Identification and characterization of latency-associated peptide-expressing γδ T cells

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Accessibility
Identification and characterization of latency-associated peptide-expressing \(\gamma\delta\) T cells

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\(\gamma\delta\) T cells are a subset of lymphocytes specialized in protecting the host against pathogens and tumours. Here we describe a subset of regulatory \(\gamma\delta\) T cells that express the latency-associated peptide (LAP), a membrane-bound TGF-\(\beta\). Thymic CD27\(^+\) IFN-\(\gamma\)\(^+\) CCR9\(^+\) \(\alpha_4\beta_7\)\(^+\) TCR\(\gamma\delta\)\(^+\) cells migrate to the periphery, particularly to Peyer’s patches and small intestine lamina propria, where they upregulate LAP, downregulate IFN-\(\gamma\) via ATF-3 expression and acquire a regulatory phenotype. TCR\(\gamma\delta\)\(^+\) LAP\(^+\) cells express antigen presentation molecules and function as antigen presenting cells that induce CD4\(^+\) Foxp3\(^+\) regulatory T cells, although TCR\(\gamma\delta\)\(^+\) LAP\(^+\) cells do not themselves express Foxp3.

Identification of TCR\(\gamma\delta\)\(^+\) LAP\(^+\) regulatory cells provides an avenue for understanding immune regulation and biologic processes linked to intestinal function and disease.
Gamma-delta (γδ) T cells are lymphocytes bearing a T-cell receptor composed of gamma and delta chains as opposed to alpha and beta chains found in conventional CD4+/CD8+ T cells. Despite comprising the majority of immune cells in niches associated with epithelial surfaces such as the intestine, only 1–2% of γδ T cells are present in secondary lymphoid tissues. γδ T cells are considered the first line of defense against pathogens as they can rapidly respond to TCR signals in an MHC-independent manner and to pattern recognition receptor signals such as Toll-like receptors. Upon activation, γδ T cells rapidly secrete IFN-γ and IL-17 and acquire cytotoxic activity. Two distinct γδ T cell subsets have been described on the basis of their cytokine production profile. γδT1 cells express CD27 and secrete IFN-γ (ref. 7), whereas γδT17 cells are CD27−, express CCR6 and secrete IL-17 (ref. 6).

In addition to their physiologic functions, γδ T cells may participate in immunopathology, including autoimmune disease models such as experimental autoimmune encephalomyelitis (EAE) and arthritis. As γδ T cells are particularly abundant in the intestinal mucosa, their participation in intestinal inflammation has also been described. IL-17+ γδ T cells play a crucial role in enhancing in vivo Th1 and Th17 differentiation and T-cell-mediated colitis in mice and exacerbate intestinal inflammation induced by dysregulated immune homeostasis.

γδ T cells have also been reported to have immunoregulatory function. For example, in inflammatory bowel disease models, γδ T-cell-deficient mice develop spontaneous colitis and are susceptible to 2,4,6-trinitrobenzene sulfonic acid-induced colitis. Transfer of intraepithelial γδ lymphocytes (IEL-γδ) ameliorates colitis in this model. In dextran sodium sulfate (DSS)-induced colitis in mice, IEL-γδ T cells help preserve the integrity of damaged epithelial surfaces by the localized delivery of keratinocyte growth factor, a potent intestinal epithelial cell mitogen. Furthermore, by secreting IL-22 as well as antimicrobial products in a retinoic acid-dependent fashion, γδ T cells play an important role in the attenuation of intestinal inflammation induced by DSS or Citrobacter rodentium infection in mice. Oral tolerance, a physiologic process that helps maintain gut homeostasis to the daily challenge of microbiota and dietary antigens is impaired in mice depleted of γδ T cells or in γδ T-cell-deficient mice.

The mechanism(s) by which γδ T cells exert regulatory function is not well understood. Forthekin box p3 (Foxp3) expression is not observed in murine γδ T cells ex vivo though they may express Foxp3 in vitro when cultured in the presence of TGF-β1 (ref. 18). There are low levels of Foxp3 expression in human γδ T cells that, like in mice, increase under Treg-inducing conditions in vitro. Moreover, Rhodes et al. reported the existence of an IL-10 + γδ T cell subset that protected the liver from Listeria-induced, CD8+ T-cell-mediated injury in mice. Interestingly, activated γδ T cells from cattle and human have been shown to express high levels of MHC-II and co-stimulatory molecules and function as antigen presenting cells (APCs). Although this APC-like function of γδ T cells has been associated with a more pro-inflammatory immune response, it is possible that regulatory subtypes of γδ T cells may occur in vivo and have immunoregulatory function.

