B) frequency of CD3⁺CD8⁺ T cells in the blood and jejunum pre- and post-SIV infection. (C) Absolute numbers of CD8⁺CD161^{hi}IL-18R α ⁺ cells and (D) CD3⁺CD8⁺ T cells per microliter of peripheral blood, calculated from population percentages and lymphocyte counts. (E) Frequency of CD8⁺CD161^{hi}IL-18R α ⁺ and (F) frequency of CD3⁺CD8⁺ T cells in the jejunum pre- and post-SIV infection. Vertical dashed line denotes day of infection. P values were calculated using a paired two-tailed student's t test.

blood (Figure 4.2F). It is important to note that alterations to CD8⁺ CD161^{hi} IL-18R α ⁺ T cell frequencies in the blood and jejunum observed at 3 days-post infection occurred prior to detectable levels of virus circulating in the blood (data not shown). Overall, these initial changes in frequency suggest that CD8⁺ CD161^{hi} IL-18R α ⁺ T cell responses are initiated very early during acute infection.

Increased activation of CD8⁺ CD161^{hi} IL-18R α ⁺ T cells during acute SIV infection

It has recently been reported that MAIT cells increase surface expression of activation markers such as CD38 and HLA-DR within the first six months of HIV infection and become activated during acute and chronic stages of other viral infections^{62,167}. To investigate activation kinetics of CD8⁺ CD161^{hi} IL-18R α ⁺ T cells during SIV infection, we assessed expression of CD69 and CD25 in the blood and jejunum. We found a significant increase in CD69-expressing CD8⁺ CD161^{hi} IL-18R α ⁺ T cells in the blood at day 14, in conjunction with peak viral load, followed by a return to below-baseline expression at day 21 (Figure 4.3A). In the jejunum, we observed a maintenance of CD69-expressing CD8⁺ CD161^{hi} IL-18R α ⁺ T cells through to day 14, with a significant decrease in CD69-expressing cells at day 28 (Figure 4.3B). To further examine the activation of CD8⁺ CD161^{hi} IL-18R α ⁺ T cells during acute infection, we analyzed co-expression of CD69 and CD25 (IL-2R α chain). Induction of CD25 expression indicates full activation of a T cell and is a more stringent biomarker of activation¹⁶⁸. We observed a significant decrease of CD69⁺CD25⁺ expressing cells in blood shortly after infection that remained below baseline through day 21 (Figure 4.3C). Importantly, we found that the frequency of CD8⁺ CD161^{hi} IL-18R α ⁺ T cells co-expressing CD69 and CD25

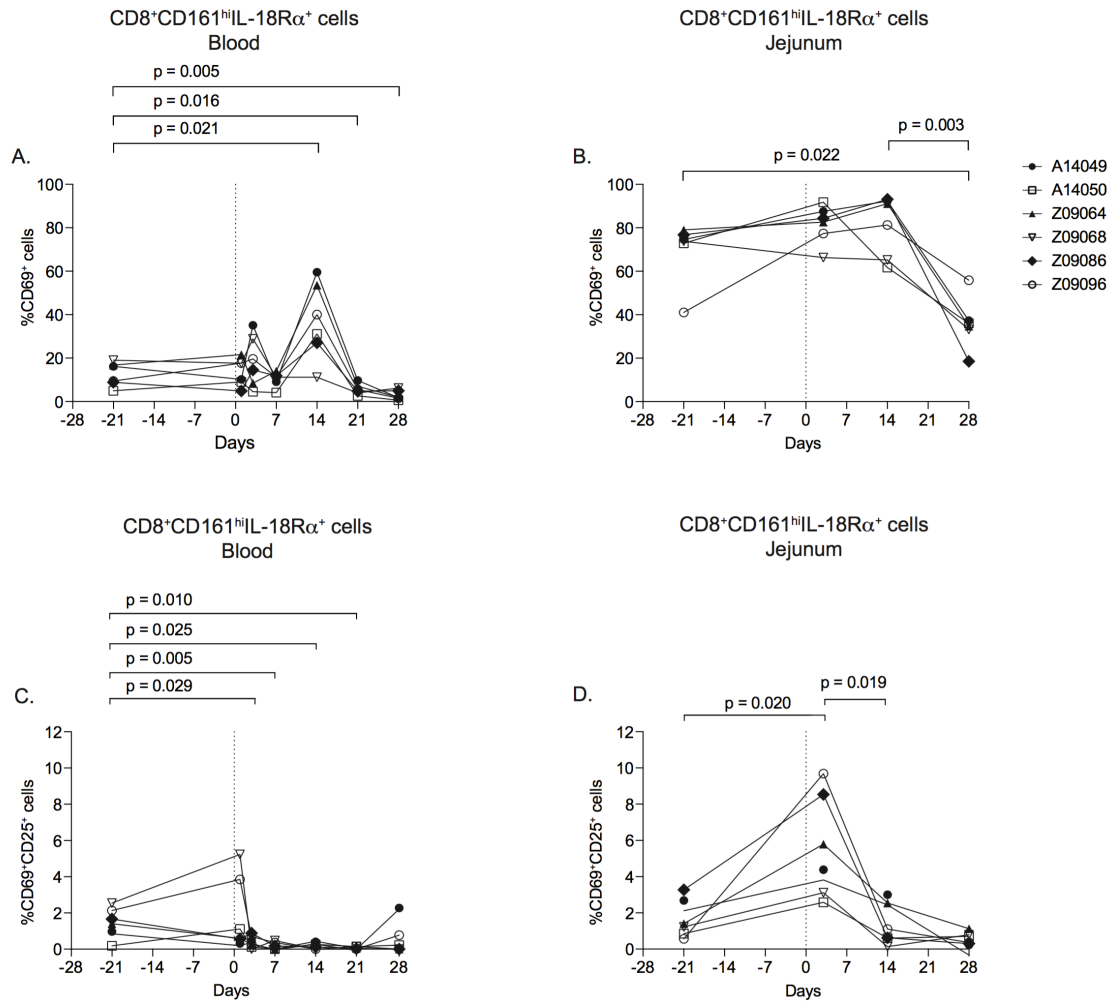


Figure 4.3. Transient activation of innate-like T cells after SIV infection.

(A). Frequency of CD69-expressing CD8⁺CD161^{hi}IL-18R α ⁺ cells in the blood and (B) jejunum pre- and post-SIV infection. (C) Frequency of CD69⁺CD25⁺ CD8⁺CD161^{hi}IL-18R α ⁺ cells in the blood and (D) jejunum pre- and post-SIV infection. Vertical dashed line denotes day of infection. P values were calculated using a paired two-tailed student's t test.

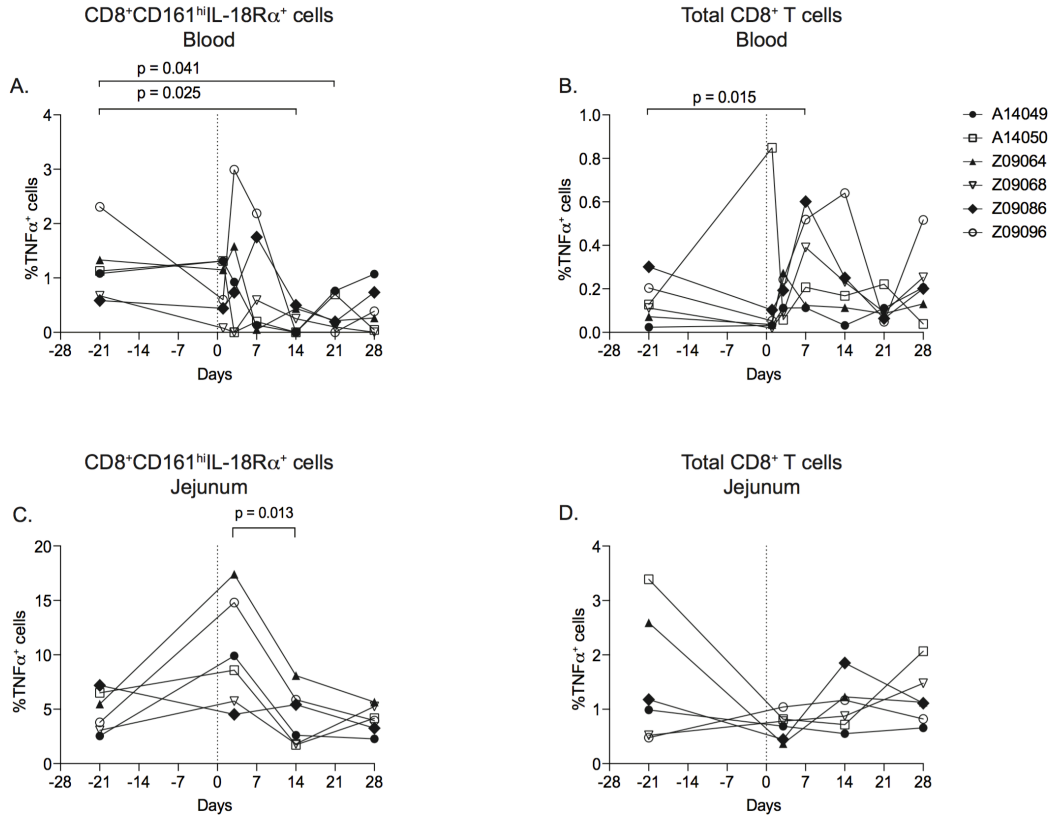


Figure 4.4. Acquisition of cytolytic effector function at acute time points during SIV infection.

(A) Granzyme B expression by CD8⁺CD161^{hi}IL-18R α ⁺ cells and (B) CD3⁺CD8⁺ T cells in the blood pre- and post-SIV infection. (C) Granzyme B expression by CD8⁺CD161^{hi}IL-18R α ⁺ cells and (D) CD3⁺CD8⁺ T cells in the jejunum pre- and post-SIV infection. Vertical dashed line denotes day of infection. P values were calculated using a paired two-tailed student's t test.

increased significantly in the jejunum at day 3 post-infection, followed by a significant drop in double-expressing cells between day 3 and day 14 before returning to baseline by day 28 (Figure 4.3D). Thus, these data suggest that CD8⁺ CD161^{hi} IL-18R α ⁺ T cells, including the MAIT cell subset, are activated very early after SIV infection in the jejunum.

