Activation and regulation of MAIT cells within mucosal tissues

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A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2019

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Program Authorized to Offer Degree:

Molecular and Cellular Biology

University of Washington

Abstract

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Mucosal-associated invariant T (MAIT) cells are a class of unconventional T cells that are defined by a limited T cell receptor (TCR) repertoire that recognize microbial riboflavin-derivative antigens presented by the major histocompatibility complex (MHC) class I-like protein, MR1. Importantly, commensal species of bacteria and yeast in a healthy microbiome generate these metabolite MAIT antigens. Thus, MAIT cells are likely mediators of commensal-specific interactions due to their broad antigen recognition, tissue location and abundance. However, since *in vivo* characterization of MAIT cells has been minimal, their role in human mucosal tissues is still poorly understood. For example, it is not clear whether functional MR1 is expressed in healthy mucosal tissues and if so, by what classes of immune cells. In this thesis, I use flow cytometric analysis to characterize MAIT cells and assess functional MR1 expression within colonic biopsies and peripheral blood samples from healthy donors. I show extensive MR1 expression among multiple colonic-resident immune cells including antigen-presenting cells and T cells. MAIT cells presented an activated phenotype within the colon with heightened expression of activation markers among the tissue-resident immune cell population. These data suggest that MAIT cells may be continually responding to the microbiota in barrier sites.

In addition to their role in normal tissue homeostasis, MAIT cells acquire potent effector function in response to proinflammatory signals, which synergize with a TCR signal. This raises the question how MAIT cells are regulated within inflamed tissues where they presumably would be receiving both of these signals. To address this, I examined the transcriptome of MAIT cells from blood and oral mucosal tissues and found that tissue MAIT cells express an immunoregulatory gene signature, which includes expression of the inhibitory receptor CTLA-4. Further, I define the requirements for surface CTLA-4 protein expression on MAIT cells and demonstrate that inflammatory cytokines are sufficient to elicit and maintain CTLA-4 protein expression on the MAIT cell surface in the absence of a TCR signal. Therefore, control of CTLA-4 expression is fundamentally different from conventional T cells, which require a TCR signal. This mechanism may serve to limit cell-mediated tissue damage in response to commensal antigen within inflamed tissues. Together, this work contributes valuable *ex vivo* and mechanistic insight into human MAIT cell immunoregulation within healthy and inflamed mucosal tissues.

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Acknowledgments

I would like to express my sincere gratitude to the entire Prlic lab for their support throughout my training as a PhD scientist. Thank you to my adviser, Martin Prlic, for all the opportunities and training he provided. I am grateful for the amazing postdocs in the lab: Amanda Woodward-Davis, Florian Mair, Jami Erickson, and Marie Frutoso. A special thank you to Amanda for her exceptional mentorship, patience, and kindness. I was lucky to share this experience with fellow graduate students Veronica Davé, Bri Traxinger, and Nick Maurice. To Veronica, I will always remember your insight during times of need and groovy dance moves when we needed to let loose. Lastly, a huge shout out to our lab manager Paula Culver.

A tremendous thank you to my committee members: Michael Emerman, Jess Hamerman, Rich Darveau, Marc Gavin, and Nikki Klatt. They were incredibly supportive throughout my PhD and met with me individually several times. I am especially grateful that Michael took me under his wing with the care and support as if I were his own student.

Thank you to my MBC squad and lifelong friends: Mollie McDonnell, Anja Ollodart, Emma Wrenn, and Dylan Udy. They were my rocks throughout this entire experience and we always looked out for one another. Thank you for all the laughs, pranks, bets, and goofy times.

Lastly, I'd like to thank my wonderful family members. My partner in life, boyfriend, and best friend, Jacob Isenberg was there for me every single day. I appreciate my wonderful siblings whom I've always looked up to, Becca and Rafi Berkson. Most importantly, thank you to my first mentors, my wonderful parents to whom I would not have not done this without.

Chapter I:
The MAIT Conundrum – How human MAIT cells distinguish between bacterial colonization from infection in mucosal barrier tissues

Introduction

Studies focusing on human mucosal-associated invariant T (MAIT) cells have gained considerable momentum in recent years. MAIT cells are found at mucosal barrier sites and their ability to quickly exert effector function indicates a potentially significant role for them in barrier immunity. Changes in MAIT cell frequency and phenotype have been reported in numerous disease settings including acute and chronic infections as well as autoimmune and malignant disorders. Recent reviews provide a comprehensive overview of these clinical studies as well as key aspects of MAIT cell function including antigen recognition, antimicrobial properties and their putative role in the liver(1-11). Here I provide an overview of the recent human MAIT cell literature to address how a cell that recognizes bacterially derived antigen and is found in mucosal barrier tissues colonized by commensal bacteria, avoids responding to these commensal-derived antigens while maintaining responsiveness to bacterial infections. I discuss the key role of inflammatory signals in regulating human MAIT cell effector function in this context and review the relationship and the functional properties of human MAIT cells in blood and mucosal tissues. In addition, I highlight gaps in our current knowledge and examine the emerging role of human MAIT cells in controlling barrier immunity tissue homeostasis.

Brief Background

MAIT cells are a fairly abundant T-cell subset in humans representing 1-10% of total T-cells in blood and mucosal tissues, and even higher abundance in the liver(12, 13). MAIT cells are characterized by a semi-invariant T cell receptor (TCR) that consists of a conserved TCRα chain(14, 15) - TRAV1-2(Vα7.2) and TRAJ33 (Jα33) - paired with a few select Vβ chains(16). This semi-invariant TCR recognizes antigen in the context of the protein MHC class I - related (MR1)(17). MR1 is well conserved across species suggesting an essential role for MAIT cells in the immune response(17-20). Instead of peptides (presented by MHC I and II) or glycolipids/lipids (presented by CD1), MAIT cells recognize metabolites and thus a different type of antigen compared to any other T cell subset(21). These metabolites are derived from vitamin B synthesis pathways and additional modifications may occur when presented in the context of MR1 and some, but not all, of these MR1 binding metabolites can activate MAIT cells(21-26). Identification of these antigens allowed the development of MR1 tetramers(22, 23), which became available to the broader scientific community through the NIH tetramer core in late 2016. In most studies published to date, MAIT cells were identified via flow cytometry by co-expression of their invariant TCR α-chain, Vα7.2, along with high expression levels of the C-type lectin CD161 (other names include KLRB1, NKR-P1A). MAIT cells in the blood are also identified by Vα7.2 expression together with expression of the cytokine receptor IL-18Rα(27, 28). Because conventional T cells can also express Vα7.2 and recent reports suggest that CD161 expression changes in certain scenarios(29, 30), using MR1 tetramers is likely the most reliable method to identify MAIT cells. However, it is important to consider that staining with anti-Vα7.2, anti-CD161 antibodies versus the MR1 tetramer shows that these populations are nearly, but not fully congruent in the blood(22, 23). This indicates that some $V\alpha7.2^{+}CD161^{hi}$ cells are

either not MR1-restricted or do not recognize the specific metabolite used to load MR1, which is plausible given evidence for differential antigen recognition by MAIT cells(31, 32). Importantly, recent studies also provide evidence for the existence of Vα7.2^{-} MR1-restricted cells(33, 34). The majority of the currently published literature and literature references in this chapter are based on the classic definition of MAIT cells (Vα7.2^{+}CD161^{hi}), but it will be necessary to keep these details in mind as the field moves forward and takes advantage of MR1 tetramers.

MAIT cell subsets, phenotypes and function in blood

MAIT cells in the blood are typically CD45RO^{+}, CD62L^{lo}, CD122^{int}, CCR7^{-} and quickly acquire effector function when stimulated with PMA and ionomycin(12). The lack of CD62L and CCR7 expression paired with the ability to quickly respond to stimulation is a hallmark of conventional effector memory T- cells(35) and MAIT cells have thus been referred to as effector memory-like(12). The reason MAIT cells have these characteristics is presumably due to near uniform expression of the transcription factor PLZF(36). Expression of PLZF is sufficient to induce acquisition of effector memory-like properties in conventional T-cells(37, 38) and is required for the innate-like effector function of NKT cells(39, 40) and certain gamma delta T-cells(41). Once activated, MAIT cells isolated from the blood (or stimulated in the presence of other peripheral blood mononuclear cells, PBMCs) secrete IFNγ, TNFα and express the cytolytic molecule granzyme B(12, 27, 30, 42, 43). Overall, it has become clear that there is more heterogeneity in the MAIT cell population than initially appreciated(44). A small fraction of MAIT cells isolated from the blood can also secrete IL-17 *ex vivo* following short term stimulation with via CD3/CD28 or PMA and ionomycin(12, 45, 46). It is noteworthy that a large fraction of MAIT cells in the blood expresses the transcription factor RORγt(36), which drives T-cells towards IL-

17 production(47). MAIT cells from patients with a loss of function mutation in Stat3 are impaired in their ability to produce IL-17 despite normal RORγt expression indicating a critical role of IL-23R signaling(36).

Additional transcriptions factors expressed in MAIT cells from the blood include Helios, Eomes, and T-bet(48). How these transcription factors specifically regulate different functional aspects of MAIT cells has yet to be fully elucidated. Finally, MAIT cells in the blood can be divided into different subsets based on CD8 and CD4 co-receptor expression. The relationship of these subsets is unclear, but single cell gene expression analysis from the two major MAIT subsets, $CD8^+$ and $CD8^-$ ($CD4^-$) cells, isolated from the blood demonstrated distinct transcriptomes(43, 49) and their respective frequencies can change independently following infection(45).

MAIT cell activation requirements and implications for their function in mucosal tissues

MAIT cells have been identified in human mucosal tissues that are colonized by commensal bacteria including intestines, rectal mucosa, buccal mucosal and vaginal tissue(12, 17, 43, 50, 51). Importantly, both commensal and pathogenic species of bacteria have intact riboflavin synthesis pathways and generate metabolites with agonist properties for MAIT cells (52). Thus, in contrast to conventional T cells(53), MAIT cells cannot use antigen specificity to distinguish commensal from pathogenic bacteria. How can MAIT cells be in close proximity to commensals in tissues without becoming activated given that MR1 is also readily available on antigen-presenting cells(25)? A recent study from our lab demonstrated that once purified, MAIT cells needed more than a TCR signal to acquire sustained effector function and that inflammatory cytokine signals (IL-12/15/18) synergized with the TCR signal to induce potent effector function(43). In this revised model of MAIT cell activation, encounter with commensal-derived

antigen (i.e. a TCR signal only) is not sufficient to activate MAIT cells unless inflammatory cytokines are present **(Figure 1)**, which are typically elicited in the context of a pathogenic infection. The synergistic interaction of cytokine and TCR signal has also been reported in the context of IL-15. IL-15 is a pleiotropic cytokine often produced early during infection by a wide range of cells with potent survival and immunomodulatory effects on T-cells(54). IL-15 expression within inflamed tissues can provide a co-stimulatory signal to drive memory T-cell effector function(55). Similar to what has been reported for conventional T cells, *Sattler et al* showed that addition of IL-15 in the context of suboptimal TCR activation increased MAIT effector function *in vitro*(56). While limited antigen availability resulted in minimal IFNγ production by MAIT cells, addition of IL-15 resulted in a synergistic effector response. A comparable effect has been reported for IL-7, which also increases MAIT cell responsiveness(13). Additional studies are needed to interrogate how MAIT cell function is modulated in various inflammatory environments. How MAIT effector function is eventually turned-off in mucosal tissues, despite the potential continuous antigen exposure from commensals, is unknown. It is important to keep in mind that even healthy mucosal barrier tissues have basal levels of inflammation and additional (MAIT cell intrinsic and extrinsic) control mechanisms are likely in place to help regulate MAIT cell functional properties. Finally, inflammatory cues such as IL-12, IL-15 and IL-18 can be sufficient to activate MAIT cells(57) and do this by directly acting on MAIT cells(43, 58) similar to what has been reported for conventional memory T cells(59). Both conventional memory T cells and MAIT cells can be bystander-activated during viral infections(60-62), but the exact role of MAIT cells during the course of a viral infection is still unclear.

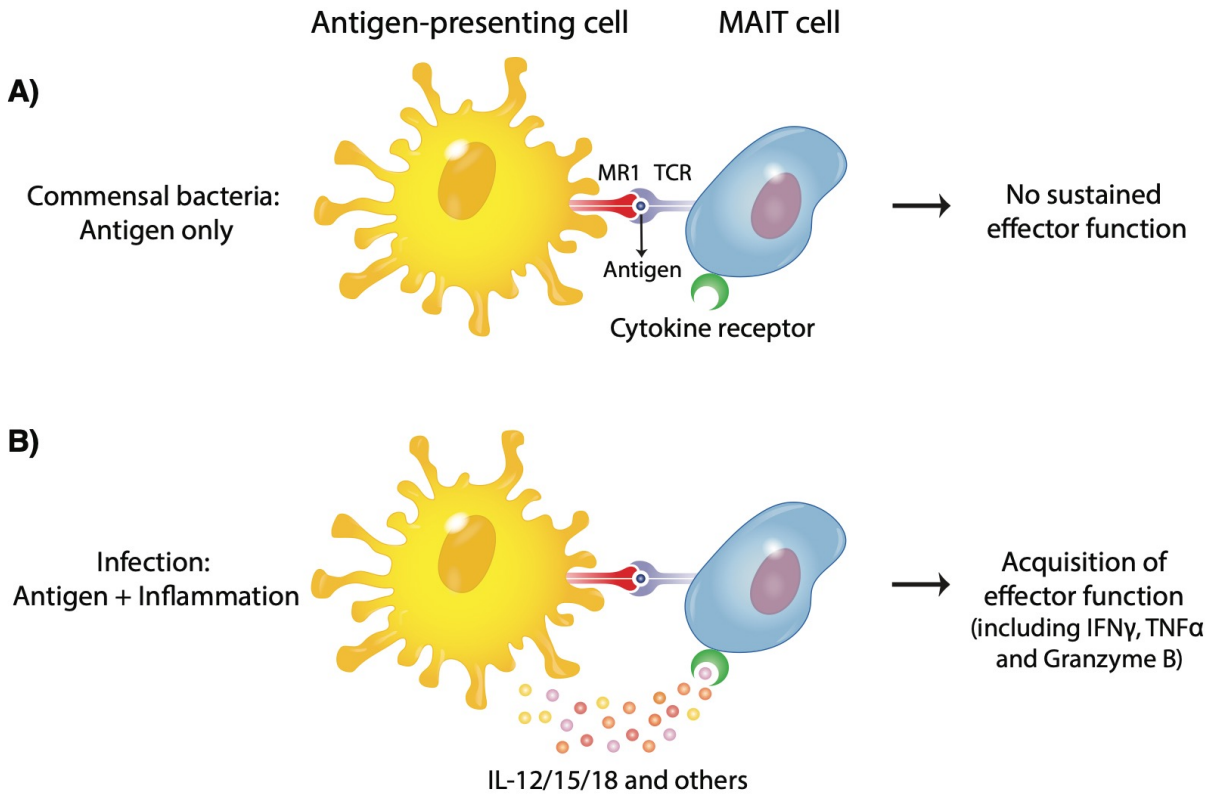


Figure 1: Model of MAIT cell effector function.

A functional riboflavin synthesis pathway is found in commensal and pathogenic species, which can lead to MR1-dependent presentation of metabolites to MAIT cells. (A) A TCR signal alone is not sufficient to elicit robust effector function, thus commensal-derived antigen in the absence of additional inflammatory cues is not sufficient to elicit MAIT cell effector function (B) Proinflammatory cytokines including IL-12/15/18 are elicited following infection and synergize with the TCR signal to elicit robust effector function.

