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Inflammatory Cytokines Induce Sustained CTLA-4 Cell Surface Expression on Human MAIT Cells

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ABSTRACT

Mucosal-associated invariant T (MAIT) cells acquire effector function in response to proinflammatory signals, which synergize with TCR-mediated signals. We asked if cell-intrinsic regulatory mechanisms exist to curtail MAIT cell effector function akin to the activation-induced expression of inhibitory receptors by conventional T cells. We examined human MAIT cells from blood and oral mucosal tissues by RNA sequencing and found differential expression of immunoregulatory genes, including CTLA-4, by MAIT cells isolated from tissue. Using an ex vivo experimental setup, we demonstrate that inflammatory cytokines were sufficient to induce CTLA-4 expression on the MAIT cell surface in the absence of TCR signals. Even brief exposure to the cytokines IL-12, IL-15, and IL-18 was sufficient for sustained CTLA-4 expression by MAIT cells. These data suggest that control of CTLA-4 expression is fundamentally different between MAIT cells and conventional T cells. We propose that this mechanism serves to limit MAIT cell-mediated tissue damage. ImmunoHorizons, 2020, 4: 14–22.

INTRODUCTION

Mucosal-associated invariant T (MAIT) cells acquire effector function when exposed to bacterial metabolites (presented by MR1) in the presence of inflammatory cues, both of which are present during bacterial infections (1, 2). Importantly, inflammatory cues are sufficient to activate MAIT cells and synergize with TCR signals to induce expression of effector molecules, including granzyme B and IFN-γ (3–5). Inflammatory cytokine-driven activation of MAIT cells has been demonstrated using primary human MAIT cells in short-term ex vivo experiments with IL-12, IL-15, and IL-18 (3, 5, 6) but also directly ex vivo in the context of viral infections including dengue, influenza, and hepatitis C (7, 8). There is also evidence that infection with the parasite Plasmodium falciparum can induce some MAIT cell activation in humans, presumably in the absence of TCR agonist signals (9). This inflammation-driven, TCR-independent mechanism of activation is akin to the phenomenon of bystander-activation in conventional memory T cells (10, 11). 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, because TCR signals control the expression of costimulatory and coinhibitory receptors.

The coinhibitory molecule CTLA-4 is induced on conventional T cells following TCR stimulation and constitutively expressed on regulatory T cells (Treg) (12, 13). CTLA-4 is noteworthy among coinhibitory molecules in that it shares its ligands B7-1 (CD80) and B7-2 (CD86) with the costimulatory 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 (13). 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 costimulatory function of CD28 (13, 14).

We 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. We report in this study 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–calcineurin–NFAT signaling axis (15) required for CTLA-4 expression by conventional human T cells (16), and thus, control of CTLA-4 expression is fundamentally different between MAIT cells and conventional T cells.

MATERIALS AND METHODS

Study approval and patient cohort
All participants provided signed informed consent, and protocols were approved by the Fred Hutchinson Cancer Research Center Institutional Review Board. Surgical procedures included gingivectomy/gingivoplasty, osseous surgery, implant uncovering, and tooth extractions. Participants (n = 39) were between 14 and 83 y old (mean = 52).

Cell isolation from mucosal tissues
Cells were isolated from tissues and blood as previously described (3).

Pathology assessment and scoring
A small portion of each oral mucosal tissue (OM) sample was embedded in Tissue-Tek O.C.T. (Sakura Finetek, Thermo Fisher Scientific) and stored at −80°C. Frozen tissue blocks were cut into 8-μm sections, and slides were stained with H&E. Histologic sections were then evaluated blinded and scored according to the following criteria: severity of inflammation (1–5), location of inflammation, type of inflammatory infiltrate, and presence of epithelial lesions. It is noteworthy that even gingival tissues that could be considered healthy have a minimal/basal level of inflammation that is referred to as homeostatic inflammation (17). Thus, tissues that received a score of 1 or 2 were defined as minimally inflamed, and tissues that received a score of 3–5 were defined as inflamed.