Effector function of CD8⁺ CD161^{hi} IL-18R α ⁺ T cells is altered during acute SIV infection

We next evaluated whether activation of CD8⁺ CD161^{hi} IL-18R α ⁺ T cells also resulted in changes in effector function, including upregulation of granzyme B, and if these alterations could be measured during acute infection. At day 3 post-infection we observed a decrease in granzyme B-expressing CD8⁺ CD161^{hi} IL-18R α ⁺ T cells in the blood, followed by a significant increase by day 14 that was not maintained by day 28 (Figure 4.4A). Total CD8 T cells demonstrated increased granzyme beginning at day 14, and this is maintained through day 28 in the blood (Figure 4.4B). Similar to our findings in the blood, granzyme B expression by CD8⁺ CD161^{hi} IL-18R α ⁺ T cells in the jejunum increased at day 14 post-infection before returning to baseline by day 28 (Figure 4.4C). The early response by CD8⁺ CD161^{hi} IL-18R α ⁺ T cells in the jejunum is unique as we observed stable granzyme B expression by total CD8 T cells in the jejunum throughout the acute phase of infection (Figure 4.4D).

In contrast to granzyme B expression, TNF α -expressing CD8⁺ CD161^{hi} IL-18R α ⁺ T cells were diminished in the blood by day 14 post-infection and remained low throughout the acute phase of infection (Figure 4.5A). Total CD8 T cells followed a distinct pattern of TNF α expression in the blood, where an initial burst of cytokine occurred at day 3 but was not maintained during the acute infection phase

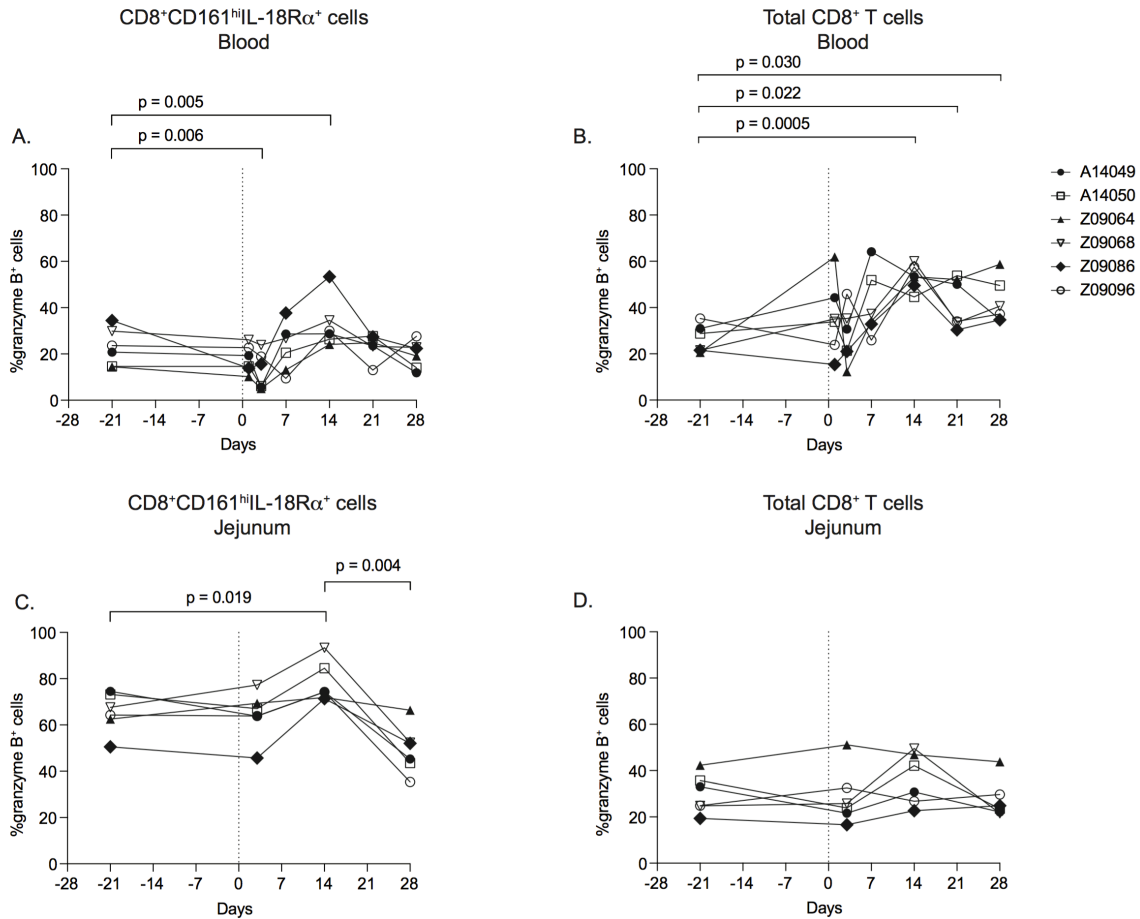


Figure 4.5. Initial burst of TNF α by innate like and conventional T cells early after SIV infection.

(A) TNF α expression by CD8⁺CD161^{hi}IL-18R α ⁺ cells and (B) CD3⁺CD8⁺ T cells in the blood pre- and post-SIV infection. (C) TNF α expression by CD8⁺CD161^{hi}IL-18R α ⁺ cells and (D) CD3⁺CD8⁺ T cells in the jejunum pre- and post-SIV infection. Vertical dashed line denotes day of infection. P values were calculated using a paired two-tailed student's t test.

(Figure 4.5B). In the jejunum, we observed an increase in TNF α -expressing CD8⁺ CD161^{hi} IL-18R α ⁺ T cells at day 3, followed by a significant drop to baseline levels or lower by day 14 (Figure 4.5C). We did not observe differences in TNF α production by conventional T cells in the jejunum (Figure 4.5D). Overall, the results described thus far indicate that CD8⁺ CD161^{hi} IL-18R α ⁺ T cells in both the blood and mucosal tissue are activated early during SIV infection, even before virus has achieved detectable levels in the blood.

Systemic depletion of IL-17- and IL-22-producing CD8⁺ CD161^{hi} IL-18R α ⁺ T cells early after SIV infection

Loss of IL-17 and IL-22 producing CD4⁺ T cells during HIV or SIV infection contributes to inflammation, viral persistence, mucosal damage, and increased microbial translocation^{153,155,169}. CD8⁺ CD161^{hi} IL-18R α ⁺ T cells, including MAIT cells, have been reported to produce IL-17 *in vitro*, and we next asked if *ex vivo* IL-17 or IL-22 production was altered during acute infection. We found a significant decrease in IL-17 producing CD8⁺ CD161^{hi} IL-18R α ⁺ T cells directly *ex vivo* in the blood as early as day 1 post-infection, which remained low throughout the viral expansion phase (Figure 4.6A). In contrast, we did not observe changes in IL-17 producing cells in the jejunum at any time points post-infection (Figure 4.6C). These kinetics were unique to CD8⁺ CD161^{hi} IL-18R α ⁺ T cells as we detected a much smaller proportion of total CD8 T cells in the blood and jejunum expressing IL-17, with significant changes in frequency occurring at day 1 post-infection in the blood before returning to baseline levels (Supplemental Figure 4.2A and Supplemental Figure 4.2C). Similar to our observations of IL-17 producing cells, we observed that IL-22 expression by CD8⁺ CD161^{hi} IL-18R α ⁺ T cells

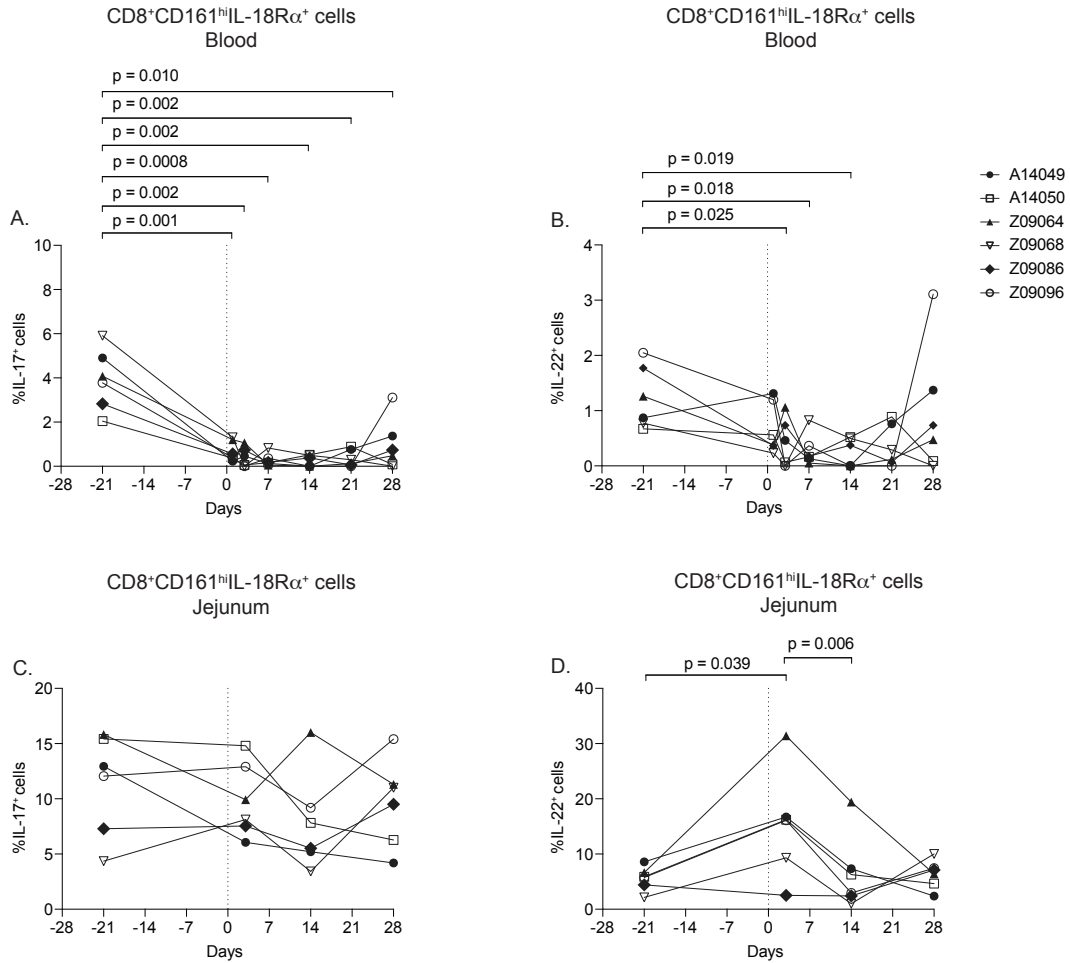


Figure 4.6. Loss of IL-17 and IL-22-expressing CD8⁺ CD161^{hi} IL-18R α ⁺ cells in the blood during acute SIV infection.