MAIT cell subsets, phenotypes and function in mucosal tissues

The tissue microenvironment varies in different anatomical locations and may influence MAIT phenotype and function. Single cell gene expression analysis comparing MAIT cells isolated from blood and rectal mucosa of healthy donors revealed that MAIT cells located in the tissue have increased expression of pro-inflammatory transcripts (but not necessarily protein) such as TNFα and this was more pronounced in the $CD8^+$ MAIT cell subset compared to the $CD8^-$ subset(43). This increase in pro-inflammatory transcripts could allow MAIT cells to respond rapidly in the tissue. Interestingly, expression of these transcripts was not uniformly high in all MAIT cells, which displayed a bimodal expression pattern for many of these genes (43) as previously reported in other single cell gene expression analysis datasets(63). The extent of functional heterogeneity within the MAIT cell population in tissues is still unclear. However, MAIT cells identified in fetal mucosal tissue and the female genital tract (FGT) were shown to have a bias towards IL-17 and IL-22 production compared to MAIT cells in the blood(50, 64). Both of these cytokines have pivotal roles in regulating barrier immunity and tissue homeostasis (65, 66) indicating that MAIT cells have a more complex role than just being a guardian against bacterial infections.

MAIT cell trafficking patterns and potential

Most MAIT cells in the blood express CCR6(12, 43, 45, 67), which is implicated in trafficking to mucosal tissues and liver, since its ligand CCL20 is expressed in steady state conditions the gut, lung and liver. Interestingly, CCL20 is typically considered to be the sole ligand for CCR6, but some evidence suggests that beta-defensins may bind to CCR6 as well(68). In general, T-cell trafficking is guided by tissue-selective adhesion and chemokine receptors that

allow cells access to specific tissues including mucosal barrier surfaces(69). The chemokine receptor expression profile of MAIT cells has not been thoroughly characterized yet, though it has important implications for homing potential. Reported expression patterns are summarized in **Table 1**. While expression of CCR6 indicates that MAIT cells can access a wide range of tissues, the lack of CCR7 and CD62L expression suggests that they lack the ability to migrate from blood to lymph nodes via high endothelial venules (HEVs)(70). Data from lymph node (LN) biopsies of cancer patients and characterization of MAIT cells in fetal tissues suggest that MAIT cells are rare in lymph nodes (LN) (12, 64). The origin of the few MAIT cells found in the lymph node is unclear, since MAIT cells have been identified in thoracic duct lymph indicating they can exit peripheral tissues(71). The signals that control these steps are not nearly as well defined as the steps required for LN entry from blood via HEVs, but two reports provided compelling evidence that CCR7 expression on conventional T-cells greatly enhances their ability to exit peripheral tissues and enter the afferent lymphatics(72, 73).

Chemokine Receptor	Fraction of MAIT population*	References
CCR2	>80%	[67]
CCR5	>80%	[12,67]
CCR6	>80%	[12,45,67]
CCR7	-	[12]
CCR9	+	[12,63]
CXCR3	<5%	[12,46]
CXCR4	60-80%	[12,67]
CXCR6	>80%	[12,45,67]

Table 1: Chemokine receptor profile for MAIT cells in human blood

*When frequencies are not reported, + and – simply indicate the overall trend of expression

Tissue-resident memory T cells (Trm) are a distinct memory population that are retained within tissues long-term without recirculating, and thus are poised for immunosurveillance at barrier tissues. Trm have been well-defined for conventional T cells using murine models of viral

and bacterial infection to demonstrate that Trm cells maintain persistent tissue residence (74). A recent murine study utilizes parabiosis, surgically joined mice, to show that a population of MAIT cells within spleen, liver, and lung were retained within tissues and do not recirculate akin to conventional Trm (75). Determining if human MAIT cells have features of Trm or a distinct Trm population has solely relied on assessing canonical markers of Trm, CD103 and CD69, which was exhibited on MAIT cells residing in buccal and gastric mucosa (76, 77). In addition, expression of cytotoxic and regulatory T-cell molecule (CRTAM), which controls residency of T-cells in the gut(78), is also enhanced in MAIT cells isolated from tissues(43). Though these studies indicate the existence of a human population of Trm MAIT cells, it has not been confirmed if these cells possess a gene signature established for human conventional T cells, indicating they are maintained long-term in tissues(79).

Conclusion

MAIT cells are located in barrier tissues to perform front-line host defense and have increased pro-inflammatory transcripts within these tissues compared to their blood counterparts highlighting that they are poised to respond rapidly at these sites. I have discussed a model (Fig. 1) for MAIT cell activation to explain how these cells control their potent effector function at barrier surfaces. Sustained TCR-mediated responses only occur in the presence of inflammatory signals that are typically indicative of an infection. The need for inflammatory signals to elicit cytotoxic effector function may serve to prevent unwanted responses at these sites to commensal-derived antigen in steady state conditions. However, it is unclear what mechanisms may control their potent effector function within inflamed tissues where they are presumably exposed to multiple inflammatory stimuli. In addition, studies characterizing MAIT cells within healthy mucosal tissues including expression of surface MR1 are limited and are necessary to

understand how the mucosal tissue environment affects their phenotype *in vivo*. Therefore, I was interested investigating MAIT cell phenotype and immunoregulation within mucosal tissues.

In this work, I will describe my efforts to assess MAIT cell phenotype and expression of MR1 within the colon from healthy donors compared to peripheral blood (Chapter III). I will also examine MAIT cells within inflamed and healthy oral mucosal in order to define mechanisms of cell-intrinsic regulation (Chapter IV). Finally, I will discuss implications of this work to appreciate the role of mucosal-MAIT cells during homeostasis and chronic inflammation (Chapter V).

Chapter II:
Material and Methods

Isolating and characterizing MAIT cells colon

Ethics statement

All participants signed informed consent and the protocols were approved by the institutional review board (IRB) at Fred Hutchinson Cancer Research Center (8636).

Human Samples

Human colonic mucosa and peripheral blood were acquired from healthy donors undergoing a flexible sigmoidoscopy from a cohort in Cape Town, South Africa (n=11, age 22-69). Up to 20 biopsies were taken from each participant after a 30 min enema salt wash. Participants used in this study were HIV negative and reported no steroid or antibiotic use.

Colonic Tissue processing

Cells from mucosal tissue were mechanically and enzymatically extracted. Tissue biopsies underwent two rounds of 30min enzymatic digestion with 700U of collagenase II (Sigma-Aldrich) in 7.5% FBS in 12.5mL of RPMI at 37°C, each followed by mechanical digestion with 30cc syringe and blunt 16-gauge needle. Single-cell suspensions were then passed through a 70um filter.

Peripheral blood mononuclear cell (PBMC) isolation

10 mL of peripheral blood were collected in ACD tubes and transferred on wet ice. PBMCs were isolated using Lymphoprep and SepMate tubes (Stem Cell) system according to manufacturer's instructions. Briefly, fresh blood was spun down 400g for 15min and plasma removed. Remaining blood was diluted with 1X PBS to 30mL of total volume, loaded onto SepMate tubes, and then spun for 1200g for 16 minutes. Buffy layer was poured, washed with PBS, and spun at 400g for 5 minutes.

DAY	Live, CD45 of Total cells	CD14+ of Live, CD45	CD19 + of Live, CD45	CD3+ of Live, CD45	CD11c+ of Live, CD45	CD123+ of Live, CD45	CD141+ of CD11c	CD1c+ of CD11c	CD141- CD1c- of CD11c
1	98.9 %	19.0 %	3.07 %	63.5 %	3.42 %	0.21 %	0.90 %	11.5 %	85.5 %
2	95.4 %	14.9 %	3.42 %	67.3 %	4.53 %	0.18 %	0.67 %	4.20 %	93.8 %
3	98.3 %	11.3 %	2.70 %	61.3 %	11.6 %	0.28 %	0.36 %	3.48 %	95.6 %
4	94.8 %	27.8 %	3.71 %	54.4 %	5.04 %	0.18 %	0.82 %	6.93 %	91.5 %
5	74.4 %	23.4 %	3.26 %	63.3 %	0.61 %	0.12 %	2.74 %	40.1 %	54.9 %
6	97.6 %	15.7 %	3.43 %	59.2 %	1.01 %	0.29 %	0.27 %	10.2 %	88.1 %
7	99.2 %	19.6 %	3.45 %	66.5 %	1.24 %	0.20 %	1.67 %	20.9 %	75.9 %
8	97.3 %	20.8 %	3.54 %	65.9 %	0.65 %	0.13 %	2.72 %	28.1 %	66.9 %

Table 2: Cell population frequencies from peripheral blood mononuclear cell (PBMC) staining control for innate panel

Cryopreserved PBMCs from the same donor were thawed and stained at the same time as fresh blood and colonic samples. Innate panel was used for analysis of Figure 1-3. Cell frequencies are recorded to show day-to-day variation.

Day	CD45, live of total cell	Lymphocytes of CD45, live	CD3+ of Lymphocytes	CD4+ of CD3	CD8+ of CD3	MR1 tet of CD3
1	95.2 %	74.6 %	84.0 %	50.5 %	43.5 %	0.42 %
2	95.7 %	76.4 %	83.3 %	51.8 %	42.5 %	0.36 %
3	78.1 %	72.0 %	83.6 %	52.5 %	42.1 %	0.38 %
4	93.5 %	69.9 %	84.0 %	48.2 %	46.4 %	0.40 %
5	57.3 %	57.0 %	50.7 %	75.5 %	20.2 %	0.56%
6	86.7 %	65.9 %	77.5 %	54.0 %	39.7 %	0.52 %
7	61.3 %	76.0 %	80.4 %	51.0 %	44.0 %	0.47 %
8	92.2 %	77.20%	82.9 %	51.6 %	44.1 %	0.36 %

Table 3: Cell population frequencies from peripheral blood mononuclear cell (PBMC) staining control for T cell panel

Cryopreserved PBMCs from the same donor were thawed and stained at the same time as fresh blood and colonic samples. T cell panel was used for analysis of Figure 3-6. Cell frequencies are recorded to show day-to-day variation.

Cryopreserved PBMC staining control

Cryopreserved PBMCs from a single donor were thawed and used as a staining control for each experiment. PBMCs were thawed in RPMI pre-warmed at 37°C and centrifuged at 400g for 10 minutes. Cells were stained at the same time as fresh blood and colon samples each day. Cell population frequencies were quantified from each panel and are listed in **Table 2** and **Table 3**.

Panel	Flourophore	Antigen	Clone	Vendor
Innate	BUV496	CD16	3G8	BD
	BUV563	CD56	NCAM16.2	BD
	BUV661	CD3	UCHT1	BD
	BUV805	CD45	H130	BD
	BV570	CD14	M5E2	BD
	BV605	CD141	1A4	BD
	BV785	CD123	7G3	BD
	PE	MR1	26.5	Biolegend
	PE-Cy5.5	CD19	SJ25-C1	ThermoFisher
	AF647	CD1c	F10/21A3	BD
	AF700	CD11c	B-ly6	BD
	APC-H7	HLA-DR	G46-6	BD
T cell	BUV395	CD8	RPA-T8	BD
	BUV496	CD3	UCHT1	BD
	BUV661	HLA-DR	G46-6	BD
	BUV737	CD69	FN50	BD
	BUV805	CD45	H130	BD
	BV480	CD103	Ber-Act8	BD
	BV605	PD1	EH12.1	BD
	BV650	CD161	DX12	BD
	PE-Cy5	CD137	4B4-1	BD
	PE-Cy5.5	CD19	SJ25-C1	ThermoFisher
	AF700	Granzyme	GB11	BD
	APC-H7	CD4	RPA-TA	BD

Table 4: Antibodies used for flow cytometry in Chapter III

Details of each antibody used for flow cytometric analysis in Chapter 3 is noted. Antibodies from each panel innate (Fig. 1-3) and T cell (Fig. 4-6) are noted.

Flow cytometry staining

Staining: Single-cell suspensions from blood, colon, and PBMC control were split for staining of antibodies using two different flow cytometry panels. The innate panel was used for Figures 1-3 and T cell panel was used for figures 4-6 **(Table 4)**. All cells were platted in a V-bottom 96 well plate and first stained with Live/Dead Fixable Dead Cell Stain (Thermo Fisher) for 15 min. Cells for the T cell panel were stained with MR1 5-OP-RU tetramer (PE, NIH tetramer core) at 1:500 in FACS wash (2% FBS in 1X PBS) for 45 min at room temperature. Cells were then stained with an optimized cocktail of surface antibodies from each panel **(Table 4)** for 20 minutes. Cells were washed between each step by centrifuging at 400g and resuspending with FACS wash (2%

FBS in 1X PBS). Intracellular staining was completed using the Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher) for the T cell panel or 4.2% PFA (Cytofix/Cytoperm, BD Biosciences) for Innate panel according to manufacturer's protocol.

Instruments: FACSSymphony (BD). Data was analyzed using Flowjo software (version 10.4.1 or higher).

Statistical Analysis

Statistical analysis was performed with Prism 8 (GraphPad) as specified in figure legends.

Isolating and characterizing MAIT cells in oral mucosa

Study approval and patient cohort

All participants signed informed consent and the protocols were approved by institutional review boards (IRBs) at the Fred Hutchinson Cancer Research Center (8335).

Human Samples

Oral mucosa was obtained from patients undergoing oral surgery where gingival tissue was removed as a normal part of the procedure. Blood was collected in the oral cavity using a syringe. Surgical procedures included gingivectomy/gingivoplasty, osseous surgery, implant uncovering and tooth extractions. Participants (n=39) were between 14 years old 83 years old (mean = 52).

Pathology assessment and scoring

A small portion of each OM sample was embedded in Tissue-Tek O.C.T. (Sakura Finetek, Thermo Fisher) and stored at -80°C. Frozen tissue blocks were cut into 8μm sections and slides

were stained with hematoxylin and eosin (H&E). Histologic sections were then evaluated blinded and scored by pathologist as "healthy" or "inflamed" according to the following criteria: severity of inflammation (1-5), location of inflammation, type of inflammatory infiltrate, presence of lamina proprial edema and epithelial lesions.

Cell isolation from mucosal tissues

After extraction, tissue and blood samples were immediately placed in RPMI 1640 (supplemented with penicillin at 100 U/ml, streptomycin sulfate at 100 μg/ml, and FBS at 10%) on ice for transport. Mucosal tissue was enzymatically and mechanically digested to isolate cells. Tissue underwent 2 rounds of mechanical digestion with 30cc syringe and blunt 16-gauge needle and enzymatic digestion with collagenase II (Sigma-Aldrich). Oral blood was treated with ACK lysing buffer to remove red blood cells. Cells were then stained for single cell sorting and RNA sequencing.

Flow cytometry

For phenotypic identification, isolated mononuclear cells from tissue or bulk peripheral mononuclear cells (PBMCs) were stained with Live/Dead Fixable Dead Cell Stain (Invitrogen) for 15 min followed by staining with a surface antibody cocktail for 20 minutes at room temperature (RT). For MR1 tetramer analysis, samples were stained with MR1 5-OP-RU tetramer (BV421, NIH tetramer core) for 40 min. followed by remaining antibodies **(Table 5)**. Cells were then washed with FACS buffer (PBS containing 2% FBS) and fixed in PBS containing 1% paraformaldehyde (Sigma-Aldrich). For samples requiring intracellular staining, cells were processed using the Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher)

according to the manufacture's instruction. Antibodies included are listed in Table 2.1. Sorting experiments were performed on a FACS Aria II (BD) and phenotyping experiments performed on a FACSSymphony (BD). Data was analyzed using Flowjo software (version 10.4.1).