In the present study, we describe and characterize a subset of regulatory γδ T cells that are Foxp3 negative and express membrane-bound TGF-β1 in the form of latency-associated peptide (LAP). These cells function as APCs and possess the ability to induce Foxp3 in CD4 T cells in vitro and in vivo.

Results

Identification of a subset of LAP-expressing γδ T cells. Given our interest in the regulatory function of T cells, which express membrane-bound TGF-β1 through its accessory-binding molecule LAP, we investigated LAP expression on γδ T cells. We found LAP-expressing γδ T cells (TCRγδ+ LAP+) in several mouse lymphoid organs, particularly those from Peyer’s patches (PPs) and small intestine lamina propria (SI-LP), where ~20% of the γδ T cells were positive for this molecule (Fig. 1a,b, Supplementary Fig. 1a). Owing to the high cellularity in spleen, the absolute number of TCRγδ+ LAP+ cells in the spleen was greater than in the other organs investigated, followed by PPs (Fig. 1a). The absolute number of TCRγδ+ LAP+ cells in SI-LP was as low as in lymph nodes, thymus, large intestine lamina propria (LI-LP) and intraepithelial lymphocytes (Fig. 1a). There was minimal expression of LAP on γδ T cells from thymus, intraepithelial lymphocytes and LI-LP (Fig. 1a; Supplementary Fig. 1b). LAP was less expressed on other lymphoid cells in the PPs and SI-LP, such as CD4 and CD8 T cells, as compared with LAP expression on γδ T cells from these same organs (Fig. 1a; Supplementary Fig. 2a,b). We also detected LAP on γδ T cells from human peripheral blood (Supplementary Fig. 3a,b), at comparable percentages found on splenic γδ T cells from mice. Because the highest frequency and absolute number of TCRγδ+ LAP+ cells were found in PPs and spleen, respectively, we performed our characterization using TCRγδ+ LAP+ cells from these organs. Consistent with their LAP expression, mouse TCRγδ+ LAP+ cells expressed more TGF-β1 than TCRγδ+ LAP− cells, as measured by messenger RNA (mRNA; Fig. 1c), and also expressed the cell-surface molecule Glycoprotein A Receptors Predominant (GARP), which is known to bind and attach LAP to the cell surface (Fig. 1d). It has been reported that under Foxp3-induction conditions in vitro, both human and mice γδ T cells express Foxp3 (refs 18,19). However, Foxp3 was not detected ex vivo in non-manipulated naïve mice. Consistent with this, we found that γδ T cells from PPs and spleen of naïve Foxp3-GFP mice did not express Foxp3 as measured either by mRNA or protein expression (Fig. 1e,f). Vγ1 and Vγ4 TCR chains were expressed on TCRγδ+ LAP+ and TCRγδ+ LAP− cells, with Vγ1 the most expressed in both cell populations (Fig. 1g,h; Supplementary Fig. 4; nomenclature based on Heilig and Togewa28). In summary, our results identify a subpopulation of γδ T cells in mice that express LAP on their surface.
IL-17A and IFN-γ as well as CCL2 and CXCL10 (chemokines involved in the recruitment of myeloid and lymphoid cells to inflammatory sites) mRNA expression. Transfer of TCRγδ + LAP + cells increased IL-10 and TGF-β1 mRNA in the SI-LP as compared with control or TCRγδ + LAP − cell-transferred animals (Fig. 2d). In the LI-LP, expression of IFN-γ mRNA was decreased in TCRγδ + LAP + cell-treated mice compared with the other two groups, though IL-17A, CCL2 and CXCL10 mRNA levels were reduced in TCRγδ + LAP + cell-treated animals as compared with TCRγδ + LAP − cell-transferred mice, but were not different from the control group (Supplementary Fig. 5a). Foxp3 mRNA was upregulated in the SI-LP, but not in the LI-LP of TCRγδ + LAP + cell-treated mice (Fig. 2d; Supplementary Fig. 5a). Consistent with this, the frequency and absolute number of CD4 + Foxp3 + cells in the SI-LP were higher in mice treated with TCRγδ + LAP + cells (Fig. 2e). PP could not be investigated because RAG-1−/− mice do not develop PP. Fluorescence-activated cell sorting (FACS) analysis demonstrated that the absolute number of total CD4 T cells and the frequency/absolute number of TCRγδ + LAP + cells were elevated compared with the LAP − counterpart in SI-LP, but not in LI-LP (Fig. 2f; Supplementary Fig. 5b). No differences were observed in either percentage or absolute number of total CD4+ and γδ T cells in the spleen (Supplementary Fig. 5c), though a significant increase of CD4 + Foxp3 + cells in TCRγδ + LAP + cell-treated mice was observed (Supplementary Fig. 5d).