(A) IL-17 and (B) IL-22 expression by CD8⁺CD161^{hi}IL-18R α ⁺ cells in the blood pre- and post-SIV infection. (C) IL-17 and (D) IL-22 expression by CD8⁺CD161^{hi}IL-18R α ⁺ cells in the jejunum pre- and post-SIV infection. Vertical dashed line denotes day of infection. P values were calculated using a paired two-tailed student's t test.

was significantly decreased in the blood as early as day 3 and continued to remain at low frequencies throughout the course of acute infection (Figure 4.6B). We detected an increase of IL-22⁺ cells in the CD8⁺ CD161^{hi} IL-18R α ⁺ T cell population in the jejunum at day 3, followed by a significant drop to baseline at day 14 (Figure 4.6D). These blood and mucosal kinetics were unique to CD8⁺ CD161^{hi} IL-18R α ⁺ T cells as we observed almost no IL-22 expression by total CD8 T cells, and no SIV-induced alterations during early infection (Supplemental Figure 4.2B and Supplemental Figure 4.2D). These observations suggest that altered functionality of peripheral CD8⁺ CD161^{hi} IL-18R α ⁺ T cells beginning early during infection may contribute to the reduction in homeostatic cytokines important for barrier immunity.

CD8⁺ CD161^{hi} IL-18R α ⁺ T cell functions correlate with markers of barrier health and reduced microbial translocation

During SIV infection, perturbations in mucosal integrity leads to increased serum cytokine levels, as well as potential for bacterial metabolites to cross the mucosal barrier and activate the MAIT TCR when presented by MR1^{129,142,170}. Given the importance of IL-17 and IL-22 in barrier health, we sought to determine if loss of IL-17 and IL-22 expressing CD8⁺ CD161^{hi} IL-18R α ⁺ T cells was associated with peripheral markers of epithelial integrity and microbial translocation. We measured plasma levels of LPS-binding protein (LBP), a peripheral indicator of microbial translocation at days 3, 7, 14, 21, and 28. We found that plasma LBP was inversely correlated with IL-17 expressing CD8⁺ CD161^{hi} IL-18R α ⁺ T cells in blood (Figure 4.7A). In addition, we observed an inverse correlation with LBP and IL-22 expressing CD8⁺ CD161^{hi} IL-18R α ⁺ T cells in blood (Figure 4.7B). These observations suggest that homeostatic cytokine

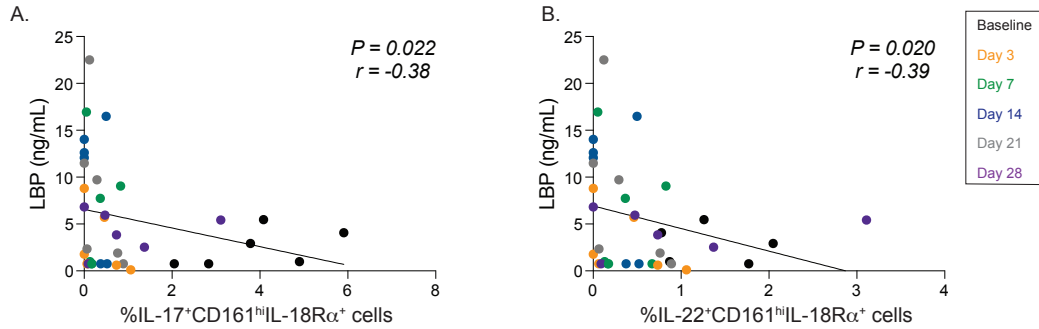


Figure 4.7. Impact of bacterial translocation on IL-17 and IL-22 producing innate-like T cells in the blood.

Correlation between (A) LPS-binding protein (LBP) in the plasma and frequency of IL-17 producing CD8⁺CD161^{hi}IL-18R α ⁺ cells at baseline and days 3, 7, 14, 21 and 28. Correlation between (C) LPS-binding protein (LBP) in the plasma and frequency of IL-22 producing CD8⁺CD161^{hi}IL-18R α ⁺ cells at baseline and days 3, 7, 14, and 28. Correlations were assessed using the Spearman's test.

production by CD8⁺ CD161^{hi} IL-18R α ⁺ T cells, including the MAIT cell subset, correlate with barrier health during the course of infection and the early loss of the functionality that we observe may contribute to mucosal dysfunction and SIV pathogenesis.

Discussion

Gastrointestinal mucosal dysfunction in HIV infection includes focal breaches in the epithelial barrier, microbial translocation, and immune activation and is associated with increased morbidities and mortality^{156,170-174}. The initiating events that lead to mucosal dysfunction during HIV infection remain unclear, thus resulting in a major hurdle for the development of effective therapeutics aimed at eliminating HIV infection and restoring barrier immunity¹⁷⁵. Recently, a decline in MAIT cell frequency has been reported during chronic HIV and SIV infections^{59-62,66,176}, but it is unclear if these changes already occur during the acute phase of infection and contribute to mucosal dysfunction following SIV infection. We hypothesized that CD8⁺ CD161^{hi} IL-18R α ⁺ T cells could contribute to mucosal dysfunction in two ways: loss of cells or cell function (such as IL-17 and IL-22) required for maintaining barrier immunity and acquisition of effector function that could damage epithelial cells. Importantly, these are not mutually exclusive events.

A significant proportion of human CD8⁺ CD161^{hi} IL-18R α ⁺ T cells in the periphery and throughout mucosal sites are MAIT cells. We show here that analogous to this human T cell subset; non-human primate (NHP) CD8⁺ CD161^{hi} IL-18R α ⁺ T cells also predominantly express the canonical MAIT TCR V α 7.2 (TRAV1-2) chain (Supplemental Figure 4.1). Importantly, CD8⁺ CD161^{hi} IL-18R α ⁺ T cells isolated from the jejunum

express IL-17 and IL-22 *ex vivo* (Figure 4.1B). The frequency of CD8⁺ CD161^{hi} IL-18R α ⁺ T cells in blood and mucosal tissues is comparable in NHPs and humans (Figure 4.2)⁶⁶. We observed an increase in the frequencies and absolute cell numbers of CD8⁺ CD161^{hi} IL-18R α ⁺ T cells in the blood as early as 3 days post-infection (Figure 2A and 2B), followed by a decrease by day 14. The decrease in frequency and absolute cell numbers in the blood and concomitant increase in the jejunum we observed 14 days post-infection may indicate that the cells are homing from the blood to mucosal sites. CD8⁺ CD161^{hi} IL-18R α ⁺ T cells, and in particular, MAIT cells in the circulation express mucosal-homing chemokine receptors at steady state⁴¹. This potential trafficking to the mucosa may explain the previously reported loss of these cells from the periphery, in particular those expressing CCR6, from the circulation in HIV-positive individuals⁶². Interestingly, CD8⁺ CD161^{hi} IL-18R α ⁺ T cell frequency was increased in the blood on day 21 (Figure 4.2) suggesting that proliferation may occur at later time points.

To define the functional properties of CD8⁺ CD161^{hi} IL-18R α ⁺ T cells during these early events, we investigated the activation status and inflammatory cytokine production of these cells in the blood and mucosa. We observed early (day 3 and day 14 post-infection) activation of CD8⁺ CD161^{hi} IL-18R α ⁺ T cells in the blood and jejunum as measured by CD69 and CD25 expression (Figure 4.3). Activation could be induced by inflammatory signals suggesting that MAIT cells are activated in a bystander fashion as recently reported for other viral infections¹⁶⁷ and similar to what has been described for conventional memory T cells²⁶⁻²⁸. However, expression of CD25 suggests the involvement of a TCR signal. Since MAIT cells recognize bacterial metabolites that are part of the riboflavin synthesis pathway, this would suggest that these cells respond to

bacterial-derived (presumably commensal) antigen. Importantly, we recently reported that MAIT cells require inflammatory cytokines for sustained effector function, and that the combination of both a TCR signal + inflammatory cytokine signal synergize to induce TNF α expression in MAIT cells, but that either one of these signals delivered alone are not sufficient for TNF α expression¹⁶⁴. Thus, SIV-induced inflammation may allow CD8⁺ CD161^{hi} IL-18R α ⁺ T cells to respond to bacterial-derived antigen. This notion is further supported by the increase in TNF α expression in the jejunum by certain animals at day 3, which returns to baseline by day 14 post-infection (Figure 4.5). The early peak in TNF α production suggests that CD8⁺ CD161^{hi} IL-18R α ⁺ T cells receive TCR + inflammatory signals within days of the infection. This response is turned off again by day 14 (Figure 4.5) indicating that the response is transient. This could be due to an inhibitory feedback loop, but how CD8⁺ CD161^{hi} IL-18R α ⁺ T cell responses are shut off is not yet understood.

Given the cytolytic capacity of MAIT cells and other CD8⁺ CD161^{hi} IL-18R α ⁺T cells, in conjunction with the early activation we observed, we investigated changes in granzyme B expression to better understand the effector response in the blood and the mucosa¹⁶¹. While there have been a variety of studies of cytolytic function of MAIT cells isolated from the blood, the effector functions of MAIT cells at the site of the mucosa remain relatively unclear. We have previously shown that MAIT cells isolated from healthy human mucosal tissue express higher levels of pro-inflammatory gene transcripts compared to their counterparts in the blood, suggesting that MAIT cells in the mucosa are poised to rapidly respond to pathogen invasion¹⁶⁴. In this study we observe that CD8⁺ CD161^{hi} IL-18R α ⁺T cells in the blood and jejunum increase granzyme

expression within the first two weeks of infection before returning to baseline levels during the chronic phase, suggesting that CD8⁺ CD161^{hi} IL-18R α ⁺ T cells exert cytotoxicity and may contribute to tissue damage. It is important to point out that in healthy human mucosal tissue, MAIT cells do not appear to express granzyme B, but that in the animals studied here we report a baseline expression of granzyme B in both the blood and mucosa (Figure 4.4)^{161,164}. This could indicate that CD8⁺ CD161^{hi} IL-18R α ⁺ T cells have more of an effector memory rather than a central memory phenotype in non-human primates, but future studies will be needed to understand these baseline differences in humans and non-human primates and how it affects target cell killing.