Flow cytometry
All flow cytometric stains were conducted at room temperature. For phenotypic identification, bulk PBMCs or mononuclear cells isolated from tissues were initially stained with LIVE/DEAD Cell Stain (Invitrogen) for 15 min in PBS. MRI tetramer (BV421; National Institutes of Health Tetramer Core) staining was performed as previously described (18). Afterwards, we conducted surface staining with optimized Ab mixes for 20 min in FACS buffer (PBS containing 2% FBS). Cells were then washed with FACS buffer and fixed in PBS containing 1% paraformaldehyde (Sigma-Aldrich). For samples requiring intracellular staining, cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) according to the manufacturer’s instructions.

Abs used for cell sorting were the following: LIVE/DEAD Fixable Aqua Viability Dye (Invitrogen), CD3 Pacific Blue (clone OKT3; BioLegend), CD8 PerCP/Cy5.5 (clone SK1; BD Biosciences), CD4 ECD (clone SFCII2T4D11; Beckman Coulter), CD161 PE/Cy5 (clone DX12; BD Biosciences), and Vα7.2 PE (clone 3C10; BioLegend).

Abs used for analysis following PBMC stimulation were as follows: LIVE/DEAD Fixable Blue Viability Dye (Invitrogen), CD25 BV421 (clone M-A251; BD), CD3 BVU661 (clone UCHT1; BD Biosciences), CD8 BVU805 (clone SK1; BD Biosciences), CD4 APCR700 (clone RPA-T4; BD Biosciences), CD161 BV605 (clone DX12; BD), Vα7.2 PE (clone 3C10; BioLegend), IL-7Rx BV786 (clone HIL-7R-M21; BD Biosciences), Foxp3 Alexa Fluor 488 (clone 259D/C7; BD Biosciences), Helios PerCP-eFluor 710 (clone 22F6; Invitrogen), and CTLA-4 PECF594 (BN13; BD Biosciences).

Concerning instruments, sorting experiments were performed on a FACSaria II (BD Biosciences), and phenotyping for CTLA-4 induction assays were performed on a FACSsymphony (BD Biosciences). Data were analyzed using FlowJo software v10.4.1 (BD Biosciences).

RNA sequencing
We performed RNA sequencing (RNA-seq) on 100–1000 sorted CD8+ MAIT cells from blood or tissue samples as previously described (18). In total, 22 samples were sequenced, 10 from blood and 11 from tissue. Single-read sequencing of the libraries was carried out on a HiSeq2500 sequencer (Illumina) with 58-bp reads, using TruSeq v4 Cluster and SBS Kits (Illumina) with a target depth of 5 million reads. Reads were aligned to the University of California Santa Cruz Human Genome assembly version 19 in Galaxy using Bowtie and TopHat (TopHat for Illumina tool, v.1.5.0). A quality filter was applied to retain libraries prepared with at least 225 cells, in which the fraction of unpaired reads examined compared with total FASTQ reads was >75%, the median coefficient of variation of coverage was <0.8, and the library had at least 500,000 reads. Sixteen of the twenty-two sequenced samples passed these quality filters. Genes with a false discovery rate of <0.05 and expression fold change of >2 between two blood and tissue samples were considered differentially expressed using the linear models for microarray data (limma) R package (19, 20).
Ex vivo stimulation of PBMCs

Cryopreserved PBMCs were thawed and left untreated in RPMI or stimulated with 100 ng/ml cytokines (IL-12 [Thermo Fisher Scientific], IL-15 [Thermo Fisher Scientific], and IL-18 [MBL International]), anti-CD3/CD28 beads (Thermo Fisher Scientific), or a combination of both treatments as previously described (3). A total of 1 × 10⁶ PBMCs were resuspended per well in 96-well plates for 6–24 h at 37°C. Indicated samples were treated with anti-MIR1 Ab (Low Endotoxin, Azide-Free clone 26.S; BioLegend) at a concentration of 50 μg/ml. To assess CTLA-4 expression after brief stimulation, PBMCs were stimulated for 6 h, and then anti-CD3/CD8 beads were removed using magnetic extraction following the manufacturer’s protocol (Thermo Fisher Scientific). Cytokines were removed by washing cell twice before resuspending in RPMI.