We also investigated TCRγδ + LAP + cells in the DSS-model, which is a T cell-independent model of colitis. Transfer of TCRγδ + LAP + cells ameliorated disease as measured by body weight with initial effects observed at day 6 and more prominent effects beginning at day 10 (Supplementary Fig. 6b). Thus there appears to be a combined effect on both disease progression and recovery. In addition, colonic length was not reduced in TCRγδ + LAP + cell-treated mice (Supplementary Fig. 6c) and histological analysis showed less tissue ulceration and inflammatory cell infiltrate in mice transferred with TCRγδ + LAP + cells (Supplementary Fig. 6d). IFN-γ mRNA was increased in LI-LP from TCRγδ + LAP + cell-treated mice (Supplementary Fig. 6e). These analyses were performed at day 14 after DSS treatment, which corresponds to the recovery phase of the colitis. Consistent with this, LI-LP from mice transferred TCRγδ + LAP + cells showed higher levels of IL-10 and TGF-β1 mRNA, cytokines important for gut homeostasis, as well as IL-22, an interleukin involved in the protection of barrier surfaces, such as the gut epithelium31 (Supplementary Fig. 6e). FACS analyses demonstrated that the absolute number of total CD4 T cells and frequency/absolute number of CD4 + Foxp3 + cells were increased in the spleen of mice treated with TCRγδ + LAP + cells as compared with the other groups (Supplementary Fig. 6f). In PP, the absolute number of total CD4 and CD4 + Foxp3 + cells was increased in TCRγδ + LAP + cell-treated mice as compared with mice that received TCRγδ + LAP − cells, but was not different from naive or control groups (Supplementary Fig. 6g). In the LI-LP, TCRγδ + LAP − and TCRγδ + LAP + cell-treated mice had increased frequency and absolute number of total CD4 and CD4 + Foxp3 + cells, but were not different from each other (Supplementary Fig. 6h).
To investigate whether TCRγδ + LAP + cells and suppressive properties in vitro, we sorted naive CD4+ T cells from Foxp3-GFP mice and stimulated them with anti-CD3e in the presence of Foxp3-APCs. As a control, nTregs were tested. We found that neither TCRγδ + LAP + nor TCRγδ + LAP – cells were suppressed in vitro as compared with nTreg cells. Instead, TCRγδ + LAP + cells induced higher proliferation than control or TCRγδ + LAP – cells even at a 1:8 responder:TCRγδ + LAP + cell ratio (Supplementary Fig. 7a,b). Furthermore, no Foxp3 induction in responder cells was observed when either TCRγδ + LAP + or TCRγδ + LAP – cells were added to the culture as compared with the Foxp3 induction by nTreg cells (Supplementary Fig. 7c,d). Consistent with this, TCRγδ + LAP + cells stimulated in vitro with plate-bound anti-CD3e and anti-CD28 acquired a pro-inflammatory profile with less TGF-β1 and more TNF-α mRNA expression (Supplementary Fig. 8a,b). To further investigate whether TCRγδ + LAP + cells had suppressive properties in vitro and to determine whether the activation of TCRγδ + LAP + cells by anti-CD3e was associated with their inability to induce CD4 + Foxp3 + cells in vitro, we sorted naive CD4 T cells from 2D2xFoxp3-GFP mice (2D2 are MOG35.55-specific TCR transgenic animals) and stimulated them with MOG35.55 peptide in the presence of TCRγδ + LAP – or TCRγδ + LAP + cells from wild-type (WT) mice in the absence of APCs. This allowed us to stimulate CD4 + T cells with MOG peptide and to assess the APC function of TCRγδ + LAP + without stimulating γδ T cells with anti-CD3e as we did above. We found that TCRγδ + LAP + but not TCRγδ + LAP – cells induced both proliferation and Foxp3 expression in CD4 T cells (Supplementary Fig. 8c,d). Thus, when TCRγδ + LAP + cells are not stimulated by anti-CD3e in vitro, they are able to induce CD4 + Foxp3 + Treg cells as they do in vivo. Moreover, because we did not add APCs to the co-culture, these results suggest that TCRγδ + LAP + cells functioned as APCs and provided co-stimulatory signals to the naive CD4 + T cells (Supplementary Fig. 8c,d). In summary, we found that in vivo TCRγδ + LAP + cells ameliorate colitis by promoting the induction of Foxp3 Treg cells. In vitro experiments demonstrate that they do not have direct regulatory function, but indirectly induce Tregs through their APC properties.