Our data thus far suggest that CD8⁺ CD161^{hi} IL-18R α ⁺T cells gain effector function early after SIV infection and may have a role in actively contributing to tissue damage and loss of the mucosal barrier function. A loss of IL-22 and IL-17 producing T cells in the blood and mucosal tissue has been linked to an increase in mucosal barrier permeability, microbial translocation, and heightened immune activation, which correlates with increased disease progression^{77,148,153,158,169,177,178}. Recent work has shown that MAIT cells in human female genital mucosa are biased towards IL-17 and IL-22 production in response to bacterial stimulation⁵², however the contribution of IL-17 or IL-22 producing MAIT cells during viral infection had yet to be investigated. To better understand the contribution of CD8⁺ CD161^{hi} IL-18R α ⁺T cells that produce the homeostatic cytokines IL-17 and IL-22, we examined frequencies of IL-17 and IL-22 producing CD8⁺ CD161^{hi} IL-18R α ⁺ T cells *ex vivo* in the periphery and jejunum, as well as their relationship with microbial translocation (Figure 4.6 and 4.7). Importantly, in the

blood we observed a significant decrease in IL-17 and IL-22 producing CD8⁺ CD161^{hi} IL-18R α ⁺ T cells as early as day 1 after infection and that this loss is maintained throughout the course of infection (Figure 4.6). At the site of the mucosa, changes in frequency of IL-17 and IL-22 producing cells were observed within the first two weeks of infection in some animals (Figure 4.6). A temporary increase in IL-22⁺ CD8⁺ CD161^{hi} IL-18R α ⁺ T cells was observed in the jejunum on day 3, but lost at later time points (Figure 6D) indicating that the decrease in IL-22 producing cells in the blood is not just due to trafficking to mucosal sites, but may also be explained by functional changes. Given the rapid depletion of the IL-17 and IL-22 producing CD8⁺ CD161^{hi} IL-18R α ⁺ T cells in the blood, we examined the association between peripheral IL-17 or IL-22 producing cells with peripheral markers of mucosal barrier health. Particularly, we measured LBP, a peripheral indicator of microbial translocation increased in HIV and SIV¹⁷⁴. We observed heightened levels of LBP in the plasma that resulted in an inverse correlation with frequency of IL-17 and IL-22 producing CD8⁺ CD161^{hi} IL-18R α ⁺ T cells in the blood, but not the jejunum (Figure 4.7). These data suggest that IL-17 and IL-22 production by CD8⁺ CD161^{hi} IL-18R α ⁺ T cells may contribute to barrier health and the loss of these subsets in the blood may contribute to barrier breakdown, microbial pathogenesis, and eventual disease progression. To our knowledge, these are the first data reported indicating that early loss of IL-17 and IL-22 producing CD8⁺ CD161^{hi} IL-18R α ⁺ T cells in the blood may contribute to the effects attributed to the loss of Th17 cells from the blood and mucosa during SIV or HIV infection^{151,177}.

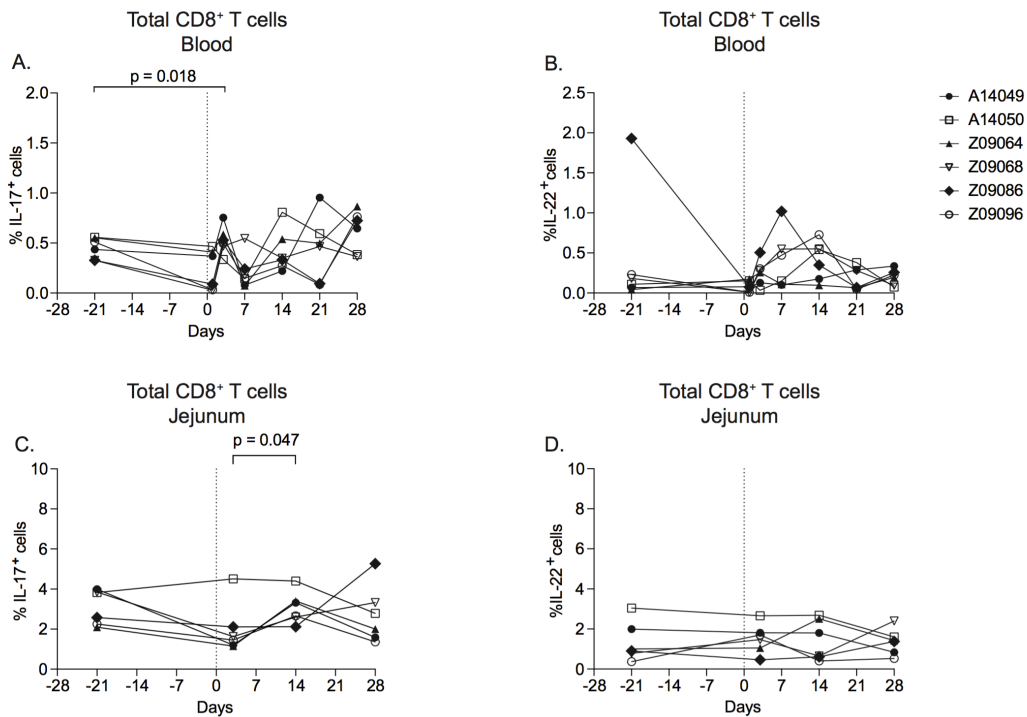
In summary, our data provide insight into the contributions of CD8⁺ CD161^{hi} IL-18R α ⁺ T cells, including the MAIT population, to barrier immunity and the functional

properties of these cells during early SIV infection. Recently, therapeutic interventions such as probiotic and fecal transplant therapy have been investigated to further understand the relationship between the microbiome and Th17/Th22 function and response¹⁷⁹⁻¹⁸¹. Given the unique antigen recognition by MAIT cells, location at the site of the mucosa, cytotoxic effector function and production of IL-17/IL-22, it will be important to study the response of this population during such therapeutic interventions to determine if IL-17 or IL-22 function can be restored while decreasing their cytotoxic properties in an effort to restore barrier immunity.

Sequence#	V-Gene	J-Gene	AA JUNCTION
1	TRAV1-1	TRAJ32	CAVRDGGSGNKLIF
2	TRAV1-1	TRAJ32	CAVRDGGSGNKLIF
3	TRAV1-2	TRAJ12	CAAMDSYKLIIF
4	TRAV1-2	TRAJ33	CAAMDSNYQLIWF
5	TRAV1-2	TRAJ33	CAAMDSNYQLIWF
6	TRAV1-2	TRAJ33	CAVRDSNYQLIWF
7	TRAV1-2	TRAJ33	CAVMDSNYQLIWF
8	TRAV1-2	TRAJ12	CAAMDSYKLIIF
9	TRAV1-2	TRAJ20	CAVRDGGYKLSF
10	TRAV1-2	TRAJ33	CAVMDSNYQLIWF
11	TRAV1-2	TRAJ12	CAVVDGGYKLIIF
12	TRAV1-2	TRAJ33	CAVRDSNYQLIWF
13	TRAV1-2	TRAJ12	CAVMDSYKLIIF
14	TRAV1-2	TRAJ12	CAVMDSYKLIIF
15	TRAV1-2	TRAJ33	CAVMDSNYQLIWF
16	TRAV1-2	TRAJ33	CAAMDSNYQLIWF
17	TRAV1-2	TRAJ33	CAVMDSNYQLIWF
18	TRAV1-2	TRAJ33	CAASDSNYQLIWF
19	TRAV1-2	TRAJ12	CAVMDSYKLIIF
20	TRAV1-2	TRAJ33	CAVMDSNYQLIWF
21	TRAV1-2	TRAJ12	CAVKDSYKLIIF
22	TRAV1-2	TRAJ12	CAVRDSYKLIIF
23	TRAV1-2	TRAJ33	CAVRDSNYQLIWF
24	TRAV1-2	TRAJ33	CAVRDSNYQLIWF
25	TRAV10	TRAJ34	CVVWGGDTDKLIIF
26	TRAV22	TRAJ27	CAVGEANADKLTF
27	TRAV22	TRAJ27	CAVGEANADKLTF
28	TRAV24	TRAJ37	CAFSSNTGKLIIF
29	TRAV29	TRAJ41	CAARNSGYALNF
30	TRAV29	TRAJ41	CAARNSGYALNF
31	TRAV36	TRAJ48	CAVESNFGNEKLTF
32	TRAV36	TRAJ48	CAVESNFGNEKLTF
33	TRAV4	TRAJ28	CLVGEGGYSGAGSYQLTF
34	TRAV9-2	TRAJ9	CARNTGGFKTVF
35	TRAV9-2	TRAJ9	CARNTGGFKTVF

Supplementary Figure 4.1. CD8⁺ CD161^{hi} IL-18R α ⁺ T cells in the blood of rhesus macaques predominantly express the canonical MAIT cell TRAV and TRAJ genes.

The V-gene and J-gene usage from 35 readable sequences identified from approximately 3,000 sorted CD8⁺CD161^{hi}IL-18R α ⁺ T cells.



Supplementary Figure 4.2. Maintenance of IL-17 and IL-22-expressing CD3⁺CD8⁺ T cells in the blood and jejunum during acute SIV infection.

(A) IL-17 and (B) IL-22 expression by CD3⁺CD8⁺ T cells in the blood pre- and post-SIV infection. (C) IL-17 and (D) IL-22 expression by CD3⁺CD8⁺ T cells in the jejunum pre- and post-SIV infection. Vertical dashed line denotes day of infection. P values were calculated using a paired two-tailed student's t test.

Chapter 5:

Activation and regulation of MAIT cells within inflamed mucosal tissue

Introduction

Maintenance of mucosal barrier function during infection followed by a return to homeostasis is required to limit pathogenesis and prevent the occurrence of new infections through mucosal sites⁸⁷⁻⁹⁰. Recently, semi-invariant T cells, including the MAIT cell subset, have been shown to play an important role in barrier immunity^{63,81}. The MAIT cell TCR recognizes metabolites derived from both commensal and pathogenic bacteria when presented by MR1⁴⁴. Given that the MAIT ligand could be derived from either commensal or pathogenic bacteria, stringent requirements for activation must exist to avoid MAIT cell responses against healthy tissue. We have recently addressed how MAIT cells at the site of the mucosa are able to avoid unwanted responses against commensal antigen while mounting an effector response against pathogenic antigen. A high TCR activation threshold of MAIT cells is overcome by inflammatory cytokines that are required for activation and sustained effector functions¹⁶⁴.