Panel	Flourophore	Antigen	Clone	Vendor
Cell sorting	Pacific Blue	CD3	OKT3	BioLegend
	PerCPCy5.5	CD8	SK1	BD
	ECD	CD4	SFCI12T4D11	Beckman Coulter
	PECy5	CD161	DX12	BD
	PE	Vα7.2	3C10	BioLegend
Phenotyping and stimulation	BV421	CD25	M-A251	BD
	BUV661	CD3	UCHT1	BD
	BUV805	CD8	SK1	BD
	APCR700	CD4	RPA-T4	BD
	PE-Cy7	CD45RO	UCHL1	BD
	UV737	CD69	FN50	BD
	YV395	CD103	Ber-ACT8	BD
	BV605	CD161	DX12	BD
	PE	Vα7.2	3C10	BioLegend
	BV786	IL-7Rα	HIL-7R-M21BD	BD
	PECF594	CTLA-4	BNI3	BD
	AF488	Foxp3	259D/C7	BD
	BV711	IFNγ	4S.B3	BD

Table 5: Antibodies used for flow cytometry in Chapter IV

Details of each antibody used cell sorting, phenotyping and stimulation in Chapter IV is noted. Antibodies shaded in yellow were used for intracellular staining.

RNAseq library generation

RNAseq was performed on 100-1000 sorted $CD8^+$ MAIT cells from blood or tissue samples. In total, 22 samples were sequenced, 10 from blood and 11 from tissue. Cells were sorted directly into SMARTer® v3 lysis reagents (Clontech). Cells were lysed and cDNA was synthesized. After amplification, sequencing libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina). Barcoded single-cell libraries were pooled and quantified using a Qubit® Fluorometer (Life Technologies).

Sequencing and alignment of libraries

Single-read sequencing of the libraries was carried out either on a HiSeq2500 sequencer (Illumina) with 58-base pair reads, using TruSeq v4 Cluster and SBS kits (Illumina) with a target depth 5 million reads. Base-calling was performed automatically in Illumina BaseSpace after sequencing; FASTQ reads were trimmed in a local Galaxy server in two steps. First, hard-trimming was applied to remove one 3'-end base (FASTQ Trimmer tool, v.1.0.0). Second, quality trimming from both ends was applied until a minimum base quality for each read of at least 30 was obtained (FASTQ Quality Trimmer tool, v.1.0.0) (80). Reads were aligned to the University of California Santa Cruz (UCSC) Human genome assembly version 19 in Galaxy using Bowtie and TopHat (Tophat for Illumina tool, v.1.5.0)(81). Read counts by Ensembl gene ID were obtained in Galaxy using htseq-count (htseq-count tool, v.0.4.1) (82).

RNAseq analysis

A quality filter was applied to retain libraries prepared with at least 225 cells, in which the fraction of unpaired reads examined compared to total FASTQ reads was >75%, the median coefficient of variation of coverage was less than 0.8, and the library had at least 500,000 reads. 16 of the 22 sequenced samples passed these quality filters. Non-protein coding genes, mitochondrial genes, and genes expressed at less than 1 count per million in fewer than 10% of samples were filtered out. Expression counts were normalized using the TMM algorithm (83). For differential gene expression analysis, we used the linear models for microarray data (Limma) R package (84, 85) after Voom transformation (86). A linear model comparing blood and tissue sample gene expression including donor identity as a random effect was used. Genes with a false discovery rate of less than 0.05 and expression fold-change of greater than 2 between two blood and tissue samples were considered differentially expressed.

Ex vivo stimulation of PBMCs

Cryopreserved PBMCs were thawed and left untreated in RPMI 1640 (supplemented as described previously) or stimulated with cytokines, anti-CD3/CD28 beads, or a combination of both treatments. Cytokine stimulation was performed using IL-12 (Invitrogen), IL-15 (Invitrogen), and IL-18 (MBL) at 100ng/mL. Dynabead Human T cell activator (Invitrogen) anti-CD3/CD28 beads were used at a 1:1 bead/T cell ratio. Whole PBMCs were left unstimulated or stimulated at a concentration of $1x10^6$ per well using 96-well plate for 6 or 24 hours at 37°C. Indicated samples were treated with anti-MR1 antibody (Low Endotoxin, Azide-Free clone 26.5, Biolegend) at a concentration of 50μg/mL. To assess CTLA-4 expression after brief stimulation, PBMCs were stimulated for 6 hours and then anti-CD3/CD28 beads were removed using magnetic extraction following manufacturer's protocol (Thermofisher). Cytokines were removed by washing cells twice by centrifugation (2000 rpm for 3 min) and resuspended in RP10.

Chapter III:
Extensive MR1 surface expression on various immune cells and MAIT cell activation within healthy colonic mucosa

Introduction

The mucosal immune system has co-evolved with an exceptionally diverse microbiome due to continuous and direct interactions at barrier surfaces. Commensal microbiota play an essential role in functional tuning and education of resident immune cells and breakdown of this relationship can result in chronic inflammatory disorders(87). This complex and dynamic relationship involves many players from the host immune system. T cells, in particular, play a pivotal role balancing both maintenance of barrier immunity and tolerance towards commensals(53, 87). $CD4^+$ T cells that have specificity for commensal antigens have been well-described, including IL-17 producing T_H17 cells and regulatory T cells that modulate mucosal barrier integrity and suppress resident immune cell functions (53, 88). Non-classical T cells are likely candidates as mediators of commensal-specific interactions due to their tissue localization and specificity to broadly conserved antigens. These innate-like T cells include NKT cells, γδ T cells, and mucosal-associated invariant T (MAIT) cells, yet their role in human mucosal tissues are still poorly understood.

MAIT cells constitute an abundant subset of human T cells that have been identified in multiple mucosal tissues including female genital tract, oral mucosa, and intestinal tract as well as in peripheral blood (50, 51, 76). They were originally defined by a semi-invariant TCR, having a single alpha chain rearrangement (TRAV1-2 and TRAJ33) paired with a limited selection of TCR beta chains(14, 15, 33). The majority of MAIT cells are $CD8^+$ (~90%) with minor $CD8^-CD4^-$ (~10%) and rare $CD4^+$ populations (12, 27). The cognate antigens for the MAIT cell TCR are metabolic intermediates from the riboflavin synthesis pathway presented by

the MHC class I-related molecule (MR1)(21). This pathway and its metabolic products are present among multiple species of bacteria and fungi, which includes known commensals (89). Because MAIT cell antigens are small organic molecules that are chemically unstable, these antigens might not endure in the environment or lumen of tissues long enough for cells to acquire, process, and present via MR1 to the MAIT TCR vitamin(21). Therefore, it is unknown if antigen-presenting cells (APCs) residing in healthy mucosal tissues are able to present exogenous antigen from commensals to neighboring MAIT cells.

MR1 surface presentation is a tightly regulated process with expression largely dependent on presence of the metabolite ligands (25, 90). Immature MR1 protein is stored in the endoplasmic reticulum in an unfolded state in the absence of ligand (91). Upon exposure to ligands derived from the extracellular environment, MR1 undergoes a molecular switch that stabilizes into a fully formed mature protein with ligand and traffics to the cell surface (91). Minimal surface MR1 protein has been detected among peripheral blood mononuclear cells likely due to the lack of microbial products, but the mature form of MR1 has been detected among $CD11c^+$ $HLA\text{-}DR^+$ DCs in the healthy female genital tract and oral mucosa (50, 51, 91). Conversely, in healthy ileum, mature MR1 was only detected on antibody-producing B cells (92). However, these studies were limited in their ability to quantify expression on these and other subsets as experiments were conducted using immunohistology. Thus, expression of functional MR1 itself has also been poorly characterized in human mucosal tissues.

I hypothesized that due to the copious presence of microbial products at barrier sites, surface expression of MR1 would be extensive among various immune cells in mucosal tissues and consequently result in MAIT cell activation. In order to appreciate the different cell types expressing surface MR1 in healthy, microbe-riche mucosal tissues, I have studied colonic tissue

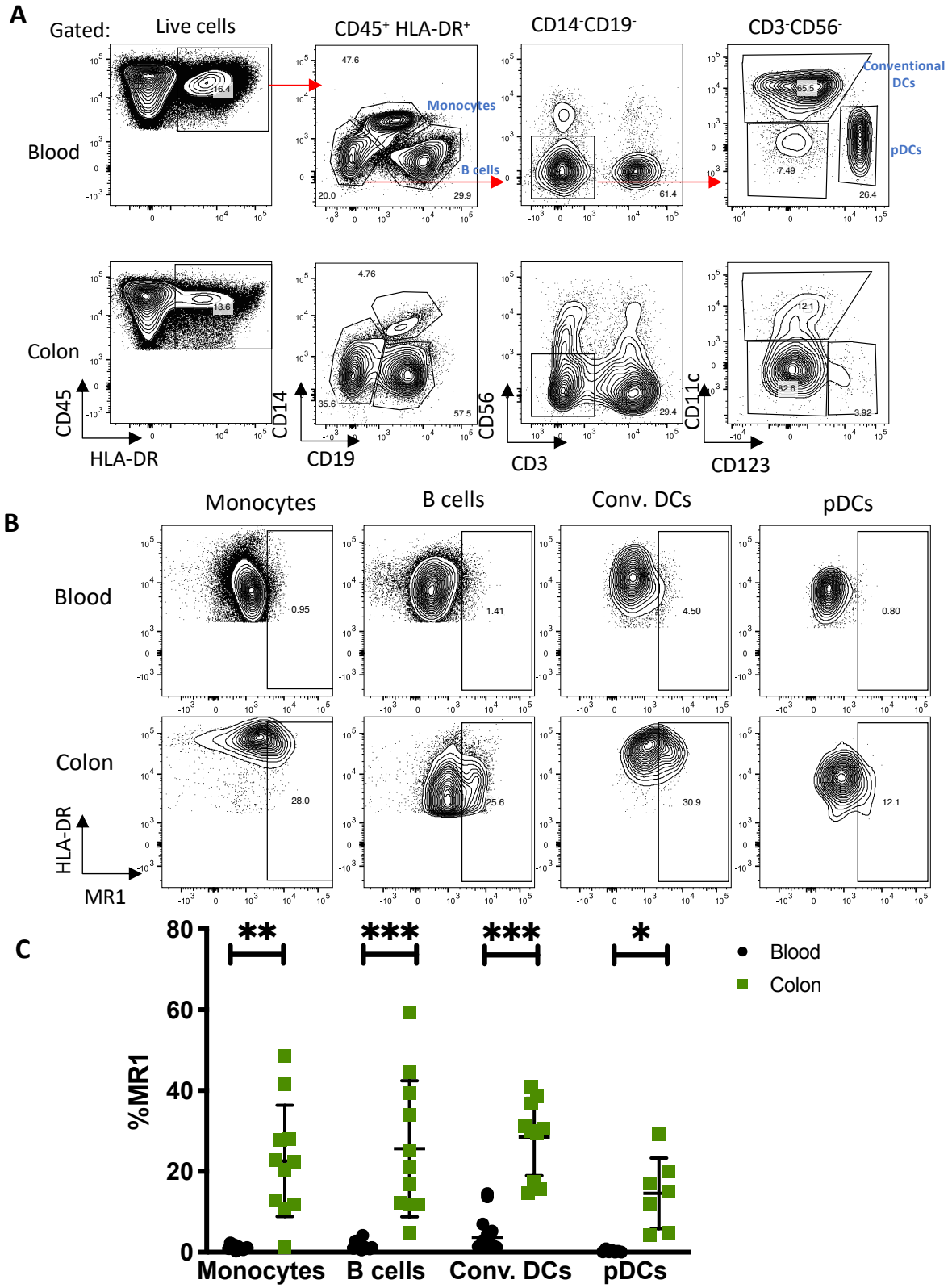
biopsies from healthy donors and compared them to donor-matched peripheral blood cells. I found that surface MR1 expression on colonic immune cells was widely expressed across many immune subsets in comparison to their blood counterparts. I also found that MAIT cells in the colon have increased expression of activation markers including known markers of recent TCR activation that was not observed on conventional T cells. Despite this, MAIT cells do not display signs of cytotoxic function but rather express markers of a tissue residency phenotype. These data suggest that MAIT cells may be continually receiving signals from the microbiota in the healthy colon and identify MAIT cells as important mediators of the interaction between the immune system and mucosal microbiome during homeostasis.

Results

Antigen-presenting cells (APCs) of healthy individuals have increased MR1 expression in the colon compared to matched blood

Although MR1 has been previously identified on B cells in the ileum(92), thorough analysis of MR1 expression on antigen presenting cells (APCs) in the colon is lacking. Therefore, I used flow cytometry to evaluate MR1 expression on APC subsets and B cells by isolating and assessing cells from matched blood and healthy colonic biopsies. The gating scheme shown for a representative donor displays how the main APC subsets were identified **(Fig. 2A)**. APCs were identified by gating live, $CD45^{+}HLA^{-}DR^{+}$ cells. Monocytes were then gated as $CD19^{-}CD14^{+}$, B cells were defined as $CD19^{+}CD14^{-}$, and DC were identified by gating $CD19^{-}CD14^{-}CD3^{-}CD56^{-}$ cells. Among dendritic cells (DC), we also differentiated plasmacytoid DCs (pDCs) from conventional DCs (cDCs) using CD123 and CD11c, respectively, due to their distinct functional roles (93) **(Fig. 2A, right)**.

I then compared surface MR1 expression on monocytes, B cells, and the DC subsets in colon and matched blood samples **(Fig 2B)**. I found that the frequency of $MR1^+$ cells was significantly higher in monocytes, B cells, cDCs, and pDCs in the colon versus the matched blood **(Fig. 2C)**. These data suggest that APCs are able to process or absorb MR1 ligands from commensals in healthy colonic tissue.


A
Gated:
Live cells
CD45+ HLA-DR+
CD14-CD19-
CD3-CD56-
Blood
Colon
Monocytes
B cells
Conventional DCs
pDCs
CD45
HLA-DR
CD14
CD19
CD56
CD3
CD11c
CD123
B
Monocytes
B cells
Conv. DCs
pDCs
Blood
Colon
HLA-DR
MR1
C
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Blood
Colon
Monocytes
B cells
Conv. DCs
pDCs

Figure 2: APCs of healthy individuals have increased MR1 expression in the colon compared to matched blood

(A) Representative FACS plot to displaying gating scheme of APC subsets including B cells from donor-matched colon biopsies and peripheral blood. (C) Representative staining and (D) frequencies showing surface MR1 expression of monocytes, B cells, conventional DCs, and plasmacytoid DCs. Black circles represent blood samples and green squares represent colon samples. n = 11 Wilcoxon matched-paired signed rank tests were performed. *p ≤ 0.05 **p ≤0.01 ***p ≤ 0.001.

Conventional DC subsets (CD141$^+$, CD1c$^+$, CD141$^-$CD1c$^-$) in colon can express MR1

Different cDC lineages are characterized based on their specialization for presenting exogenous or endogenous antigen(94), which may have implications for how MR1-dependent T cells are presented antigen. CD1c is an isoform of CD1, a family of antigen-presenting proteins that are non-polymorphic and present non-peptide antigen to invariant T cells, similar to MR1. Expression of CD1c on cDCs denotes a specific cDC subset (cDC2) associated with priming of CD4$^+$ T cells to extracellular pathogens (95). In comparison, the subset defined by CD141 expression (cDC1) is known for cross-presenting antigen to CD8$^+$ T cells in response to tumors and pathogens that do not directly infect DCs (96). CD11c$^+$ cDC subsets were identified based in CD141 and CD1c expression **(Fig 3A)** and examined for surface MR1 expression **(Fig 3B)**. First, we observed no enrichment for MR1 expression in the blood within any cDC subset **(Fig 3C),** mirroring the MR1 expression on total cDCs. Importantly, all three subsets displayed MR1 expression in the colon, although the CD141$^-$CD1c$^-$ subset had a significantly higher frequency of MR1$^+$ cells compared to the cDC1 and cDC2s subsets. Nonetheless, my data show all three cDC populations in the colon can express MR1 signifying that, regardless of subset specialization, cDCs are able to process, absorb and likely present MR1 ligand to MR1-restricted T cells.