Ex vivo stimulation of OM

Cells from OM were resuspended at a concentration of 8 × 10⁵–1 × 10⁶ T cells per condition (CD3-expressing cells in the single-cell suspension) and from healthy/minimally inflamed to severely inflamed. To assess the extent of inflammation, a piece of each analyzed tissue was saved for a blinded evaluation by a pathologist (see Materials and Methods). We assigned tissues with a score of 1 and 2 to a minimally inflamed group and tissues with a score of 3–5 to an inflamed group. We use the term minimally inflamed for tissues with a score of 1 or 2, because even healthy gingival tissues have a minimal/basal level of inflammation that is referred to as homeostatic inflammation (17). The remaining tissue was processed to acquire a single-cell suspension for subsequent analysis by flow cytometry. MAIT cells were identified as live, CD3⁺, Vα7.2⁺ [the invariant TCR α-chain that allowed initial identification of MAIT cells (21, 22)], and CD161hi cells in the OM and matched blood (Fig. 1A).

In some samples, MAIT cells were also identified using the MR1 tetramer (23) in addition to using the Vα7.2⁺CD161hi definition, which yielded a largely, but not fully, congruent population of MAIT cells (Supplemental Fig. 1). To provide consistency with previous studies of MAIT cells from human tissues, including our own (18), we used the Vα7.2⁺CD161hi definition of MAIT cells in this study and acknowledge that this population does not include all MR1-restricted cells. We next assessed the distribution of CD4 and CD8 coreceptor expression within the MAIT cell population in the OM. We found that a majority expressed CD8 (Fig. 1B) similar to MAIT cells from blood (24), lymph (18), and other mucosal barrier tissues (25), including buccal tissue (26), indicating that the general distribution of MAIT cell subsets in the OM is comparable to other tissues. MAIT cell frequencies (as a percentage of CD3+ T cells) in both minimally inflamed (pathology score 1 and 2) and inflamed (scores of 3–5) were overall similar to donor-matched blood (lines between data points indicate donor-matched blood and tissue) (Fig. 1C), although we observed a slight, but statistically significant, decrease of MAIT cell frequencies in inflamed tissues. We next examined if MAIT cells in inflamed OM (scores of 3–5) expressed CD69 and CD103, which are biomarkers that can indicate recent activation (CD69) and/or tissue-residence (CD69 and CD103) (27). We found that CD8⁺ MAIT cells in inflamed OM expressed one or both of these proteins, indicating a possible tissue-resident phenotype similar to conventional CD8⁺ T cells (Fig. 1D, Supplemental Fig. 2A). 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 may require cell-intrinsic immunoregulatory mechanisms.

Statistical analysis

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

RESULTS

MAIT cells are present in OM

To identify cell-intrinsic regulatory mechanisms of MAIT cells that are physiologically relevant, we examined MAIT cells isolated from human OM. We specifically used gingival tissues obtained from routine periodontic surgeries, yielding tissues that range from healthy/minimally inflamed to severely inflamed. To assess the extent of inflammation, a piece of each analyzed tissue was saved for a blinded evaluation by a pathologist (see Materials and Methods). We assigned tissues with a score of 1 and 2 to a minimally inflamed group and tissues with a score of 3–5 to an inflamed group. We use the term minimally inflamed for tissues with a score of 1 or 2, because even healthy gingival tissues have a minimal/basal level of inflammation that is referred to as homeostatic inflammation (17). The remaining tissue was processed to acquire a single-cell suspension for subsequent analysis by flow cytometry. MAIT cells were identified as live, CD3⁺, Vα7.2⁺ [the invariant TCR α-chain that allowed initial identification of MAIT cells (21, 22)], and CD161hi cells in the OM and matched blood (Fig. 1A).