In the periphery MAIT cells express various chemokine receptors, including CCR6, which may allow them to gain access to inflamed mucosal sites⁴¹. Once MAIT cells enter inflamed tissue sites it is unclear how the MAIT cell response is regulated, or the mechanism by which their response is shut off. Inhibition of conventional T cells occurs through cell-intrinsic and extrinsic mechanisms. Upon T cell activation, inhibitory receptors such as PD-1, Fas, and CTLA-4 are expressed and provide cell-intrinsic

signals to disrupt activation pathways (PD-1 and CTLA-4)⁹¹ or initiate apoptosis (Fas)⁹² as a means to limit overt immune responses and prevent aberrant tissue damage by activated cells^{93-96,182,183}. Additionally, cell-extrinsic mechanisms, such as suppression by regulatory T cells (Tregs), function to limit conventional T cell responses^{184,185}. Tregs suppress immune responses through secretion of inhibitory cytokines such as IL-10, sequestration of IL-2, engagement of B7 ligands through CTLA-4 on antigen presenting cells, or cytolysis of immune cells⁹⁷⁻¹⁰¹.

MAIT cells in the blood constitutively express the Fas receptor, suggesting that they are susceptible to Fas-mediated apoptosis⁴¹. In healthy tissues MAIT cells express PD-1, and during chronic inflammatory diseases such HIV, HCV, and tuberculosis, an increase in PD-1 expression by MAIT cells in the blood has been reported^{52,58,102-104}. During chronic HCV infection, MAIT cells in the blood display increased expression of inhibitory ligands including PD-1, Tim3, and CLTA-4¹⁰⁵. It remains unclear if expression of these inhibitory ligands confers MAIT cell suppression, or if Tregs have the potential to suppress MAIT cell responses within mucosal tissues.

We reasoned that once the activation threshold of MAIT cells had been met, there must be cell intrinsic or extrinsic mechanisms to regulate their potent effector functions and limit tissue destruction. Here, we demonstrate that MAIT cells in the blood migrate in a CCR6-dependent manner, which may allow MAIT cells to infiltrate inflamed mucosal sites, become activated, and exert effector functions. To better understand MAIT cell functions and regulation within the inflammatory environment, we probed MAIT cell functional and transcriptional properties within inflamed mucosal tissue. We observe that MAIT cells within inflamed tissues display direct *ex vivo* function,

specifically expression of TNF α and granzyme B. In addition, MAIT cells within inflamed tissue remain responsive to TCR signaling, suggesting that provided the correct activating signals MAIT cells are able to exert normal effector responses. Using next-generation sequencing methods we demonstrate that MAIT cells express complex gene networks important for cellular activation, migration, and suppression. This includes high gene expression of the inhibitory receptor, CTLA-4. Moreover, we show that inflammatory cytokines are sufficient for induction of CTLA-4 expression on MAIT cells, but a TCR signal is not. We propose that due to the potent effector functions of MAIT cells, intrinsic mechanisms including expression of CTLA-4 exist to prevent aberrant immune responses and facilitate a return to tissue homeostasis. Our findings have important implications for understanding regulation of MAIT cell functions within inflamed mucosal tissues sites during chronic inflammatory diseases that disrupt mucosal barrier integrity including HIV infection and inflammatory bowel diseases.

Results

CCR6 mediates MAIT cell migration

MAIT cells are easily identified in the blood and throughout various mucosal tissues. In the circulation MAIT cells express mucosal-homing chemokine receptors at steady state. Trafficking to the mucosa may explain the previously reported loss of MAIT cells from the periphery in various disease states including chronic HIV infection⁵⁹⁻⁶³. In particular, MAIT cells express high levels of CCR6⁴¹, an important chemokine receptor that binds to CCL20, and results in trafficking of lymphocytes to mucosal tissues (Figure 5.1A).

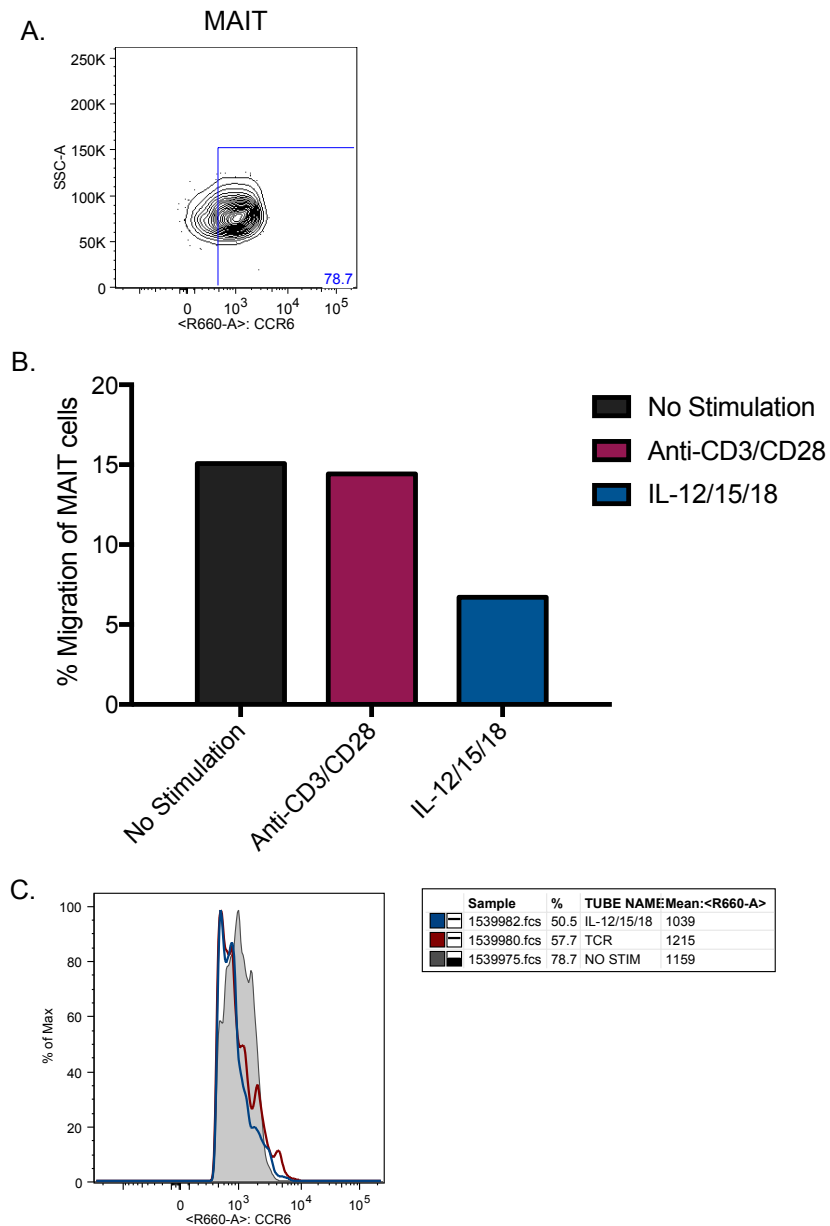


Figure 5.1. CCR6-mediated MAIT cell migration.

(A) Representative flow plot from the blood of one donor showing CCR6 expression by MAIT cells defined as live CD3⁺CD8⁺CD161^{hi}V α 7.2⁺ cells. (B) The frequency of unstimulated, anti-CD3/CD28 or IL-12/15/18 stimulated MAIT cells that migrated in response to CCL20 as compared to the input number of cells for each condition. (C) Representative histogram of unstimulated or activated MAIT cells expressing CCR6.

Damaged or infected epithelial cells recruit T cells and dendritic cells that express CCR6 by secreting CCL20¹⁸⁶. It has previously been shown that CCL20 is sufficient for migration of CCR6-expressing memory T cells, but not CCR6-expressing B cells, in the absence of activation¹⁸⁷. Due to the differences in migration of CCR6-expressing T and B cells, we questioned whether CCR6-expressing MAIT cells would migrate in response to CCL20. To investigate the migratory capacity of MAIT cells, MAIT cells were isolated from the blood of healthy human donors and activated with a combination of inflammatory cytokines (IL-12/15/18) or a TCR signal (anti-CD3/CD28). *In vitro* transwell assays of activated MAIT cells revealed that upon TCR stimulation or without stimulation MAIT cells migrated in response to CCL20. However, the number of cytokine-activated MAIT cells that migrated in comparison to unstimulated or TCR-stimulated MAIT cells was decreased (Figure 5.1B). To determine what was driving the observed differences in MAIT cell migration, we analyzed CCR6 expression on unstimulated, cytokine-, or TCR-activated MAIT cells and found that upon activation with cytokines CCR6 is downregulated (Figure 5.1C). This is true for a subset of TCR-activated MAIT cells as well, albeit to a lesser extent.

These data suggest that MAIT cells in the circulation do not require activation in order to migrate and have the potential to enter healthy and inflamed mucosal tissues provided the correct chemokine signals.