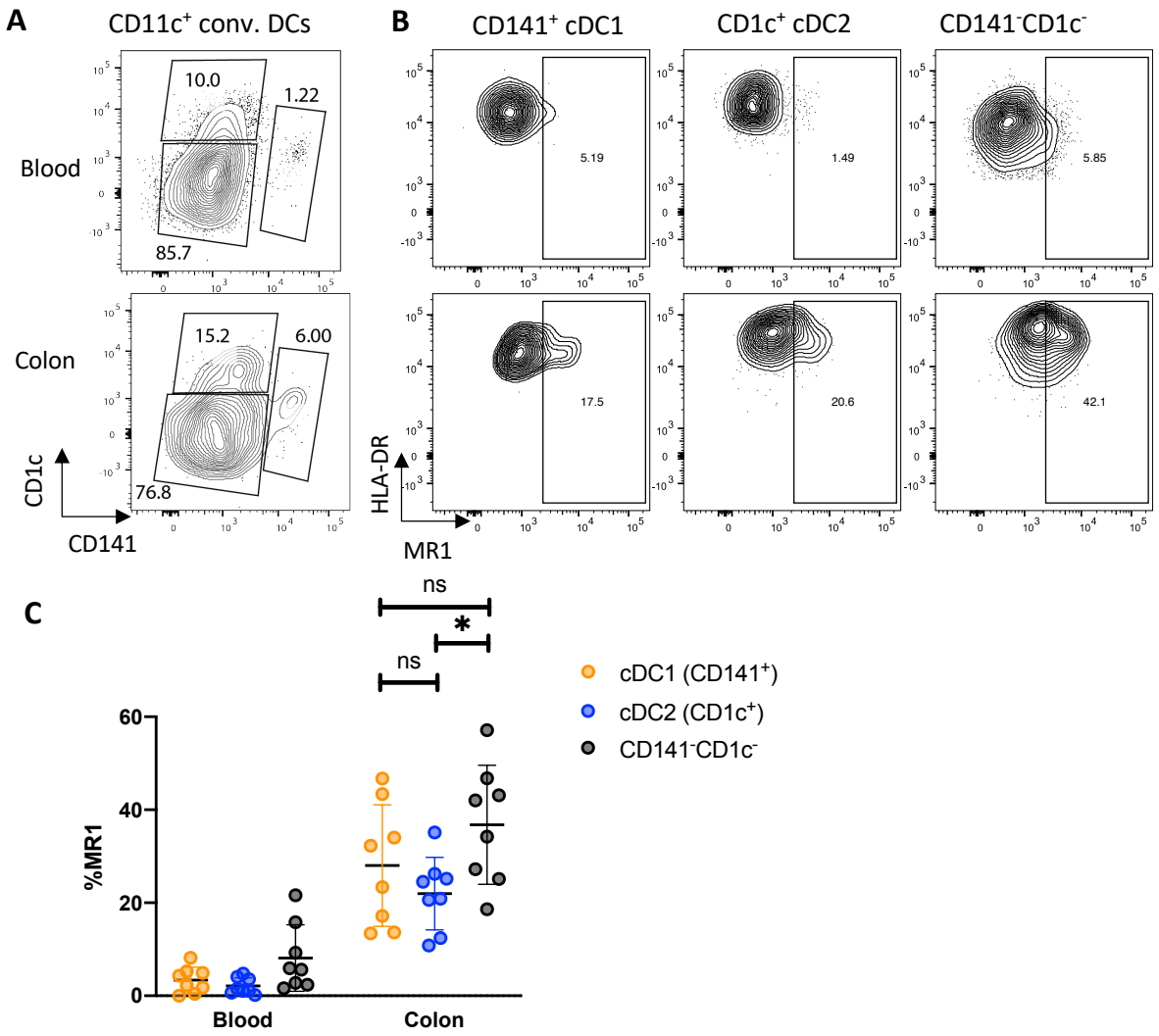

Figure 3: Conventional DC subsets in colon have increased MR1 expression

(A) Representative FACS plot showing CD11c+ conventional DCs gated as CD1c+, CD141+, and CD1c-CD141- subsets and (B) assessed for MR1 in blood and tissue. (C) Quantification of MR1 expression frequency on cDCs subsets in blood and colon is shown. Orange circles are CD141+ cDC1 cells, blue circles are CD1c+ cDC2, black circles are CD141-CD1c-. n=8 Two-way ANOVA was implemented with Tukey post-hoc test *p ≤ 0.05 **p ≤0.01 ***p ≤ 0.001.

Activated T cells in the colon express MR1

It is unknown if MR1 expression on T cells also occurs *in vivo* within healthy mucosa. However, it has been shown that conventional T cells can activate MAIT cells in a MR1-restricted manner when exposed to a high concentration of exogenous antigen (riboflavin metabolite 5-OP-RU) *ex vivo* (97). These experiments suggest that T cells do have an ability to

acquire and present exogenous antigen to MAIT cells. Therefore, in addition to quantifying expression of surface MR1 on various APC subsets (**Fig. 3**), I also asked if non-professional APCs, such as a T cells, could express surface MR1. To this end, T cells were gated as live, $CD45^{+}CD19^{-}CD14^{-}CD3^{+}$ with low side scatter **(Fig 4A)** and were then assessed for MR1 expression in blood and matched colon samples **(Fig 4B)**. I found increased frequency of MR1-expressing T cells in the colon compared to matched blood revealing even non-phagocytic cells can present MR1 ligands in healthy mucosal tissue **(Fig 4C)**. Notably, we observed MR1 preferentially co-expressed with HLA-DR on T cells **(Fig 4B)**. While constitutive expression of HLA-DR is a hallmark of professional APCs, this antigen-presenting molecule is also upregulated on T cells after activation (98). Thus, we studied how MR1 expression compared between $HLA\text{-}DR^{+}$ and $HLA\text{-}DR^{-}$ cells in the colon. These data reveal that MR1 expression was increased among the $HLA\text{-}DR^{+}$ cells compared to $HLA\text{-}DR^{-}$ cells **(Fig. 4D),** suggesting MR1 might be shuttled to the surface after activation. My data reveal an unexpected finding that not only is there MR1 expression among T cells in the colon, but that the majority of $HLA\text{-}DR^{+}$ activated conventional T cells in the colon express MR1.

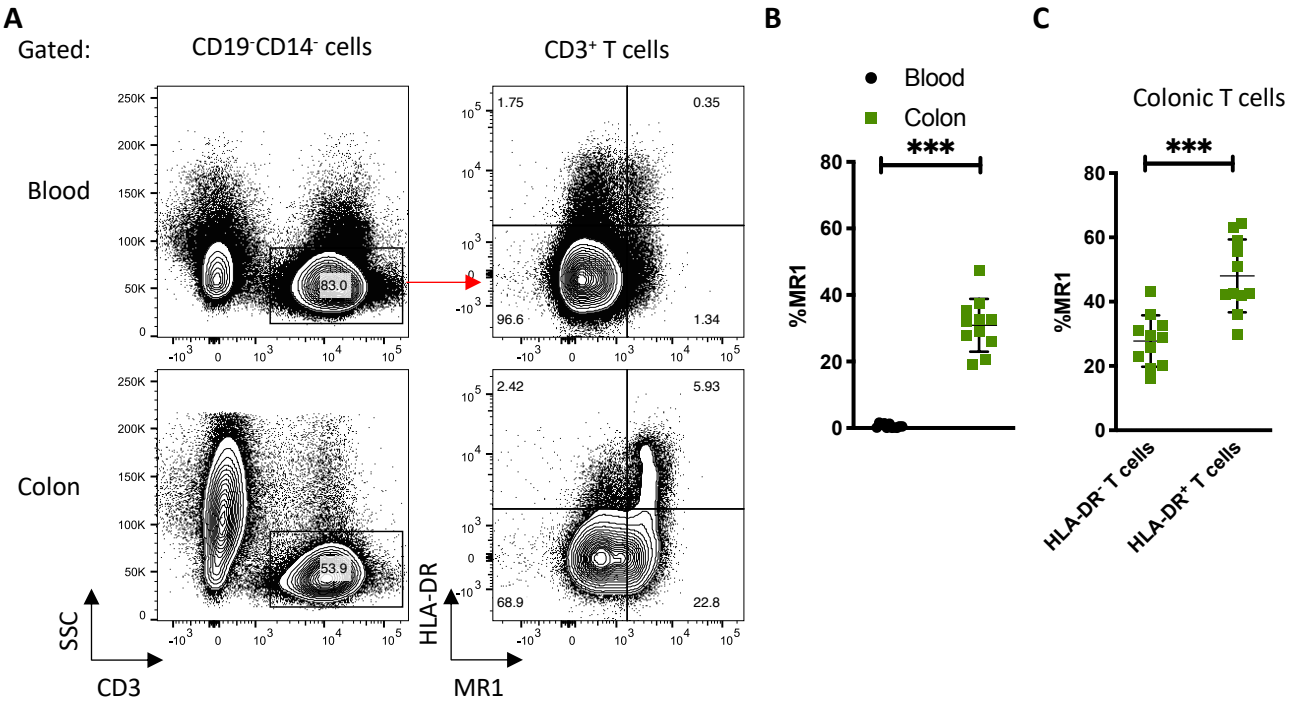

Figure 4: Activated T cells express MR1 in the colon

(A) CD3+ T cells were assessed for surface MR1 and HLA-DR expression. (B) Quantification of MR1 expression on T cells in the blood and colon (C) HLA-DR+ and HLA-DR+ T cells in the colon were assessed for surface MR1 expression n=11 Wilcoxon matched-paired signed rank tests were performed. *p ≤ 0.05 **p ≤0.01 ***p ≤ 0.001.

Colonic MAIT cells display signs of recent TCR activation

The MR1 surface expression observed in healthy colon across immune subsets (Figs. 1-3) suggests that microbial products can penetrate the mucosal barrier for presentation to MR1-dependent T cells such as MAIT cells. MAIT cells are abundant in mucosal barrier tissues and can respond *in vitro* to secreted products from bacterial species of the human microbiota (97). However, it is unknown whether the MR1 surface protein I observe on immune cells in the colon is actively presenting antigen to MAIT cells. Therefore, I hypothesized that colonic MAIT cells *in vivo* would display signs of TCR engagement. The costimulatory molecule, CD137 (4-1BB) has been reported to be induced on antigen-specific T cells post-TCR signaling and then downregulated by 72 hours post-stimulation (99). Moreover, antigen stimulation with

Mycobacterium tuberculosis induced CD137 expression on MAIT cells(100). Thus, I sought to investigate the levels of CD137 expression on MAIT cells in colon and matched blood to ask if these cells could have recently received a TCR signal.

First, I identified MAIT cells among the total T cells compartment in blood and colon by using an MR1 tetramer loaded with potent MAIT antigen 5-OP-RU and co-staining with a known MAIT marker, CD161 **(Fig. 5A)**. MAIT cells in the colon constituted a similar frequency of the total T cell compartment as in the blood **(Fig. 5B)**, which has also been reported for other mucosal tissues (12, 50, 76, 77). I then compared CD137 expression on MAIT cells in blood and colon as well as non-MAIT $CD8^+$ and $CD4^+$ cells, as reference populations **(Fig. 5C)**. I found that MAIT cells in the colon had significantly increased CD137-expressing cells compared to MAIT cells in matched blood (Fig. 4D). In contrast, the percentage of cells that were CD137+ was not different between colon and blood in conventional T cell populations **(Fig. 5D)**. These data suggests that MAIT cells in the healthy colon are receiving a TCR signal, possibly in response to commensal microbiota.

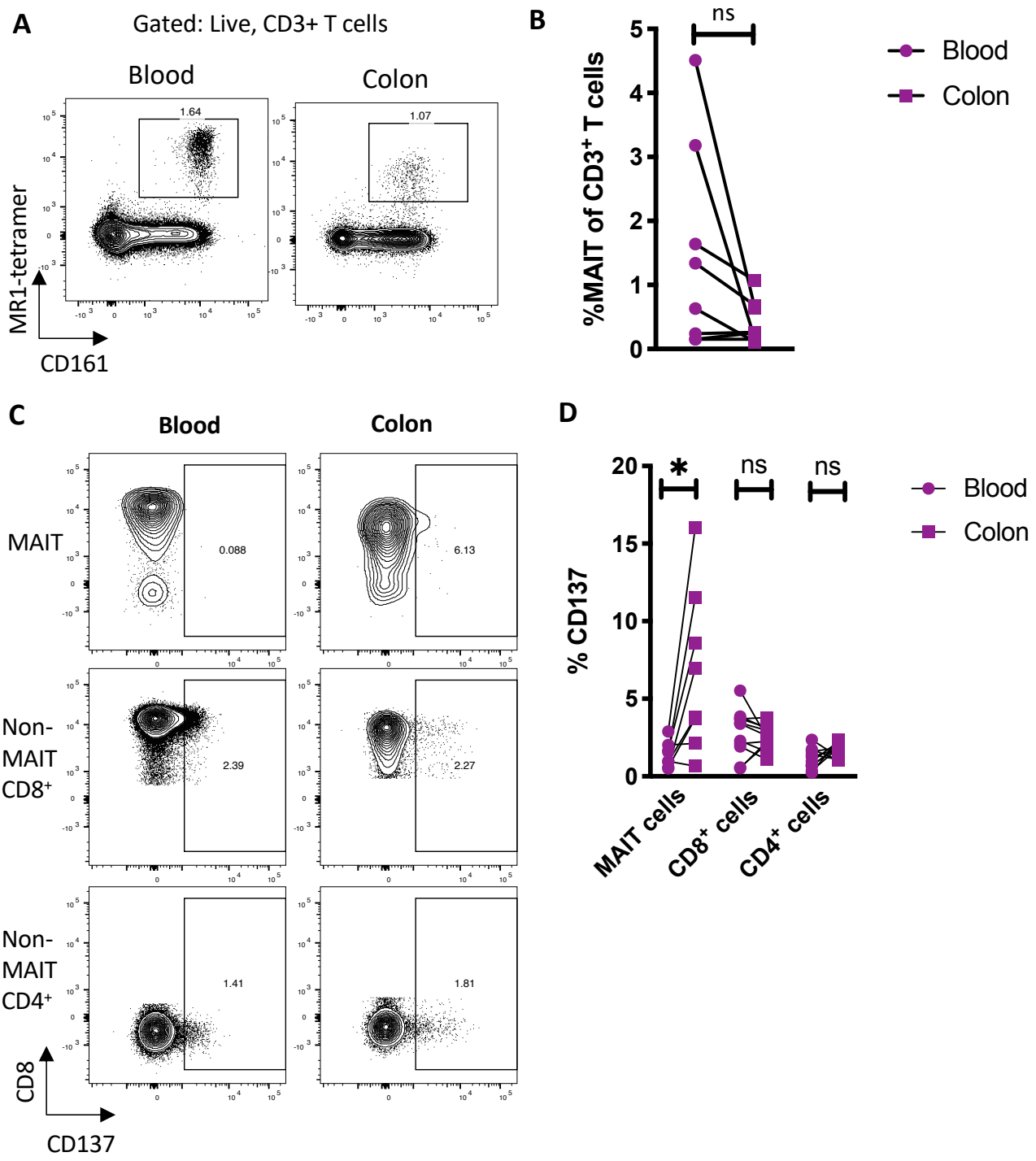

Figure 5: MAIT cells in colon display signs of recent TCR activation

(A) Representative FACS plots of the total T cell compartment for 5-OP-RU MR1 tetramer+ and CD161+ MAIT cells of the are shown from donor-matched blood and colon. (B) MAIT cell frequencies in matched blood (purple circles) and colon (purple squares) (C) Representative FACS plot and (D) quantification of frequencies of CD137 on MAIT cells, non-MAIT CD8+ T

cells, non-MAIT CD4+ T cells. Lines connect donor-matched samples. n=8 Wilcoxon matched-paired signed rank tests were performed. *p ≤ 0.05 **p ≤0.01 ***p ≤ 0.001.