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Statistical analysis

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

Distinct transcriptional profiles of CD8⁺ MAIT cells isolated from OM and donor-matched blood

To identify immunomodulatory genes expressed by MAIT cells in the OM, we examined the CD8⁺ MAIT cell population in the blood versus minimally inflamed and inflamed OM by RNA-seq. Principal component analysis did not reveal any striking differences between CD8⁺ MAIT cells isolated from minimally inflamed versus inflamed OM (Fig. 2A). In contrast, the differences between donor-matched blood and tissue MAIT cells yielded 56 differentially expressed genes between CD8⁺ MAIT cells from OM and blood (an arbitrary patient identifier is listed at the bottom of the heatmap to allow pairing of donor-matched blood and tissue) (Fig. 2B). Finally, we found that CD8⁺ MAIT cell isolated from the OM had a gene expression signature (compared with blood) indicative of tissue residence (28) (Supplemental Fig. 2B), which aligned with our flow cytometry data (Fig. 1D).

We next screened the differentially expressed genes between CD8⁺ MAIT cells from OM (minimally inflamed and inflamed tissues combined) and blood for genes associated with regulatory or inhibitory functional properties (Fig. 2C). We found genes that negatively regulate TCR costimulation or activation, including CTLA4 and TIGIT (29) as well as genes (IRF4 and BATF), that mediate expression of inhibitory receptors in conventional CD8⁺ T cells (30). 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. Finally, we compared the expression levels of transcripts encoding for proteins with well-defined costimulatory or coinhibitory function as well as the gene expression levels of the signaling components for IL-12, IL-15, and IL-18 in blood versus OM CD8⁺ MAIT cells. Applying k-means clustering to our samples, we found...
near-uniform separation between blood and OM CD8+ MAIT cells (Fig. 2D). We also observed that OM CD8+ MAIT cells had higher expression of genes with inhibitory, as well as costimulatory, function (Fig. 2D). Further, components of the IL-12, IL-15, and IL-18 signaling pathways appeared to be expressed at comparable levels, suggesting that MAIT cells in the tissues maintain responsiveness to these cytokines (Fig. 2D). These data indicate that there is heterogeneity in the expression of costimulatory and coinhibitory molecules in the OM MAIT cell population and that OM MAIT cells retain their ability to respond to activating cytokines (3).

**Inflammatory cytokines are sufficient to trigger CTLA-4 expression on the MAIT cell surface**

CTLA-4 protein is expressed by conventional T cells following TCR stimulation and constitutively expressed by Treg (31, 32). 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 (33). Because 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 with blood (lines between data points indicate donor-matched blood and tissue), which was observed in minimally inflamed or inflamed OM (white squares) (minimally inflamed OM, n = 9; inflamed OM, n = 18). Treg were included as a positive control, and conventional memory CD8+ T cells (defined as non-MAIT CD45RA+) were included as a reference population (Fig. 3A–C). These data demonstrate ex vivo CTLA-4 protein expression on the cell surface of an MAIT cell population in OM and highlight a potential regulatory mechanism for MAIT cell activation.

We next wanted to define which signals are necessary to induce CTLA-4 expression on the MAIT cell surface. To address this, PBMCs from healthy donors were stimulated for 24 h ex vivo with either anti-CD3/CD28 beads, a combination of the cytokines IL-12/15/18, or both signals. IL-18 is a potent inducer of IFN-γ together with IL-12 or IL-15 in conventional memory T cells and NK cells (34, 35). Similarly, we previously reported that IL-18 elicited IFN-γ production by MAIT cells in the presence of IL-12 or IL-15, but not by itself (3). Because we furthermore observed that a combination of all three cytokines elicited the strongest IFN-γ and granzyme B response (3) and our RNA-seq data...
indicated that OM CD8+ MAIT cells express the necessary receptors (Fig. 2D), we chose to stimulate MAIT cells with a combination of these three cytokines to assess CTLA-4 expression. CTLA-4 expression on CD8+ MAIT cells was compared with conventional memory CD8+ T cells (CD45RO+) and Treg. All three T cell populations increased expression of CTLA-4 after incubation with anti-CD3/CD28 beads (Fig. 3D), but only CD8+ MAIT cells also induced surface expression of CTLA-4 following incubation with IL-12/15/18 for 24 h (Fig. 3D). Stimulation of T cells with anti-CD3/CD28 beads resulted 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 cannot be directly compared with the IL-12/15/18 stimulation condition in regards to its ability to induce CTLA-4 surface expression. Importantly, addition of an anti–MR-1–blocking Ab 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. 3E). Together, these data provide strong evidence that inflammatory signals are sufficient to induce expression of CTLA-4 on the cell surface of MAIT cells.