Functional characterization of MAIT cells in healthy and inflamed mucosal tissues

We recently reported that MAIT cells require inflammatory cytokines for sustained effector function, and that the combination of both a TCR signal + inflammatory cytokine

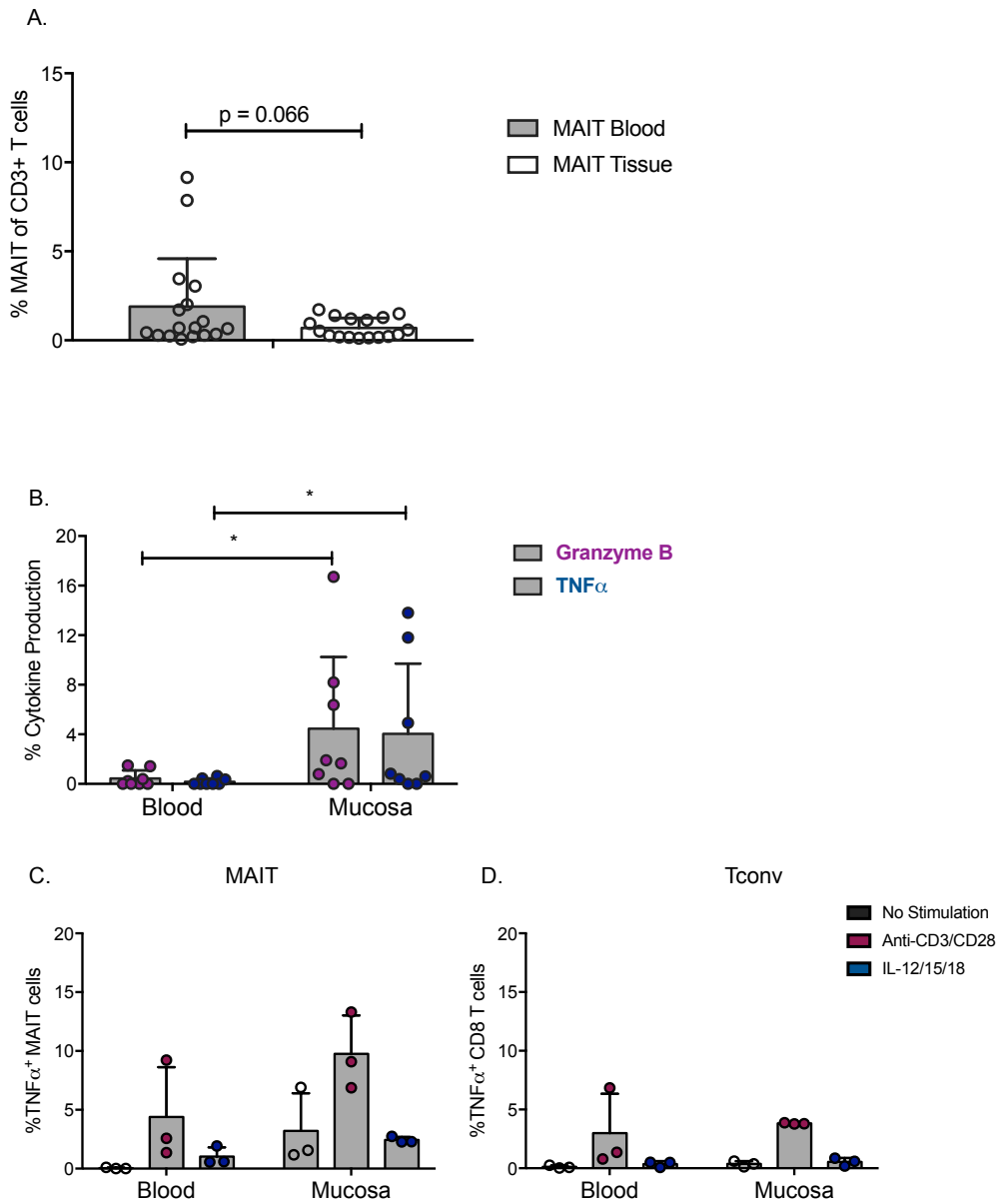


Figure 5.2. Function and activation of MAIT cells within inflamed mucosal tissue.

(A) The frequency of CD3⁺CD8⁺CD161^{hi}V α 7.2⁺ MAIT cells within the CD3⁺ T cell subset in blood and inflamed gingival tissue (n=17). (B) Frequency of MAIT cells expressing granzyme B or TNF α in the blood or inflamed mucosal tissue directly ex vivo (n=8). (C) TNF α expression by CD3⁺CD8⁺CD161^{hi}V α 7.2⁺ MAIT cells in the blood and mucosa with no stimulation, anti-CD3/CD28 or IL-12/15/18 stimulation. (D) TNF α expression by CD3⁺CD8⁺ T cells in the blood and mucosa with no stimulation, anti-CD3/CD28 or IL-12/15/18 stimulation.

signal synergize to induce TNF α expression in MAIT cells¹⁶⁴. Additionally, we have reported that inflammatory cytokines in the absence of a TCR signal are sufficient for MAIT cell expression of granzyme B. To better understand MAIT cell activation and contributions to immune responses within inflamed mucosal tissue, we obtained inflamed gingival tissue and matched blood from donors undergoing periodontic surgery for various inflammation-related diseases. Donors enrolled in this study were not on any current antibiotics or long-term immunosuppressive drugs (Table 5.1), thus allowing us to obtain mucosal tissues in the absence of immune-modulatory therapies. The frequency of MAIT cells in the blood and mucosal tissues from these donors is in line with what has been previously reported for other mucosal tissues (Figure 5.2A)^{41,63}.

Despite high expression of pro-inflammatory gene transcript, MAIT cells within healthy mucosal tissue do not express inflammatory proteins¹⁶⁴. To determine the activation status of MAIT cells within inflamed tissues, we interrogated MAIT cell activation directly *ex vivo* from inflamed gingival mucosa. MAIT cells within inflamed tissue significantly expressed more granzyme B and TNF α compared to those in the blood (Figure 5.2B). These data suggest that MAIT cells within inflamed mucosal tissues are responsive to inflammatory signals generated *in vivo*.

Previously published data work demonstrated that during certain chronic infections, MAIT cells are less responsive to activation by *E.coli*-fed APCs but not to exogenous cytokine stimulation^{60,188}. To determine if MAIT cells in inflamed tissues retain functional responses to exogenous stimuli, we stimulated mucosal-mononuclear cells (MMC) isolated from inflamed oral mucosa or blood matched from the same donors. MMC were stimulated for 6 hours with a combination of inflammatory cytokines

(IL-12/15/18) or a TCR signal (anti-CD3/CD28). We analyzed both the MAIT cell and total CD8 T cell subset by intracellular cytokine staining. Similar to our previously published results, MAIT cells in the blood expressed TNF α after stimulation with anti-CD3/CD28 but not recombinant IL-12/15/18 (Figure 5.2C). Furthermore, MAIT cells within inflamed mucosal tissue remained responsive to TCR signaling, but displayed a limited response to inflammatory cytokine signals. Total CD8 T cells in the blood and inflamed tissue followed a similar pattern (Figure 5.2D).

Together, these data reveal that a TCR signal is sufficient for short-term mucosal MAIT cell stimulation but that inflammatory cytokine signaling is not. As there were not significant activation differences in mucosal MAIT cells compared to blood, but we did observe significant differences in direct *ex vivo* function, we sought to further define the activation status of MAIT cells within inflamed mucosal tissues.

Gene expression networks of MAIT cells isolated from inflamed mucosal tissue

We next evaluated gene expression profiles of CD8⁺ MAIT cells isolated from the peripheral blood and inflamed mucosal tissue of four donors. MAIT cell populations within inflamed mucosal tissue displayed a distinct gene expression signature compared to MAIT cells in the blood (Figure 5.3A). Additionally, a variety of chemotaxis and activating genes including CCL3, CCL4, CRTAM, JUN, FOS, and CD69 were upregulated in mucosal MAIT cells (Figure 5.3B). Interestingly, we observed a highly significant upregulation of inhibitory genes including CTLA-4. Network analysis

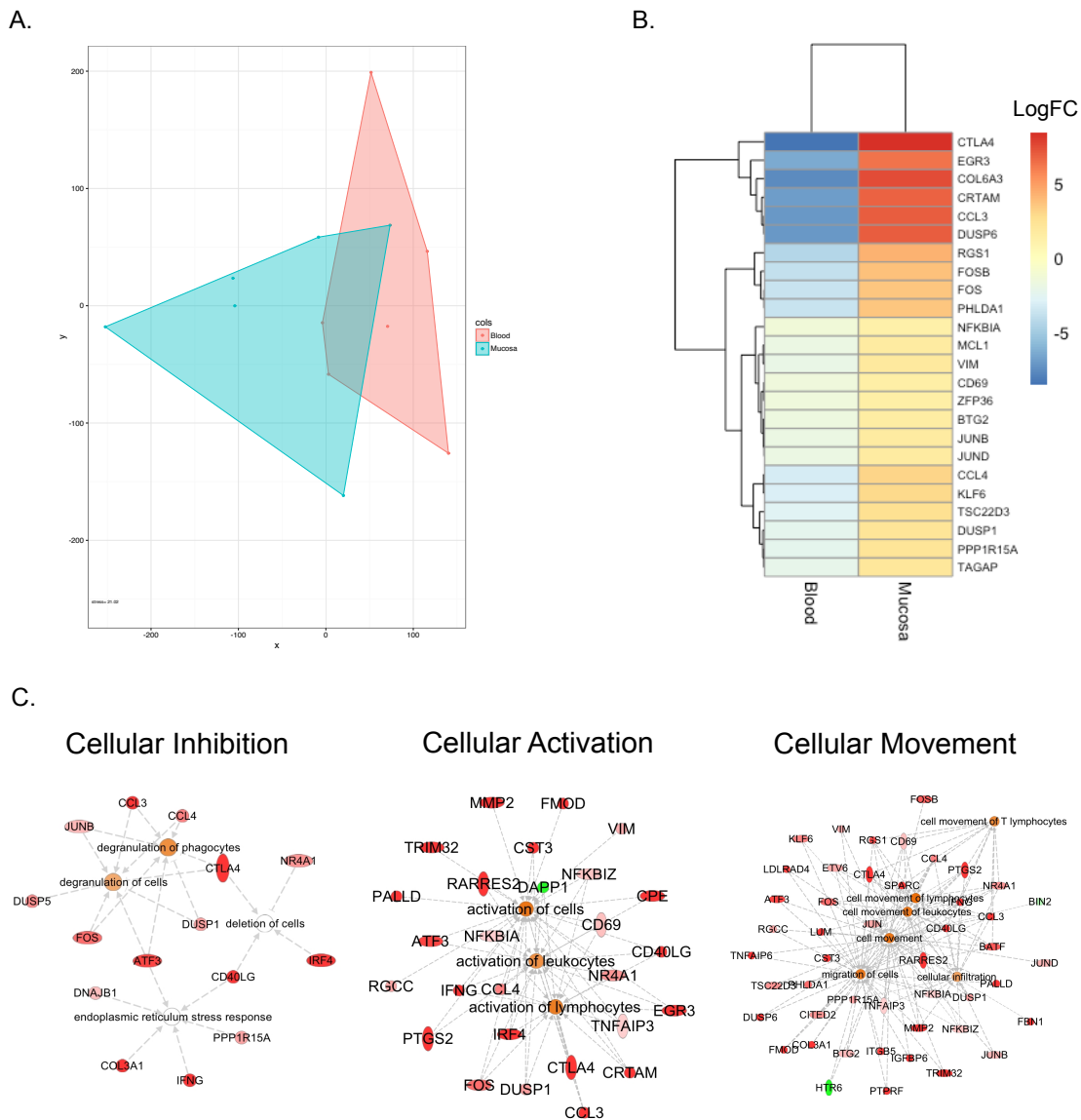


Figure 5.3. Gene expression analysis of MAIT cells in the blood and inflamed mucosal tissue.