Colonic MAIT cells are activated without a cytotoxic phenotype

Because my data suggest that MAIT cells in the colon are receiving a TCR signal (**Fig. 5D**), I wanted to further phenotype these cells compared to donor matched blood for additional markers of activation. HLA-DR upregulation on T cells has been extensively studied as a general marker of activation from multiple stimuli. I found that MAIT cells in the colon had increased HLA-DR-expressing cells compared to matched blood, while both non-MAIT $CD4^+$ and $CD8^+$ T cells in the colon had similar HLA-DR-expressing cells between blood and tissue **(Fig. 6 A, B)**. This suggests the colonic tissue environment increases MAIT cells activation.

The checkpoint inhibitor PD-1 is expressed on memory T cells after persistent TCR signaling in the context of chronic viral infection and cancer (101). I next tested for PD-1 expression on MAIT cells and in reference T cell populations in the blood and colon to understand if these cells could be continuously receiving a TCR signals in the healthy mucosa. MAIT cells in the colon had increased PD-1 expression compared to blood, while PD-1 expression was lower and not differentially expressed between blood and tissue on non-MAIT $CD8^+$ T cells **(Fig. 6C, D).** Interestingly, despite low CD137 expression observed on non-MAIT CD4+ T cells, this T cell compartment exhibited heightened expression of PD-1 suggesting these cells could still be seeing cognate antigen within healthy tissues **(Fig. 6D)**. These data suggest that MAIT cells and non-MAIT $CD4^+$ T cells in the colon could be continuously responding to commensal products from the microbiota.

MAIT cells require both antigen and proinflammatory cytokines including IL-12, IL-15, and IL-18 for sustained cytotoxic function (43). Healthy intestinal tissues possess a basal level of inflammation (102), but it is unknown if levels are sufficient to initiate an effector program in

MAIT cells *in vivo.* Since these data suggest MAIT cell activation occurs in the colon, I next inquired if colonic MAIT cells could exhibit a cytotoxic phenotype. Thus, I tested production of the intracellular cytolytic molecule granzyme B on MAIT cells in colon compared to non-MAIT CD8s as a positive control since the effector memory subset is known to be cytotoxic (103) **(Fig. 6E)**. MAIT cells had markedly less granzyme B-producing cells than non-MAIT $CD8^+$ T cells **(Fig. 6F)**. Thus, these data suggest that the healthy colonic tissue environment increases MAIT cell activation, yet the vast majority of these cells remain non-cytotoxic.

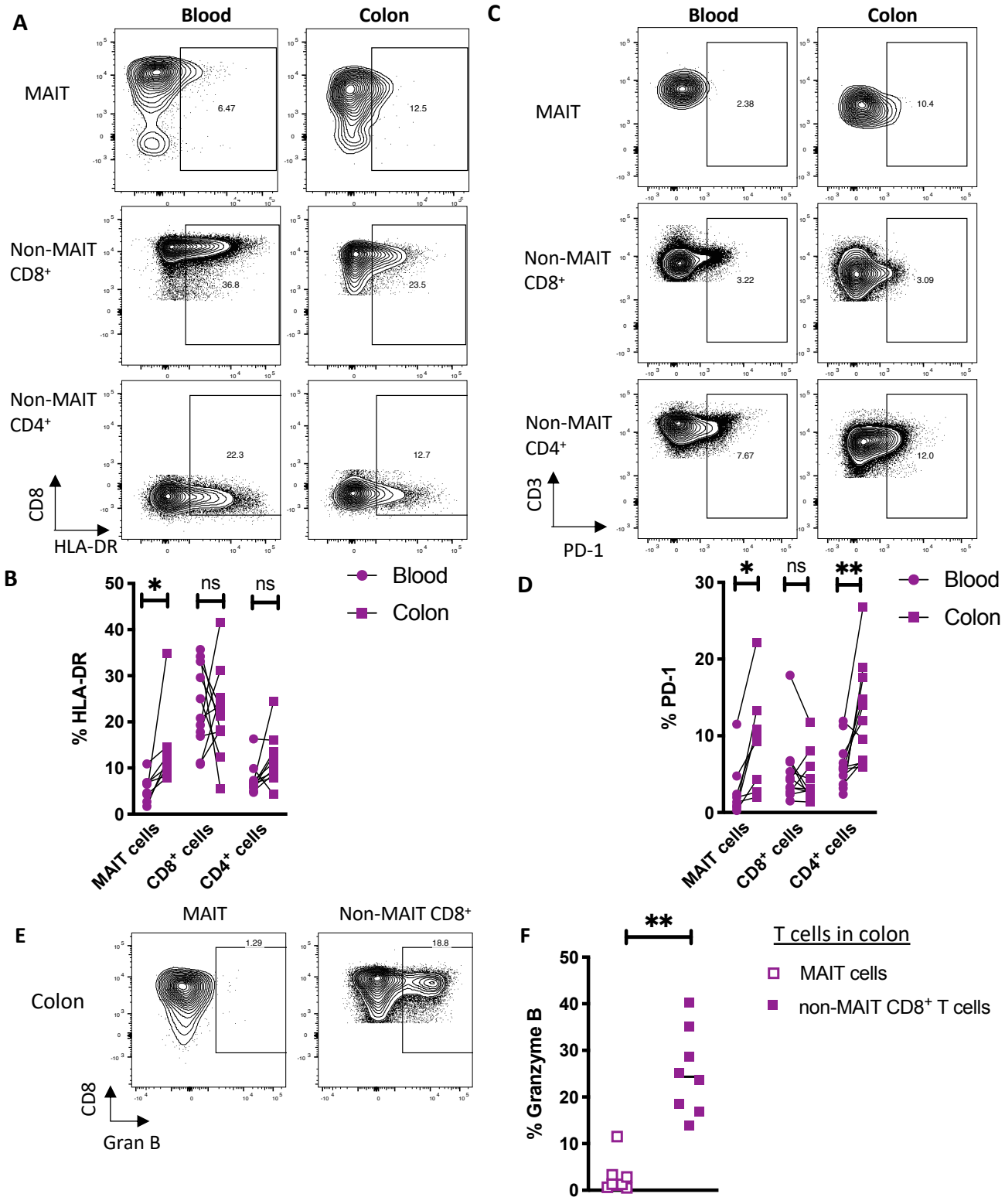

Figure 6: Colonic MAIT cell are activated without a cytotoxic phenotype

Representative FACS plot and quantification of frequencies of HLA-DR (A, B) and PD-1 (C,D) on MAIT cells, non-MAIT CD8$^+$ T cells, non-MAIT CD4$^+$ T cells. (E,F) Intracellular Granzyme

B expression on MAIT cells and non-CD8$^+$ T cells in the colon. Open purple squares represent MAIT cells from colon while closed purple squares represent non-CD8$^+$ T cells in the colon. Lines connect donor-matched samples. n=8 Wilcoxon matched-paired signed rank tests were performed. *$p \leq 0.05$ **$p \leq 0.01$ ***$p \leq 0.001$.

Tissue resident MAIT cells express markers of activation

Antigen exposure in peripheral tissues can initiate a program for tissue resident memory T cells (Trm), a functionally distinct subset that are retained in tissues long-term, which provides localized protective immunity and surveillance (104-106). Because my data suggest colonic MAIT cells exhibit markers of activation (**Figs 5 and 6**), I next asked if these cells could display a phenotype of tissue residence. Biomarkers for Trm are controversial with some studies showing CD69 expression is sufficient to identify this population (79), while others include co-expression of both CD69 and CD103 (107, 108). Importantly, CD69 also serves as a marker of activation (109) and I have shown MAIT cells possess an activated phenotype in the tissue. Therefore, for this study, I chose to focus our analysis on the CD69$^+$CD103$^+$ Trm population keeping in mind that we could be underestimating the Trm compartment. Non-MAIT T cells and MAIT cells in the blood and colon were assessed for CD69 and CD103 expression **(Fig. 7A)**. Some MAIT cells in the blood expressed CD69 which indicates some of these cells could be activated. In the colon, the CD69$^+$CD103$^+$ Trm population (red bars) on average constituted 26.34% (19.39 SD) of the MAIT population compared to 61% (7.3 SD) of non-MAIT CD8$^+$ T cells and 6.65% (2.5 SD) of non-MAIT CD4$^+$ T cells **(Fig. 7C)**. These data suggest that at least a proportion of MAIT cells possess a Trm phenotype and could be retained in the tissue indefinitely or for prolonged periods.

I next interrogated the MAIT cell population in the colon to understand if the cells with a Trm phenotype were activated compared to non-Trm MAIT cells. Cells were split into a Trm and

non-Trm phenotype defined by CD103 expression since the majority of cells in the tissue express CD69 **(Fig. 7C)**. $CD103^-$ and $CD103^+$ MAIT cells and non-MAIT T cells were assessed for CD137, HLA-DR, and PD-1 expression in the colon **(Fig 7 D,E,F)**. Colonic $CD103^+$ MAIT cells had a significantly higher frequency of CD137 expressing cells compared to $CD103^-$ MAIT cells suggesting MAIT cells with a Trm phenotype may be receiving a TCR signal in healthy colon **(Fig 7D)**. While no significant difference was observed in HLA-DR expression between $CD103^-$ and $CD103^+$ MAIT cells, the frequency of PD-1 expressing cells was significantly increased among $CD103^+$ MAIT cells **(Fig 7E, F)**. Similarly, non-MAIT $CD8^+$ T cells showed increased PD-1 expression among the $CD103^+$ cells compartment **(Fig 7F)**. These data therefore indicate that a population of MAIT cells in the healthy colon may be residing long-term in the tissue and continuously responding to commensals during homeostasis.

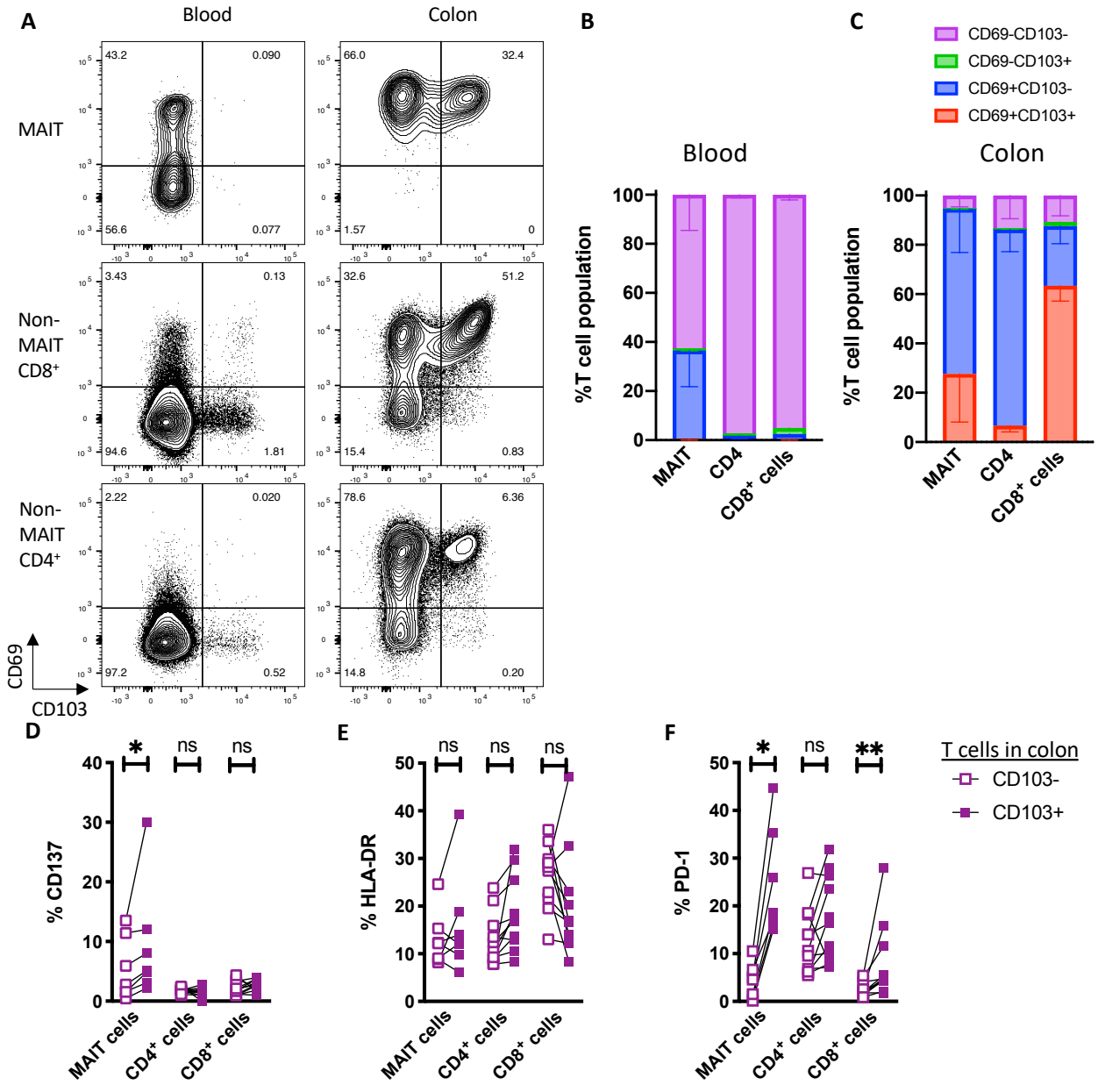

Figure 7: Tissue resident MAIT cells express markers of activation

(A) Expression CD103 and CD69 is shown for MAIT cells, non-MAIT CD4$^+$ T cells, and CD8$^+$ T cells populations. Quantification of CD103 and CD69 populations on T cell populations is displayed for blood (B) and colon (C) samples. T cell populations in the colon were gated as CD103$^+$ (closed purple squares) and CD103$^-$ (open purple squares) and assessed for CD137 (D), HLA-DR (E), PD-1 (F) expression. Open purple squares represent MAIT cells from colon while closed purple squares represent non-CD8+ T cells in the colon. Lines connect donor-matched samples. n=8 Wilcoxon matched-paired signed rank tests were performed. *$p \leq 0.05$ **$p \leq 0.01$ ***$p \leq 0.001$.

Discussion

It is becoming more appreciated that the microbiota and resident immune cells within mucosal tissues have a continuous dialogue and the results of our study indicate that MR1 expressing cells and MAIT cells actively participate at this interface. My analysis shows that among all immune cell subsets characterized (monocytes, B cells, conventional and plasmacytoid DCs, and T cells), MR1 surface expression was heightened among cells residing in the colon compared to matched peripheral blood. In addition, our data suggest that some $MR1^{+}$ cells in the colon are presenting MAIT cell antigen because colonic MAIT cells displayed markers of activation with increased expression among those with a Trm phenotype. These results indicate that MAIT cells reside long-term in the colonic tissue, are continuously receiving signals through the TCR or cytokines, and are therefore engaged in barrier immunity within healthy issues.

These data suggest that multiple immune subsets in the colon showed the capability to take up exogenous ligands for stabilization of surface MR1 including monocytes, dendritic cells (pDCs and cDCs), B cells and T cells. MR1 expression among multiple subsets, including non-phagocytic T cells, suggests a universal ability of immune cells to acquire microbial small molecules to potentially present to MR1-depedent T cells. Across these various cell types, MR1 surface expression was increased in the colon compared to peripheral blood which points to an abundance of MR1 ligands in the mucosa. Ligands for MR1 encompass a range of microbial-derived and synthetic metabolites though I do recognize the possible existence of undiscovered endogenous MR1 ligands (110, 111). The dense microbial colonization on mucosal tissues, especially within the colon, point to a more likely scenario that the MR1 expression we observe in the colon is due to microbial products from commensals. Importantly, natural MR1 ligands

have been described that do not stimulate MAIT cells. These ligands include those derived from degradation products of folic acid(21, 32). Follow-up studies should test whether colonic immune cells can activate MAIT cells isolated from the blood to understand whether MR1-expressing cells in the tissue possess MAIT antigen with agonist properties. Blocking MR1 with anti-MR1 antibody would be essential to show if activation was MR1-dependent.