Brief exposure to inflammatory cytokines is sufficient to induce sustained CTLA-4 expression by MAIT cells
To determine whether cytokine-induced CTLA-4 surface expression was maintained following cytokine withdrawal, we incubated...
PBMCs for 6 h with either IL-12/15/18, anti-CD3/CD28 beads, or both stimuli (Fig. 4A). After 6 h, the stimuli were removed by washing, and cells were either analyzed (Fig. 4B) or cultured for another 12 or 18 h without further stimulation before examining CTLA-4 surface expression (Fig. 4C, 4D). We found that CTLA-4 was not expressed after 6 h of stimulation (Fig. 4B), but this brief (6 h) exposure to cytokines in the absence of a TCR signal was sufficient to induce surface expression of CTLA-4 on CD8+ MAIT cells 12 h (Fig. 4C) and 18 h later (Fig. 4D). These data suggest that a program for surface expression of CTLA-4 is quickly exerted and at least temporarily sustained after exposure to inflammatory stimuli.

Finally, we sought to determine if CTLA-4+ MAIT cells isolated from OM 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 h. We chose 7 h to minimize de novo expression of CTLA-4 in these experiments and used both stimuli to induce maximum MAIT-effector function (Fig. 4B). 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). We found that MAIT cells that expressed CTLA-4 had reduced production of IFN-γ after stimulation (Fig. 4E) even though CTLA-4 itself was not engaged by anti-CD3/CD28 beads.

**DISCUSSION**

Immunoregulatory mechanisms that serve to limit the effector response of conventional T cells are well documented (13, 36). We asked if similar immunoregulatory mechanisms are in place to curtail or prevent MAIT cell effector function following activation of MAIT cells by either TCR-mediated signals or inflammatory cytokines. We considered that the signals eliciting expression of immunoregulatory proteins such as CTLA-4 could differ between
MAIT cells and conventional T cells. CTLA-4 is a protein with critical immunoregulatory properties and expressed on Treg as well as conventional CD4+ and CD8+ T cells following activation via their TCR (13). In addition to cell-intrinsic inhibition that is mediated by CTLA-4 via interaction of phosphatases with its cytoplasmic domain, it has been suggested that CTLA-4 could also act cell extrinsically. In this scenario, CTLA-4 inhibits priming of other T cells in trans by competing for availability of B7-1 and B7-2 on APCs (14). Whether the role of CTLA-4 on MAIT cells is to restrain their own proinflammatory responses or to preclude neighboring T cells from receiving costimulation similar to what has been proposed for Treg will require further studies. CTLA-4 must be expressed on the cell surface for these cell-intrinsic and -extrinsic immunoregulatory mechanisms, but most of the protein is located intracellularly in resting T cells (33). Importantly, we report, in this study, that cell surface expression of CTLA-4 on MAIT cells is induced by inflammatory cytokines independently of a TCR signal (Fig. 3). 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. Expression of CTLA-4 is strictly dependent on the transcription factor family NFAT in conventional T cells, as shown by the loss of CTLA-4 and IFN-γ with intracellular cytokine staining. Left, Representative gating for IFN-γ and total CTLA-4 expression in CD8+ MAIT cells after stimulation. Right, IFN-γ expression in CTLA-4+ and CTLA-4- OM CD8+ MAIT cells after TCR and IL-12, -15, and -18 stimulation (n = 6 donors). Two-way ANOVA with Dunnett multiple comparisons test were performed in (C), whereas Wilcoxon matched-paired signed-rank tests were used in (E). *p ≤ 0.05, **p ≤ 0.01.