(A) Multi-dimensional scaling plot of MAIT cells isolated from the blood (red) or inflamed gingival mucosa (blue). (B) The top genes differentially expressed at $p \leq 0.01$ between MAIT cells isolated from the blood compared to MAIT cells isolated from inflamed mucosal tissue. (C) Network analysis of differentially expressed genes at $p \leq 0.05$ between MAIT cells isolated from the blood and inflamed mucosal tissue. Red indicates upregulated genes, while green denotes downregulated genes. Arrows represent known gene interactions, based off of Ingenuity pathway analysis.

confirmed up-regulation of pathways associated with cellular inhibition, activation, and migration (Figure 5.3C). Together, these data suggest that once MAIT cells enter inflamed tissue they may function to secrete chemokines to aid in immunosurveillance, as well as possess cell-intrinsic mechanisms to avoid overt effector responses within inflamed mucosal sites.

Intrinsic regulation of MAIT cell responses by CTLA-4

To date, there have been few studies regarding MAIT cell inhibitory receptor expression, and it remains unclear as to how the MAIT cell response is turned off following activation. Due to the high expression of CTLA-4 transcript we detected in mucosal MAIT cells, in conjunction with previous reports of inhibitory receptor expression, including CTLA-4, by MAIT cells in the circulation during chronic infections^{58,105,188}, we considered the possibility that MAIT cell expression of inhibitory receptors within inflamed tissues function to modulate the MAIT cell response. Upon TCR activation, conventional T cells upregulate inhibitory receptors including CTLA-4⁹³. However, the activating signals required for MAIT cell expression of CTLA-4 have yet to be investigated. Hence, we first sought to determine the signals required for CTLA-4 expression on MAIT cells by stimulating sorted MAIT cells with inflammatory cytokines (IL-12/15/18) or a TCR signal (anti-CD3/CD28) for 24 hours. Surprisingly, MAIT cells stimulated with inflammatory cytokines upregulated CTLA-4 (Figure 5.4A). This was in stark contrast to MAIT cells stimulated through TCR signaling, where we did not observe any increase in CTLA-4 expression compared to unstimulated MAIT cells. These data demonstrate that unlike conventional T cells, which require TCR signaling for expression of CTLA-4¹⁸⁹⁻¹⁹³, inflammatory cytokine stimulation alone is sufficient for

MAIT cell expression of CTLA-4. The effects of a combined inflammatory and TCR signal on MAIT cell expression of CTLA-4 is currently under investigation. Thus, expression of CTLA-4 may be a possible mechanism for MAIT cell suppression within inflamed tissues and avoidance of tissue damage or unwanted effector responses.

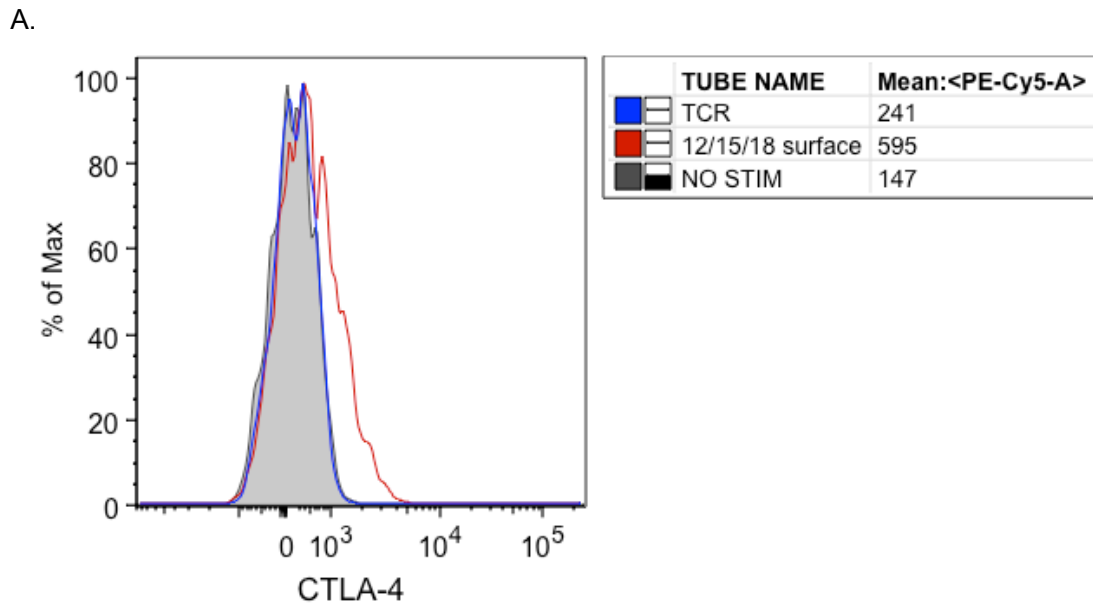


Figure 5.4. CTLA-4 expression of stimulated MAIT cells.

(A) Surface expression of CTLA-4 by sorted MAIT cells ($CD8^+CD161^{hi}V\alpha 7.2^+$) left at rest (no stim) or stimulated with anti-CD3CD28 (TCR) or cytokines (IL-12/15/18) for 24 hours.

Patient ID	Procedure	Tissue	Size (mm ³)	Disease Status	Probing Depth	Sex	Age	Ethnicity	Race	BMI	HIV+	HCV+	Diabetes	Antibiotics
OM 19	Osseous	sulcular keratinized gingiva	20x2x2	Severe Periodontitis	7mm	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
OM 20	Distal Wedge	sulcular keratinized gingiva	7x10x5	Severe Periodontitis	8mm	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
OM 21	Osseous	sulcular keratinized gingiva	20x5x2	Severe Periodontitis	4-7mm	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
OM 22	Distal Wedge	Distal Wedge	4x10x4	Severe Periodontitis	6mm	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
OM 27	Osseous	retro molar pad + sulcular	4x7x2	Severe Periodontitis	6mm	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
OM 28	Osseous	palatal sulcular	5x5x10	Severe Periodontitis	10mm	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
OM 29	Peri-implant	granulomatous tissue	5x5x7	Peri-implantitis	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A
OM 32	Osseous	Distal Wedge	5x5x10	Severe Periodontitis	6mm	F	40	Not Hispanic	Asian	28.5	no	no	no	no
OM 33	Osseous	granulomatous tissue	5x5x3	Severe Periodontitis	5mm	M	57	Not Hispanic	White	25.8	no	no	no	no
OM 39	Distal Wedge	Distal Wedge	6x3x2	Severe Periodontitis	7mm	F	36	Not Hispanic	White	18.6	no	no	No	no
OM 44	Osseous	sulcular keratinized gingiva	5x5x5	Severe Periodontitis	7mm	M	59	Not Hispanic	Black or African	N/A	N/A	N/A	N/A	N/A
OM 48	Osseous	sulcular keratinized gingiva	10x5x5	Severe Periodontitis	6mm	M	61	Unknown	White	28.1	no	no	No	no
OM 49	Osseous	sulcular keratinized gingiva	5x5x5	Moderate Periodontitis	4mm	M	65	Asian	Asian	22.4	no	no	no	no
OM 51	Distal Wedge	sulcular keratinized gingiva	10x7x8	Severe Periodontitis	6mm	M	65	N/A	White	24.4	no	no	no	no
OM 52	Osseous	sulcular keratinized gingiva	5x5x5	Severe Periodontitis	6mm	M	59	Not Hispanic	White	31.6	no	no	no	no
PM 53	Osseous	sulcular keratinized gingiva	8x4x4	Severe Periodontitis	9mm	F	73	Not Hispanic	White	29.7	no	no	no	no
OM 54	Osseous	sulcular keratinized gingiva	8x8x8	Severe Periodontitis	7mm	F	54	Asian	Asian	26.2	no	no	no	no

Table 5.1. Overview of blood and inflamed tissue donors.

Clinical and self-reported data from 17 donors enrolled in the study of MAIT cell activation and function in inflamed mucosal tissues. N/A refers to no data collection regarding the participant or an incomplete self-reporting form.

Discussion

MAIT cells are abundant in the blood, liver, and throughout mucosal tissues and display potent effector functions upon activation^{41,46,164}. Much of the work on MAIT cell contributions to immunity has been done using peripheral blood from healthy human donors. Recently, it was demonstrated that MAIT cells in healthy mucosal tissue express high levels of inflammatory transcripts, and may be biased towards IL-17/IL-22 production in the female reproductive tract^{52,164}. In this study, we sought to define the MAIT cell response within mucosal tissues during an ongoing inflammatory response to better understand MAIT cell contributions to immunity.

To address these questions, we first wanted to clearly define 1) the signals that are sufficient for MAIT cell migration 2) MAIT cell activation and effector responses within inflamed tissue sites and 3) the mechanisms in place to prevent pathogenesis. Here, we demonstrate that resting or TCR-activated MAIT cells are able to migrate in response to CCL20 due to high expression of CCR6 (Figure 5.1). The CCL20-mediated migration we observe is similar to what has been previously published for CCR6-expressing memory CD8 T cells¹⁸⁷. In addition, MAIT cells within inflamed mucosal tissue display effector responses when analyzed directly *ex vivo* (Figure 5.2). This is in contrast to our previous work on MAIT cells isolated from healthy tissue, which express pro-inflammatory gene transcripts in the absence of effector molecules. Hence, we hypothesize that MAIT cells are poised effectors located at barrier sites, and when provided the correct signals act as potent effectors to eliminate pathogen. The direct *ex vivo* function (Figure 5.2) observed in MAIT cells within inflamed mucosal tissue could be induced by either inflammatory signals alone, similar to memory T cell subsets that

respond to inflammation in a bystander fashion^{167,26-28}, or by the combination of an inflammatory + TCR signal. The nature of the activating ligand (commensal or pathogenic) in this study is unknown, and it will be of further interest to determine if commensal derived antigen could activate MAIT cells within an inflammatory environment.