MAIT cells in the colon presented an activated phenotype suggesting that some of the surface MR1 expressed by colonic immune cells could possess MAIT antigen. Similarly, MAIT cells isolated from different healthy mucosal tissues sites have also presented an activated phenotype including from female genital tract and oral mucosa(50, 77). However, it is important to note that initiation of T cell activation markers could result from TCR-independent signals such as exposure to cytokines. Cytokines have been shown to induce expression of T cell activation markers including PD-1 and HLA-DR (112, 113). Assessing gene expression data between MAIT cells in the tissue and matched blood could potentially reveal differential expression of genes indicative of TCR signaling in the tissue.

$CD103^+$ Trm MAIT cells in the colon had increased expression of CD137 and PD-1 suggesting these cells could be continuously stimulated in healthy colon. The discrepancy in activation markers between $CD103^+$ Trm and $CD103^-$ non-Trm MAIT could be for various reasons. Though the MAIT cell population has a highly restricted TCR repertoire and appears to be activated by that same potent antigen, TCR variation does exist in this population that could cater to some antigenic variation (34, 114). TCR sequencing of $CD103^+$ Trm and $CD103^-$ non-Trm MAIT cells within peripheral tissues might reveal bias in certain clonotypes between these populations. Alternatively, given the importance of CD103 to binding the epithelium (115), another explanation could be that $CD103^+$ MAIT cells and $MR1^+$ APCs are located in the

epithelium and consequently, closer to the microbiota. Investigating the tissue localization of these subsets could illuminate which cells are physically interacting in the tissue though follow-up studies using *ex vivo* stimulation should confirm any potential APC-to-MAIT cell interactions.

Our work supports a growing body of literature that the immune system and microbiota are continuously interacting during homeostasis. MAIT cells do not just serve as antibacterial cells poised to eliminate intracellular pathogens but are also actively involved as sentinels of the commensal microbiota. MAIT cells are likely active players in the constitutive sensing of commensals due to their tissue resident phenotype and activation status in healthy mucosa.

Chapter IV:
Inflammatory cytokines induce sustained CTLA-4 cell surface expression on MAIT cells

Introduction

Mucosal-associated invariant T (MAIT) cells acquire effector function when exposed to bacterial metabolites (presented by MR1) in the presence of inflammatory cues (116), both of which are present during bacterial infections. Importantly, inflammatory cues are sufficient to activate MAIT cells and induce expression of effector molecules including granzyme B and interferon-γ (IFN-γ). This has been demonstrated using primary human MAIT cells in short term *ex vivo* experiments using IL-12, IL-15, and IL-18 (56, 116, 117), but also directly *ex vivo* in the context of viral infections including dengue, influenza and hepatitis C (62). There is also evidence that infection with the parasite *Plasmodium falciparum* can induce some MAIT cell activation in humans, presumably in the absence of T cell receptor (TCR) agonist signals (45). This inflammation-driven, TCR-independent mechanism of activation is akin to the phenomenon of "bystander-activation" in conventional memory T cells (59, 118). The physiological significance and the consequences of inflammation-driven activation of MAIT cells are still largely unclear. However, the distinction between TCR-mediated versus inflammation-driven T cell activation is important for conventional T cells, since TCR signals control the expression of co-stimulatory and co-inhibitory receptors. Inflammation-driven activation does not elicit expression of the same co-inhibitory receptors (118), which could explain how "bystander-activated" conventional memory T cells contribute to tissue damage and pathology for prolonged time periods.

The co-inhibitory molecule CTLA-4 is induced on conventional T cells following TCR stimulation and constitutively expressed on regulatory T cells (119, 120). Among co-inhibitory

molecules CTLA-4 is noteworthy in that it shares its ligands B7-1 (CD80) and B7-2 (CD86) with the co-stimulatory molecule CD28. CTLA-4 binds these ligands with greater avidity and affinity than CD28, which allows the inhibitory signal to outcompete the stimulatory signal and turn off the T cell response (120). It has been proposed that CTLA-4-mediated inhibition can act cell-intrinsically via interaction of phosphatases with its cytoplasmic domain as well as cell-extrinsically by reducing B7-1 and B7-2 availability and thus interfering with the co-stimulatory function of CD28 (120, 121). Continual TCR signals as encountered in the context of chronic infections or cancer eventually lead to T cell dysfunction including the loss of effector function (122).

I asked if CTLA-4 or other immunoregulatory mechanisms are in place to curtail MAIT cell effector function following activation of MAIT cells by either TCR-mediated signals or inflammatory cytokines. I report here that surface expression of CTLA-4 on MAIT cells is induced by inflammatory cytokines independently of the TCR. This indicates that the signals controlling surface CTLA-4 expression on MAIT cells occur independently of the TCR – NFAT signaling axis and thus control of CTLA-4 expression is fundamentally different between MAIT cells and conventional T cells.

Results & Discussion

MAIT cells are present in healthy and inflamed oral mucosal tissue

To identify cell intrinsic regulatory mechanisms of MAIT cells that are physiologically relevant, we chose to examine MAIT cells isolated from human oral mucosal (OM) tissues. I specifically used gingival tissues obtained from surgically discarded tissue. I chose gingival tissues to address this question since periodontic surgeries are routine procedures and yield tissues with a range of inflammation (healthy/minimally inflamed to severely inflamed). A piece of each analyzed tissue was saved for a blinded evaluation by a pathologist to assess the extent of inflammation (see Methods). The remaining tissue was processed to acquire a single-cell suspension for subsequent analysis by flow cytometry. MAIT cells were identified as live, $CD3^+$, $V\alpha7.2^+$ and $CD161^{hi}$ cells in the OM and matched blood (**Fig 8A**). MAIT cells in some blood and mucosal samples were also identified using the MR1 tetramer (loaded with the 5-OP-RU) (123), which yields a largely but not fully congruent population with the $V\alpha7.2^+CD161^{hi}$ definition of MAIT cells (**Fig 9**) as previously reported (124). To provide consistency with previous studies, I defined MAIT cells as $V\alpha7.2^+CD161^{hi}$ in our study and acknowledge that this that this population does not include MR1-restricted $V\alpha7.2^-$ cells. I next assessed the distribution of CD4 and CD8 expression within the MAIT cell population and found that a majority expressed CD8α (**Fig 9B**), as previously reported from blood (12), lymph (124) and other mucosal barrier tissues (50) including buccal tissue (125), indicating that general distribution of MAIT cell subsets in OM is comparable to other tissues. MAIT cell frequencies (as a % of $CD3^+$ T cells) in both healthy and inflamed OM tissues were overall similar to donor-matched blood (**Fig 9C**), although we observed a slight, but statistically significant decrease of MAIT cell frequencies in inflamed tissues. I next examined if MAIT cells in inflamed tissues expressed biomarkers indicative of activation and/or tissue-residence, CD69 and CD103 (126). I found that

CD8$^+$ MAIT in inflamed OM expressed one or both of these receptors indicating a possible tissue-resident phenotype similar to conventional CD8$^+$ T cells (**Fig 8D, Fig10A**). The presence of CD8$^+$ MAIT cells with a tissue-resident phenotype also suggests that MAIT cells remain in tissues for long periods of time and thus require cell intrinsic immunoregulatory mechanisms.

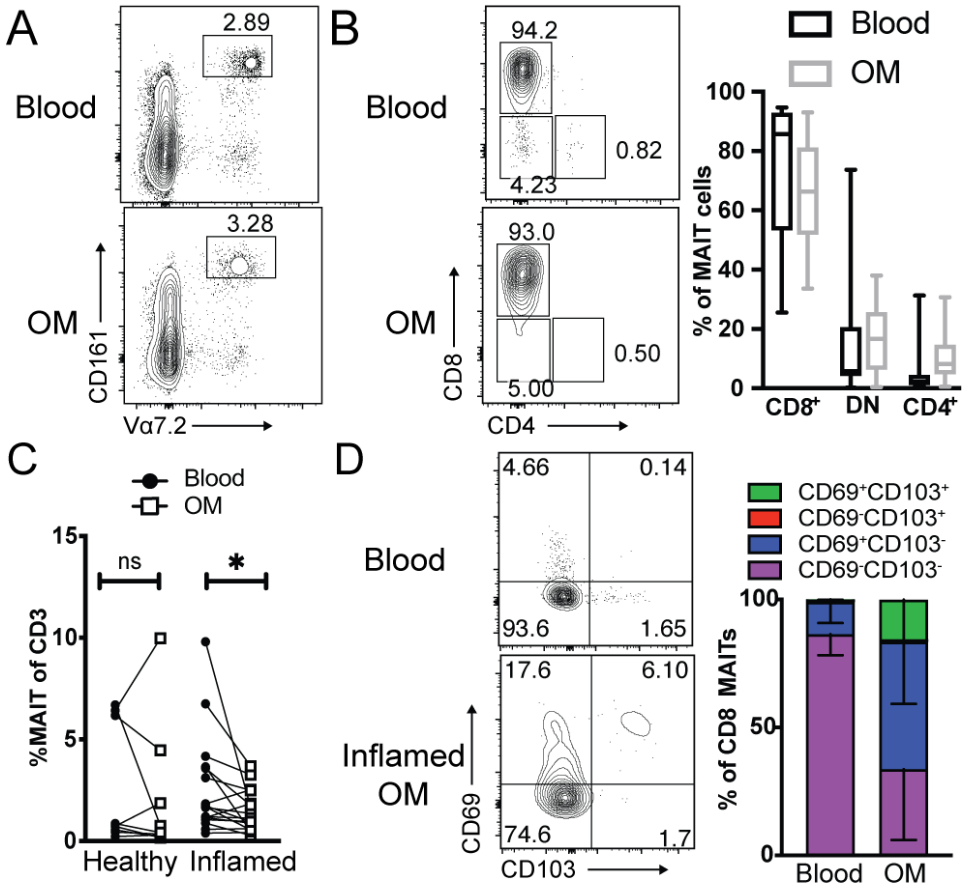

Figure 8: CD8$^+$ MAIT cells are present in healthy and inflamed tissues and express markers of tissue residency

(A) Representative FACS plots of Vα7.2$^+$CD161^{hi} MAIT cells of the total T cell compartment are shown from donor-matched blood and oral mucosa (B) MAIT cell subsets are displayed based on CD4 and CD8 co-receptor staining. (C) MAIT cell frequencies in matched blood (black circles) and inflamed or healthy OM (white squares) (healthy n=9, inflamed n=18) (D) CD69 and CD103 expression on CD8$^+$ MAIT cells from inflamed tissues (n= 11). Wilcoxon matched-paired signed rank tests were performed. *p ≤ 0.05 **p ≤0.01 ***p ≤ 0.001.

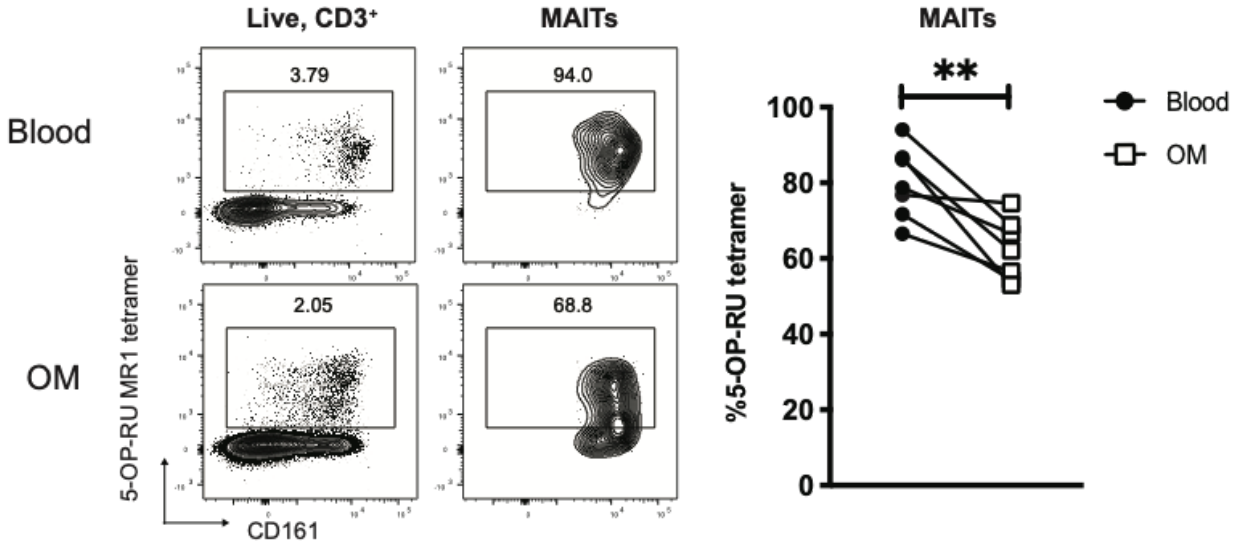

Figure 9: 5-OP-RU MR1 tetramer expression on MAIT cells in oral mucosa and matched blood

Representative staining of 5-OP-RU MR1 tetramer on total CD3+ cells (to determine gates) and MAIT cells ($V\alpha7.2^{+}CD161^{hi}$) from OM and matched blood. Tetramer staining on the total MAIT cell population quantified (n=7). Wilcoxon matched-paired signed rank tests were performed. *p ≤ 0.05 **p ≤0.01 ***p ≤ 0.001.

Distinct transcriptional profiles of $CD8^{+}$ MAIT cells from inflamed oral mucosa and matched blood

To identify immunomodulatory genes expressed by MAIT cells in the OM, I examined the $CD8^{+}$ MAIT cell population in the blood vs. healthy and inflamed OM by RNA-seq. PCA analysis did not reveal any striking differences between $CD8^{+}$ MAIT cells isolated from healthy vs. inflamed tissues (**Fig 11A**). In contrast, the differences between donor-matched blood and tissue MAIT cells were apparent as shown in a heat map showing the 56 differentially expressed genes between $CD8^{+}$ MAIT cells from OM and blood (**Fig. 11B**). I was initially surprised by the similarity of expression profiles of MAIT cells isolated from healthy and inflamed OM. However, it is noteworthy that even healthy gingival tissue has a basal level of inflammation that is referred to as "homeostatic inflammation" (127). Thus, these similarities of gene expression

may reflect the exposure of MAIT cells to a steady state of inflammatory processes. Furthermore, we found that MAIT cell isolated from the OM had a gene expression signature (compared to blood) indicative of tissue-residence (79) (**Fig 11B**), which aligned with our flow cytometry data (**Fig. 8D**). These data highlight that healthy tissues are a more appropriate reference baseline than blood to truly discern the effect of tissue pathologies on MAIT cells and that single-cell resolution is likely required to identify MAIT cell populations with varying degrees of dysfunction during inflammation.

I next screened the differentially expressed genes between MAIT cells from OM (healthy and inflamed tissues combined) and blood for genes associated with regulatory or inhibitory functional properties (**Fig 11C**). I found genes that negatively regulate TCR co-stimulation or activation including *CTLA4* and *TIGIT* (128) as well as genes (*IRF4* and *BATF*) that mediate expression of inhibitory receptors in conventional CD8 T cells (129). *CTLA4* was one of the most highly differentially expressed genes suggesting it may play a key role in regulating MAIT cell function similar to its role in conventional T cells.