FIGURE 4. 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 h stimulation of PBMCs either (B) immediately or (C) 12 h and (D) 18 h postremoval of beads and cytokines (n = 4). (E) Single-cell suspensions isolated from the OM were stimulated with recombinant cytokines IL-12, -15, and -18 (100 ng/ml each) and anti-CD3/CD28 TCR beads for 7 h and assayed for CTLA-4 and IFN-γ with intracellular cytokine staining. Left, Representative gating for IFN-γ and total CTLA-4 expression in CD8+ MAIT cells. (Center) CTLA-4 expression in OM CD8+ MAIT cells after stimulation. Right, IFN-γ expression in CTLA-4+ and CTLA-4- OM CD8+ MAIT cells after TCR and IL-12, -15, and -18 stimulation (n = 6 donors). Two-way ANOVA with Dunnett multiple comparisons test were performed in (C), whereas Wilcoxon matched-paired signed-rank tests were used in (E). *p ≤ 0.05, **p ≤ 0.01.
by these cells may also act on MAIT cells and contribute to the induction of CTLA-4 expression on the MAIT cell surface. We limited our stimulation experiments to 24 h to minimize such indirect effects. Importantly, our main conclusion that this expression occurs in the absence of a TCR signal is not affected by possible indirect effects, because TCR signals were prevented by the addition of anti-MR1 Ab. We have also not observed MAIT cell proliferation in this experimental setup, indicating that CTLA-4 expression is not dependent on MAIT cells entering cell cycle.

Our data also raise the question of why CTLA-4 expression is regulated by inflammatory signals in MAIT cells. Importantly, our data demonstrate that even a brief exposure to inflammatory stimuli is sufficient to induce CTLA-4 expression (Fig. 4). This suggests that MAIT cells may have a temporally restricted effector phase that is followed by a phase that is refractory to stimulation, which could be essential to ensure that inflammatory responses eventually subside in tissues. It is also important to consider that MAIT cells are located in mucosal tissues with a microbiome and, thus, are presumably exposed to their Ag. Because a TCR plus costimulatory signal alone is not sufficient to activate MAIT cells but synergizes with inflammatory cues to elicit a potent effector response (3), inflammation-driven induction of CTLA-4 expression could be in place to minimize responses against commensal-derived Ag by preventing costimulation. For example, viral infections or physical tissue damage elicit inflammatory responses, but in these cases, a misdirected immune-response against commensal-derived Ag would impair resolution of inflammation and prevent return to tissue homeostasis. Thus, we propose that this cytokine-driven, TCR-independent regulation of CTLA-4 expression ensures that MAIT cells in inflamed tissues restrain deleterious inflammatory responses against commensal organisms.

Finally, our ex vivo MAIT cell stimulation data (Fig. 4) also suggest that CTLA-4 expression by MAIT cells in tissues may be a biomarker for overall hyporesponsiveness given that CTLA-4+ MAIT cells failed to produce cytokines even when CTLA-4 itself was not engaged by stimulating MAIT cells with anti-CD3/CD28 Ab–coated beads in the presence of IL-12/15/18. 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 (38). Whether CTLA-4–expressing MAIT cells are just temporarily hyporesponsive or in a dysfunctional state similar to conventional T cells remains to be determined. Similarly, if functionally hyporesponsive MAIT cells have lost their ability to interact with other immune subsets such as B cells (39) or can still provide some help will need to be addressed in future studies.

In summary, our data provide strong evidence that inflammatory cytokines induce sustained CTLA-4 cell surface expression on MAIT cells in a TCR-independent manner. We propose that this TCR-independent induction of CTLA-4 expression in MAIT cells serves to limit MAIT cell–mediated tissue damage, which may be of interest for therapeutic intervention for a wide range of inflammation-driven tissue pathologies, including autoimmune lesions and tumors.

**DISCLOSURES**

The authors have no financial conflicts of interest.

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Supplement 1: 5-OP-RU MR1 tetramer expression on MAIT cells in oral mucosa and matched blood.
(A) Representative staining of 5-OP-RU MR1 tetramer on total CD3⁺ cells and MAIT cells (Vα7.2⁺CD161⁺) from OM and matched blood. (B) Frequency of MR1 tetramer staining on the total MAIT cell population (n=7).
Wilcoxon matched-paired signed rank tests were performed. *p ≤ 0.05  **p ≤0.01 ***p ≤ 0.001.
Supplement 2: 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+ conventional memory T cells (non-MAIT 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.