We have previously shown that a TCR signal alone is not sufficient for prolonged effector functions, but rather elicits secretion of the chemokines CCL3 and CCL4. Within inflamed mucosal tissues MAIT cells express high levels of CCL3 and CCL4 transcript, suggesting that the cells are receiving a TCR signal *in vivo* (Figure 5.3). Secretion of chemokines could function to recruit effector cells, including MAIT cells, from the periphery as well as Tregs to the site of inflammation^{194,195}. It is plausible that Treg recruitment could function as another regulatory mechanism by which MAIT cell responses are controlled, although further work will be needed to explore this possibility.

Despite reports of MAIT cell reduced functionality and expression of exhaustion markers such as PD-1 in healthy tissue and during chronic infection^{58,102,104,105,188}, we observed that MAIT cells within inflamed tissue maintained responsiveness to TCR signaling (Figure 5.2). This is in line with recent reports highlighting PD-1 and other inhibitory receptors on human T cells as markers of activation or differentiation rather than exhaustion¹⁹⁶. Thus, our data demonstrate that MAIT cells within inflamed mucosal tissues are activated and retain the potential to respond to activating signals. Whether MAIT cells within inflamed mucosal tissues are more or less responsive to exogenous signals compared to those found within healthy tissues remains unclear.

Given that MAIT cells recognize antigen derived from both commensal and pathogenic bacteria, we have hypothesized that the MAIT cell activation threshold must be high in order to avoid unwanted responses against commensals that would result in tissue damage and destruction of barrier sites. How the MAIT response is fine-tuned to avoid these unwanted responses during an ongoing inflammatory response is currently unknown. Gene expression analysis revealed high expression of CTLA-4 transcript in MAIT cells isolated from inflamed mucosal tissues compared to their counterparts in the blood (Figure 5.3). We propose that in addition to strict signaling requirements of MAIT cells¹⁶⁴, a high activation threshold is maintained during the inflammatory response by cell-intrinsic mechanisms such as expression of CTLA-4. Importantly, we find that a TCR signal alone is not sufficient for expression of CTLA-4, but rather inflammatory cytokines induce expression of CTLA-4 (Figure 5.4). This is a fundamental difference between MAIT cells and conventional T cells, and demonstrates that regulation of the MAIT cell response is distinct from other T cell subsets^{93,189,192,197,198}. Investigations into synergy of TCR + cytokine signals on MAIT cell expression of CTLA-4 are currently underway. Future studies will need to examine if CTLA-4 expression alone is sufficient for suppression of MAIT cell responses or if other inhibitory signals are required.

In summary, our data provide, to our knowledge, a novel mechanism by which MAIT cell responses are regulated within inflammatory mucosal tissues. Since MAIT cells located in inflamed tissue sites display potent effector function directly *ex vivo*, we propose that once MAIT cells have been activated (via inflammation in the presence or absence of a TCR signal), tight regulation of MAIT responses must occur to maintain barrier integrity while preventing gross tissue damage (Figure 5.5). We postulate that

regulation of inhibitory proteins through inflammatory cytokine stimulation avoids expression of these receptors through commensal-antigen presentation alone, which may occur within healthy human tissues. A fundamental understanding of MAIT cell interactions with the microbiome in healthy mucosal tissues will provide further information on the basic biology and subsequent activation of MAIT cells during acute and chronic inflammatory diseases. Given the increase in immune-modulatory molecules in the clinic to boost responses against tumors it will be of great interest to study the effects of checkpoint blockade on MAIT cell responses in the tumor environment^{82,83,199}.

Importantly, our study investigates MAIT cell activation and suppression in an inflammatory context, and provides potential targets for improving responses not only to chronic diseases but also tumor-specific responses.

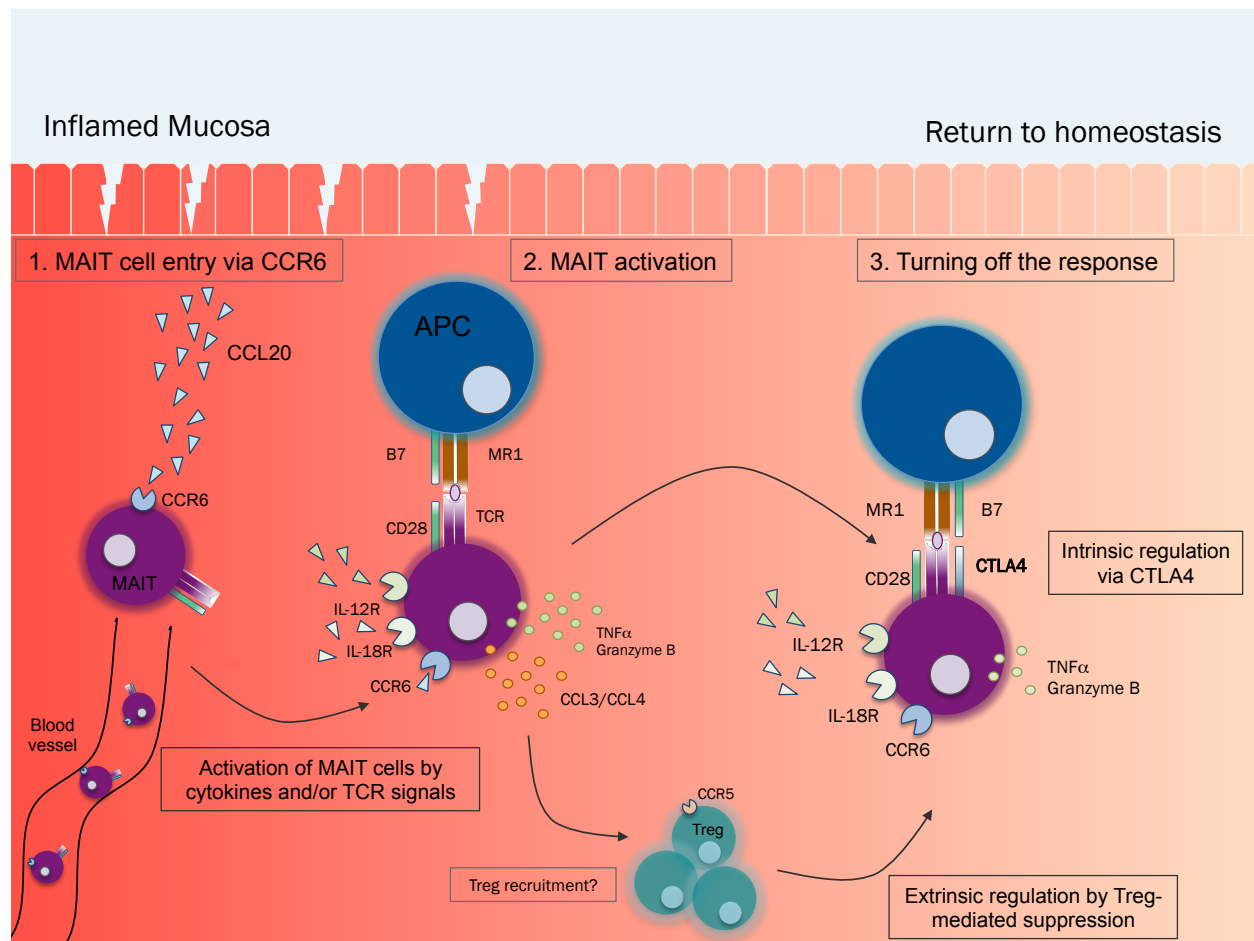


Figure 5.5. Model of MAIT cell activation and suppression at inflamed mucosal sites.

1. Upon damage or infection to the mucosa, chemokines including CCL20 are released by epithelial cells. Through expression of CCR6, MAIT cells migrate from the blood to the inflamed tissue site in response to a chemokine gradient. **2.** Once MAIT cells have reached the tissue, they become activated by the inflammatory environment in the presence or absence of a TCR signal. Activation of MAIT cells results in secretion of effector molecules such as TNF α and granzyme B, as well as secretion of chemokines including CCL3 and CCL4, which may function to recruit regulatory T cells (Tregs) through CCR5 receptor expression. **3.** Once activated by inflammatory cytokine signals, MAIT cells upregulate CTLA-4, thereby conferring suppression of the MAIT cell response. A combination of CTLA-4 expression and Treg recruitment facilitates a shutting down of the MAIT cell response, followed by a return to tissue homeostasis and maintenance of barrier function.

Chapter 6:

Concluding Remarks and Future Directions

Here, we have used a number of experimental approaches to identify the activation requirements and effector functions of MAIT cells in relation to human health and disease. Despite the conflicting roles for TCR and cytokine activation in regulating MAIT cell activity in the literature, we demonstrate that inflammatory cytokines are necessary and sufficient for MAIT cell functions. This activation is apparent during acute and chronic inflammation as demonstrated by MAIT cell activation during acute SIV infection and within chronically inflamed oral mucosal tissue.

As MAIT cell function has been implicated in the progression of cancer, autoimmunity, and infectious disease, a basic understanding as to how MAIT cell homeostasis is regulated in these immune contexts provides the potential for therapeutic intervention in these disease states. Importantly, the homing capacity of MAIT cells has yet to be explored, and could be an incredibly important tool for cancer immunotherapy. Considering how potent MAIT cell effector responses are in the periphery and at the mucosa, it is imperative to understand how their function is regulated and turned off once the immune response has resolved infection. Our results suggest that once MAIT cells receive the correct activating signals in the tissue, they function to secrete inflammatory mediators while also upregulating inhibitory ligands such as CTLA-4. This cell-intrinsic mechanism only occurs when MAIT cells receive cytokine but not TCR signals, and appears to be an important regulatory element of MAIT cell responses. We hypothesize that regulation of inhibitory ligands by inflammation rather than a TCR signal allows MAIT cells to recognize commensal-

derived antigen in the absence of an ongoing inflammatory response and function to secrete chemokines that may potentially aid in immunosurveillance of the tissues. Thus, our work demonstrating the activation and subsequent suppression of MAIT cell responses in mucosal tissues has important implications for understanding the development and progression of a breadth of infections and cancer-related diseases. Further work will be required to determine additional mechanisms of MAIT cell activation, including other inflammatory cytokines such as IL-1 β , as well as inhibition of the MAIT response through other cell-intrinsic or extrinsic pathways.

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