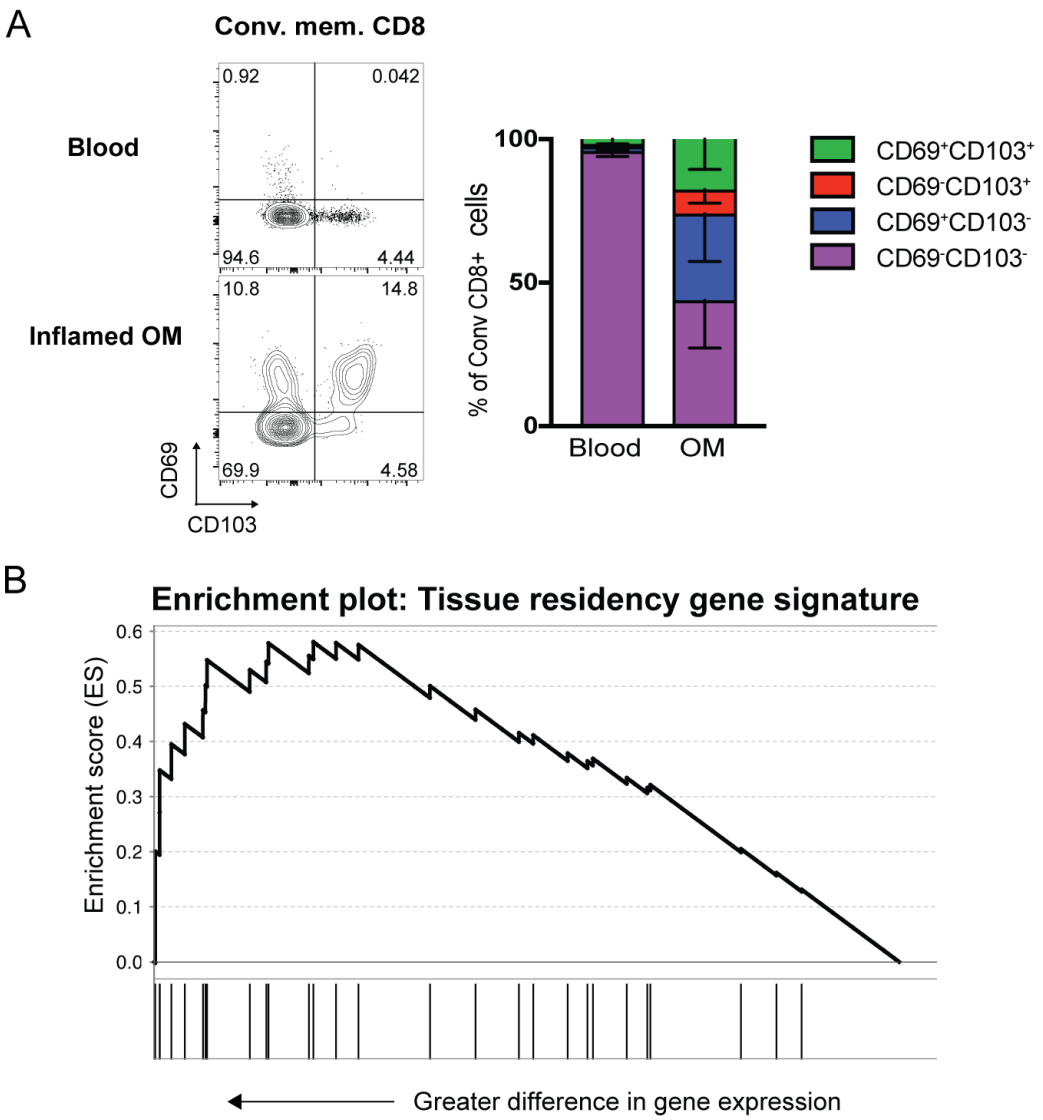

Figure 10: Tissue-resident memory population among CD8+ conventional and MAIT cells in oral mucosa

(A) Representative staining and quantification of CD69 and CD103 expression on CD8+ non-MAIT T cells (CD45RA-) within inflamed oral mucosa and matched blood (n= 18). (B) Gene set enrichment analysis for human resident memory T-cell gene signature (Kumar et al) on CD8+ MAIT cells between blood and OM. The Y-axis is the enrichment score and the X-axis is genes ranked according to the absolute value of the log fold change between the CD8+ MAIT OM vs. blood sample. $p < 0.001$ for the core signature enrichment

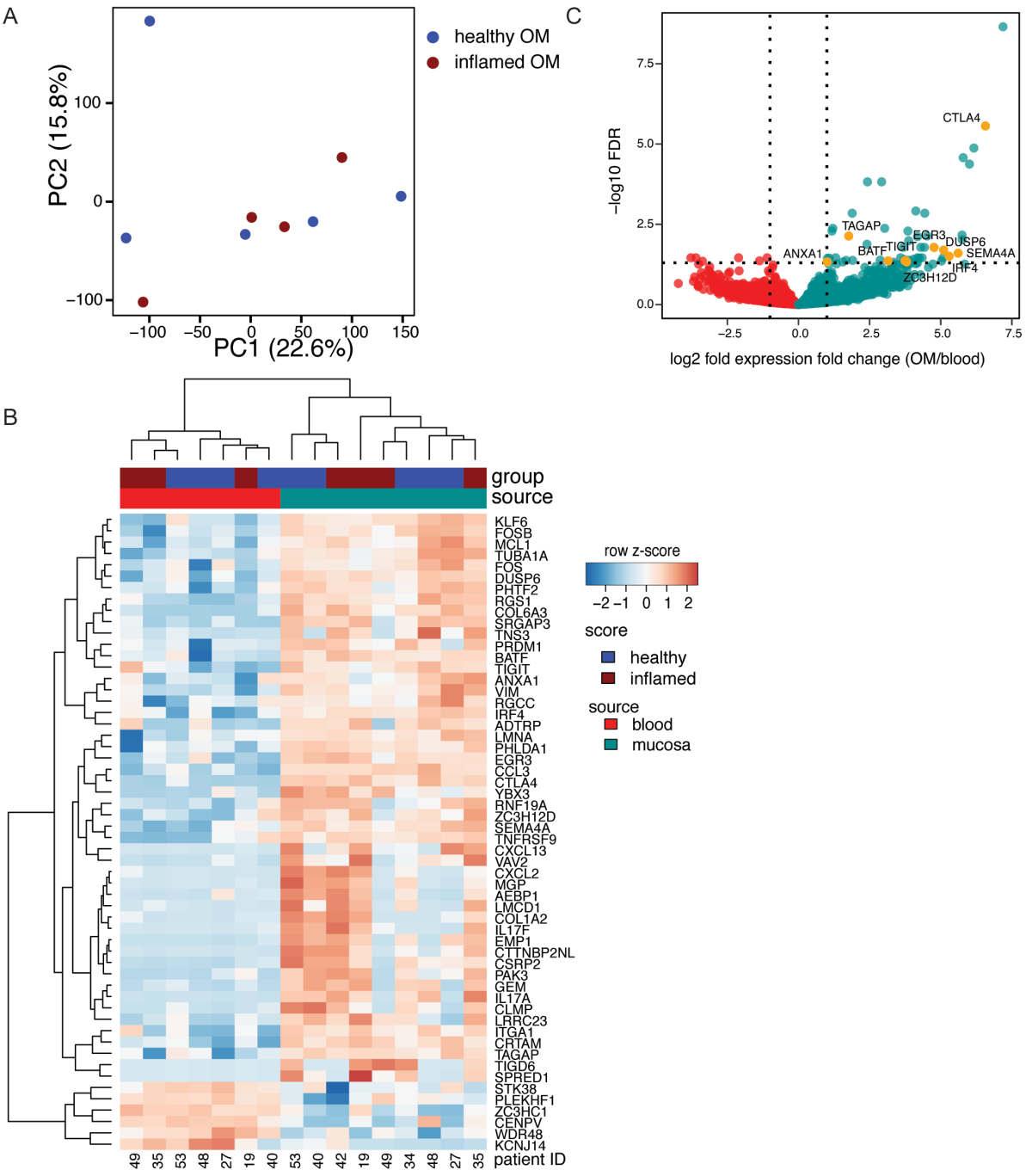

Figure 11: CD8^{+} MAIT cells in oral mucosa express transcripts associated with immunomodulatory function including CTLA-4

CD8+ MAIT cells were sorted from healthy or inflamed oral mucosa and donor-matched blood for bulk RNA-sequencing. (A) Principal component analysis of global gene expression of oral mucosa samples is color-coded based on inflammation category. n= 8 donors (B) Heatmap showcasing top 56 differentially expressed genes using fold-change cutoff of at least 2 (FDR ≤ 0.05). Red indicates an increase in gene expression in one sample relative to the other, while blue indicates lower expression. (C) Volcano plot showing FDR on Y axis and log2 fold change on X axis with genes associated with immunoregulatory function highlighted in orange.

Inflammatory cytokines are sufficient to trigger CTLA-4 expression on MAIT cells

CTLA-4 protein is expressed by conventional T cells following TCR stimulation and constitutively expressed by regulatory T cells (130, 131). Importantly, expression of CTLA-4 protein on the cell surface is regulated by the phosphorylation status of its intracellular domain with most of the protein being located intracellularly in resting T cells (132). Since expression of CTLA-4 on the cell surface is critical for exerting its regulatory function, we assessed the cell surface expression of CTLA-4 on $CD8^+$ MAIT cells in blood and OM directly *ex vivo*. Analysis by flow cytometry revealed significantly increased expression of CTLA-4 on the cell surface of $CD8^+$ MAIT cells isolated from OM compared to blood, which was observed in healthy as well as inflamed tissues (**Fig 12A, B**). Regulatory T cells (Tregs) were measured as a positive control and conventional CD8 memory T cells (defined as non-MAIT $CD45RA^-$) were included as a reference population (**Fig 12A-C**). These data demonstrate *ex vivo* CTLA-4 protein expression on the cell surface of a MAIT cell population in OM tissues and highlight a potential regulatory mechanism for MAIT cell activation.

I next wanted to define which signals are necessary for CTLA-4 expression on the MAIT cell surface. To address this, peripheral blood mononuclear cells (PBMCs) from healthy donors were stimulated for 24 hours *ex vivo* with either anti-CD3/CD28 beads, a combination of recombinant IL-12/15/18 or both signals. CTLA-4 expression on $CD8^+$ MAIT cells was compared to conventional memory $CD8^+$ ($CD45RO^+$) and Tregs. All three T cell populations increased expression of CTLA-4 after incubation with anti-CD3/CD28 beads (**Fig 12D**), but only $CD8^+$ MAIT cells also induced surface expression of CTLA-4 following incubation with IL-12/15/18 (**Fig 12D)**. Stimulation of T cells with anti-CD3/CD28 beads did result in TCR downregulation, which affected subsequent detection by flow cytometry (data not shown). Thus,

the anti-CD3/CD28 experimental condition serves primarily as a positive control for conventional T cells, but should not be directly compared to the IL-12/15/18 condition in regards to its ability to induce CTLA-4. Importantly, addition of an anti-MR-1 blocking antibody did not affect CTLA-4 expression in IL-12/15/18 stimulated MAIT cells formally excluding the possibility that MAIT cells received a TCR signal during the culture **(Fig 12E)**. Together, these data provide strong evidence that inflammatory signals are sufficient to induce expression of CTLA-4 on the cell surface of MAIT cells. A TCR-independent induction of CTLA-4 cell surface expression on MAIT cells is noteworthy as it indicates that CTLA-4 expression is regulated by distinct signaling pathways in MAIT and conventional T cells. This cytokine-driven, TCR-independent regulation of CTLA-4 expression could ensure that MAIT cells in inflamed tissues restrain deleterious inflammatory responses against commensal organisms.

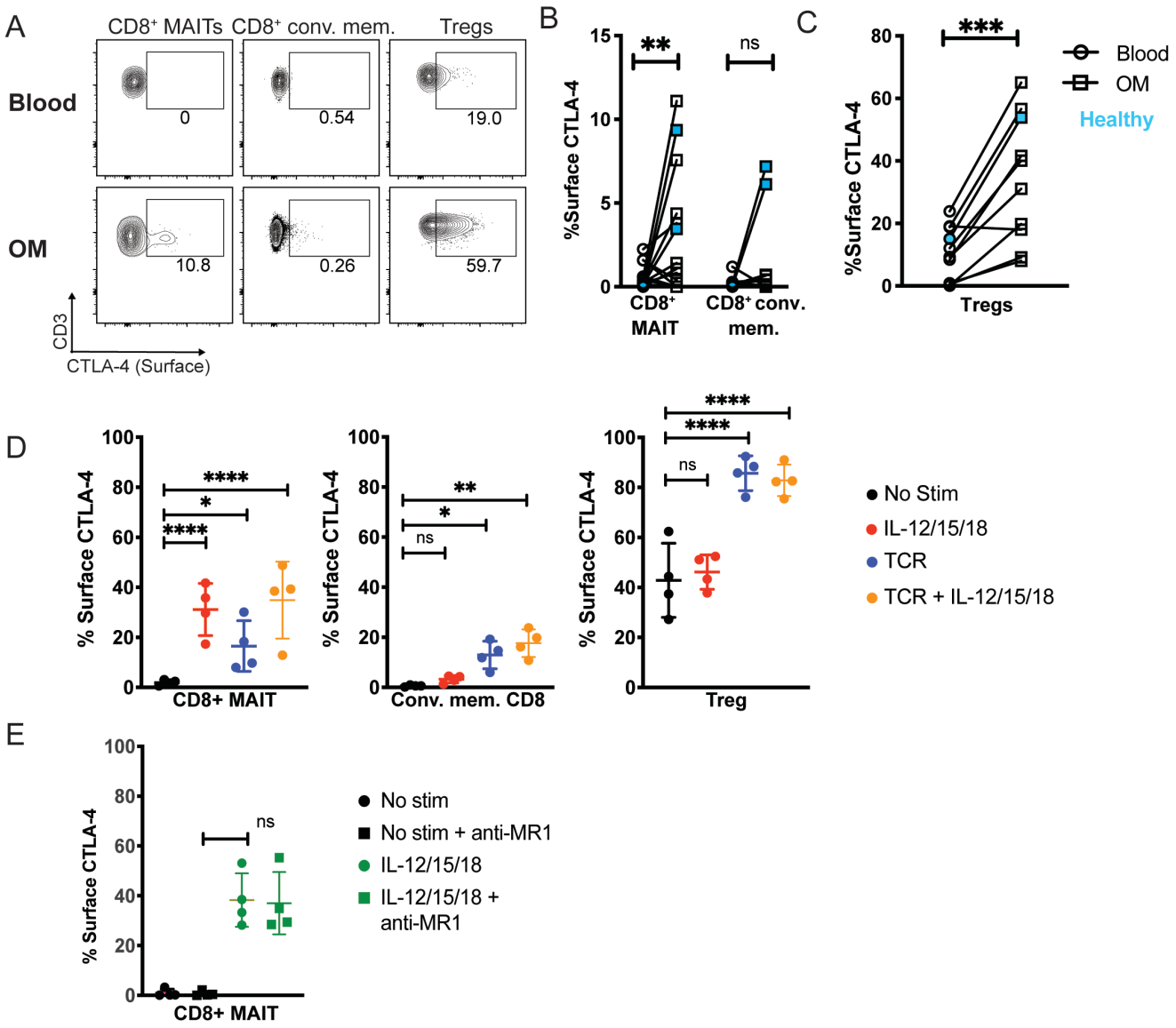

Figure 12: Inflammatory cytokines induce surface expression of CTLA-4 on CD8⁺ MAIT cells

(A-C) Ex vivo surface CTLA-4 expression on CD8⁺ MAIT cells, CD8⁺ memory T cells (non-MAIT, CD45RA⁻), and regulatory T cells (Treg) ($CD3^+CD4^+IL\text{-}7R\alpha^{lo}CD25^{hi}Foxp3^+$) in donor-matched blood (black circles) and OM (white squares) from n=12 patients (D) Peripheral blood mononuclear cells (PBMCs) were rested (black circle) stimulated with recombinant cytokines IL-12, 15, and 18 (100ng/mL) (red circle), anti-CD3/CD28 beads (blue circle) or anti-CD3/CD28 beads + recombinant cytokines IL-12, 15, and 18 (orange circle). CTLA-4 expression on the cell surface of CD8⁺ MAIT cells, CD8⁺ memory T cells (non-MAIT, CD45RO⁺), and Tregs was measured by flow cytometry. (E) PBMCs were left untreated or treated with cytokines and either with or without anti-MR1 (50µg/mL) Two-way ANOVA with Dunnett's multiple comparisons test were performed in D while Wilcoxon matched-paired signed rank tests were used in B and C. *$p \leq 0.05$ **$p \leq 0.01$ ***$p \leq 0.001$.

Brief exposure to inflammatory cytokines is sufficient to induce sustained CTLA-4 expression by MAIT cells

If CTLA-4 on mucosal MAIT cells was critical for maintaining healthy mucosal homeostasis, I hypothesized that its expression might be maintained for an extended time frame following inflammatory cues in order to restrain damaging immune responses through cell intrinsic and/or extrinsic mechanisms. To determine whether cytokine-induced CTLA-4 surface expression was maintained following cytokine withdrawal, I incubated MAIT cells for 6 hours with either IL-12/15/18, anti-CD3/CD28 beads or both stimuli **(Fig. 13A)**. After 6 hours the stimuli were removed by washing and cells were either analyzed **(Fig. 13B)** or cultured for another 12hrs or 18hrs in absence of stimulation before examining CTLA-4 surface expression **(Fig. 13C, D)**. I found that CTLA-4 was not expressed after 6hrs of stimulation **(Fig. 13A),** but this brief (6hrs) exposure to cytokines in the absence of a TCR signal was sufficient to induce expression of CTLA-4 on $CD8^+$ MAIT cells 12hrs **(Fig. 13C)** and 18hrs later **(Fig. 13D)**. These data suggest that a program for surface expression of CTLA-4 is quickly exerted after exposure to inflammatory stimuli. In addition to the cis-acting inhibitory properties of CTLA-4, it has been suggested that CTLA-4 trafficking and expression on Treg can also inhibit priming of other T cells in trans by competing for availability of B7-1/2 on APCs (121, 133). Whether the role of CTLA-4 on MAIT cells is to restrain their own proinflammatory responses or to preclude neighboring T cells from receiving co-stimulation similar to what has been proposed for Tregs will require further studies.

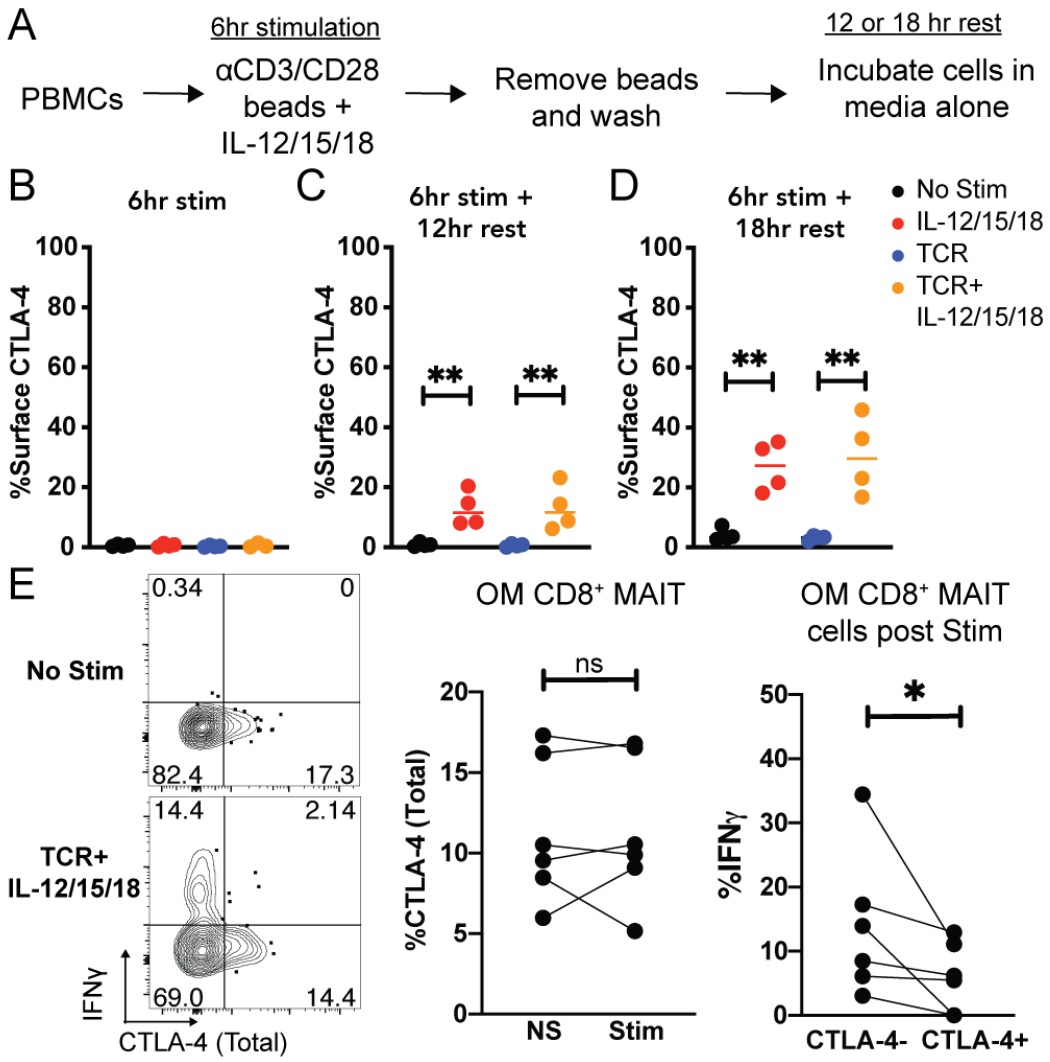

Figure 13: Brief exposure to inflammatory cytokines is sufficient to induce CTLA-4 expression by MAIT cells

(A) Outline of the experimental workflow (B) CTLA-4 expression on cell surface of $CD8^+$ MAIT cells after 6 hour stimulation of PBMCs either (B) immediately, or (C) 12hr and (D) 18hr post removal of beads and cytokines. n = 4 (E) A single cell suspension of cells isolated from the oral mucosa was stimulated with recombinant cytokines IL-12, 15, and 18 (100ng/mL) and anti-CD3/CD28 beads for 7 hours. CTLA-4 and IFNγ expression by $CD8^+$ MAIT cells was measured with intracellular cytokine staining, (n=6 donors).

Finally, I sought to determine if CTLA-4^+ MAIT cells isolated from OM tissue could respond to activating stimuli when CTLA-4 is bypassed by direct engagement of CD28. To test this, we stimulated cells isolated from the OM with anti-CD3/CD28 beads and cytokines (IL-12/15/18) for 7 hours. I chose 7 hours to minimize de novo expression of CTLA-4 in these

experiments and with both stimuli to induce maximum MAIT effector function **(Fig. 13B).** For this *ex vivo* stimulation experiment, we assessed overall CTLA-4 expression (including intracellular CTLA-4) to facilitate acquisition of a sufficient number of CTLA-4-expressing MAIT cells. The detection of MAIT cells was again affected by the anti-CD3/CD28 bead stimulation (data not shown). I found that MAIT cells that expressed CTLA-4 had reduced production of IFNγ after stimulation (**Fig. 13E**) even though CTLA-4 itself was not engaged by anti-CD3/CD28 beads. These data suggest that there are other mechanisms in place that control MAIT cell function and that CTLA-4 expression by MAIT cells may be a biomarker for overall hyporesponsiveness. This functionally altered MAIT cells may also affect other immune subsets that MAIT cells interact with including B cells (134).

Together, our data provide strong evidence that inflammatory cytokines induce sustained CTLA-4 cell surface expression on MAIT cells in a TCR-independent manner, which is noteworthy given that a TCR signal is necessary for CTLA-4 expression by conventional T cells. I propose that this TCR-independent induction of CTLA-4 expression in MAIT cells serves to limit MAIT cell-mediated tissue-damage in response to commensal antigen and may be of interest for therapeutic intervention for inflammation driven tissue pathologies.

Chapter V:
Final perspectives and future directions

The thesis work presented here has investigated the MAIT cells that reside in mucosal tissues and their peripheral blood counterparts in order to elucidate how MAIT cells function within microbe-rich and inflamed tissue environments. I showed surface protein expression of MR1 on multiple immune subsets from healthy colon and matched blood, indicating that these immune cells are continuously absorbing and capable of presenting secreted byproducts from commensal microbiota that could in turn, activate MR1-restricted T cells such as MAIT cells. Moreover, MAIT cells within healthy colon express markers of activation and tissue residency. I then showed that MAIT cells located in the healthy and inflamed oral mucosa (OM) compared to matched blood express gene signatures associated with tissue residency and immunoregulation including the known inhibitory receptor CTLA-4. *Ex vivo* work revealed that inflammatory cytokines alone without a TCR signal induce CTLA-4 expression uniquely in the MAIT cell population, which is not the case for conventional T cells. These results raise additional questions about MAIT cell function within mucosal barrier tissues: What is the role of MAIT cells during tissue homeostasis? What are the implications of cytokine-induced CTLA-4 on MAIT cells? This chapter will address these outstanding questions and future directions of MAIT cell research.

Role of MAIT cells during tissue homeostasis

MAIT cells have been well-described for their role as proinflammatory and cytotoxic mediators against pathogens during infectious disease (7, 9, 10, 135). However, these cells likely also have an important role coordinating the response to commensals during homeostasis due to their strategic location in barrier tissues and ability to respond to a wide variety of

microorganisms. This places MAIT cells at the center of the homeostatic immunological dialogue between the host and the microbiota, which has profound implications if any dysbiosis ensues, such as what occurs during inflammatory bowel disease. In Chapter 1, I proposed a model that showed a TCR signal alone may represent a situation where MAIT cells are responding to commensals capable of providing MAIT antigen. Data from Chapter 3 that showed increased MAIT cell activation and MR1-expressing immune cells within the colon supports this model whereby MAIT cells could be continuously presented MR1 antigens in tissues exposed to a dense microbiota. Alternatively, activation markers could be initiated in a TCR-independent manner and it is unknown if functional MR1 expression on these colonic immune cells is a result of ligands that are specific MAIT antigens with agonist properties. Therefore, the field must pursue what signals MAIT cells within healthy tissues are receiving as well as any functional consequences in order to unravel their role during tissue homeostasis. Recently published studies have analyzed global gene expression of MAIT cells after signaling through the TCR, exposure to cytokines, and a combination of both stimuli (136, 137). Future studies are needed comparing gene expression profiles of mucosal-resident MAIT cells to MAIT cells stimulated via their TCR or cytokine signaling to better understand what stimuli they may be getting within the tissue.

MAIT cells residing in the tissue may serve an important function maintaining tissue homeostasis distinct from a proinflammatory or cytotoxic role. Supporting this, human MAIT cells stimulated through their TCR showed a similar transcriptional profile to murine IL-17-producing CD8+ T cells (Tc17) that were specific for commensal flora including enrichment of a tissue repair gene signature (136-138). Moreover, MAIT cells were shown to accelerate wound healing in an *in vitro* model of epithelial wound repair, which suggests an important role in local homeostatic functions (136). It would be important to test if MAIT cells residing in tissues truly

have this function by running a gene set enrichment for the tissue repair signature on global gene expression of isolated MAIT cells from tissues compared to blood MAIT cells, as well as measuring expression of proteins associated with wound repair (Furin, VEGFA). In addition, MAIT cells residing in peripheral tissues exposed to microbial products presented a bias for production of IL-17 (13, 50, 125, 139). This is reminiscent of commensal-specific $CD4^+$ T cells in the intestine that produce IL-17 (Th17) during steady state conditions and play a dominant role maintaining barrier integrity by inducing tight junction proteins, producing antimicrobial peptides, and preventing opportunistic infections by commensals (140). MAIT cells may polarize to a type 17 program similar to T_C17 or TH17 cells within mucosal tissues in order to serve a similar role during tissue homeostasis. It is currently unknown what signals specifically induce IL-17 production by MAIT cells. To test this further, future studies should assess MAIT exposure to cytokines associated with inducing IL-17 (IL-1β, IL-6, IL-21, IL-23, and TGFβ(141)) both with and without a TCR signal.

Data showed here also support the growing body of literature that a population of MAIT cells within peripheral tissues have a Trm phenotype. In addition to identifying co-expression of Trm markers CD69 and CD103 on mucosal MAITs, I showed OM MAIT cells enriched for a gene signature of Trm, indicating long-term residence within tissues. The significance of a distinct population of MAIT cells seeded into peripheral tissues for long-term residence, is that they may be the primary MAIT population interreacting with commensals during homeostasis. Supporting this, $CD103^+$ MAIT cells in the colon had increased CD137 and PD-1-expressing cells, while $CD103^+$ MAIT cells in buccal mucosa had increased HLA-DR expression compared to $CD103^-$ cells (51). However, additional studies are needed to confirm if CD103 actually defines a distinct Trm population. Differential gene expression between $CD103^+$ and $CD103^-$

MAIT cells isolated from tissues should be investigated in order to understand if $CD103^+$ cells have enrichment of a Trm gene signature. These populations should also be assessed for additional protein markers recently identified with conventional Trm including CD101, CXCR6, CD49a (142). Since MAIT cells have been identified in afferent lymph and thus, egress from peripheral tissues (71), it would be valuable to parse out if a distinct MAIT Trm cell populations exists and if they are in fact activated during homeostasis.

Implications of cytokine-induced CTLA-4 on MAIT cells

My analysis showed MAIT cells expressing surface CTLA-4 in response to inflammatory cytokines without the need for a TCR signal, which is in contrast to our conventional understanding of CTLA-4 induction. The mechanism of inflammation-induced CTLA-4 expression on MAIT cells is important to define and could be critical for how we understand MAIT cell regulation. Studies from different chronic inflammatory settings, especially within the liver, have observed CTLA-4 expression on MAIT cells, which also frequently present with an exhausted phenotype or reduced effector functions (113, 143-145). Future studies could examine whether inflammatory cytokine-induced CTLA-4 expression on MAIT cells is sufficient to diminish their robust effector function in response to subsequent TCR stimulation. A well-established model to test this uses monocytes exposed to fixed *E. coli*, which utilizes a physiological TCR stimulus that includes expression of CD80 and CD86, molecules that are ligands for CD28 and CTLA-4. MAIT cells could first be exposed to inflammatory cytokines and then subsequently co-cultured with fixed *E. coli*-stimulated monocytes, with or without anti-CTLA-4 to block this inhibitory receptor. Production of inflammatory cytokines such as IFNγ and TNFα by MAIT cells would be the readout for effector function.

Alternatively, expression of CTLA-4 on MAIT cells could serve to function extrinsically to prevent co-stimulation of other T cells, which is an established role for regulatory T cells (121, 128). In that regard, intestinal MAIT cells have been shown to serve a regulatory or protective role during diabetes and graft-versus-host disease (146, 147). MAIT cell expression of CTLA-4 during these conditions is unknown but should be explored as a potential mechanism of suppression. CTLA-4 expression by Tregs has been shown to prevent conventional T cells from receiving CD28-mediated co-stimulation by clustering around antigen-presenting cells (148). To understand if MAIT cells cluster around APCs, CTLA-4^{+}MAIT and HLA-DR^{+} CD11c^{+} DCs could be assessed using immunhistochemistry on inflamed livers known to harbor CTLA-4-expressing MAIT cells.

By characterizing the MAIT cell phenotype within mucosal tissues and mechanisms of immunoregulation, I have demonstrated potential mechanisms for how MAIT cells can be controlled within healthy and inflamed human mucosal tissues to highlight their functional implications. This work contributes valuable insight into how MAIT cells behave *in vivo* and aids pursuits to manipulate these cells therapeutically.

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