To further characterize TCRγδ + LAP + cells, we performed RNA-Seq of both TCRγδ + LAP + and TCRγδ + LAP – cells (Table 1; Supplementary Data 1 and Supplementary Data 2). We identified a signature of 407 genes that were enriched in TCRγδ + LAP + versus TCRγδ + LAP – cells with P < 0.05. Among the upregulated genes, we found increased expression of genes related to TCRγδ + LAP + cells (Table 1; Supplementary Data 1 and Supplementary Data 2). We identified a signature of 407 genes that were enriched in TCRγδ + LAP + versus TCRγδ + LAP – cells with P < 0.05. Among the upregulated genes, we found increased expression of genes related to TCRγδ + LAP + cells (Table 1; Supplementary Data 1 and Supplementary Data 2).
LAP+ cells from PPs had higher expression of MHC-II and CD86 than both thymic γδT1 cells and TCRγδ+ LAP− cells. They also expressed CD40 (Fig. 3a–d). MHC-I was detected on all γδ T cells analysed (Fig. 3a–d).

To determine whether TCRγδ+ LAP+ cells could function as antigen presenting cells, we cultured TCRγδ+ LAP+ or TCRγδ+ LAP− cells with Alexa Fluor 488-conjugated ovalbumin (OVA). TCRγδ+ LAP+ cells took up twice as much OVA as their LAP− counterparts (Fig. 4a). When OVA323–339 peptide-pulsed TCRγδ+ LAP+ were cultured with naive CD4+ T cells from OT-IxFoxp3-GFP (OVA323–339-specific TCR transgenic) mice, we observed proliferation to a similar extent as with OVA323–339 peptide-pulsed CD103+ CD11c+ dendritic cells (DC; Fig. 4b). No proliferation was observed when TCRγδ+ LAP− cells were pulsed with OVA323–339 peptide and were cultured with CD4+ T cells (Fig. 4b).

The decreased proliferative response seen when T cells were cultured with CD103+ CD11c+ DCs versus CD103− CD11c− DCs is consistent with their well-known tolerogenic properties and their ability to induce Foxp3 in CD4+ T cells32. We thus measured Foxp3 expression in naive CD4+ T cells co-cultured with OVA323–339 peptide-loaded TCRγδ+ LAP+ or TCRγδ+ LAP− cells. We found that TCRγδ+ LAP+ but not TCRγδ+ LAP− cells induced Foxp3 expression in a fashion analogous to CD103+ CD11c+ DCs (Fig. 4c). Because LAP has been reported to be important for Foxp3 induction in a cell-contact-dependent manner33, we investigated the requirement for LAP to induce Foxp3 by TCRγδ+ LAP+ cells in vitro. Using a monoclonal anti-LAP antibody developed in our laboratory34, we found that the induction of Foxp3 in CD4+ T cells by TCRγδ+ LAP+ cells was reduced by three-fold when LAP was blocked (Fig. 4d). To investigate whether the proliferative activity and Foxp3 induction by TCRγδ+ LAP+ cells were dependent on MHC-II, we sorted TCRγδ+ LAP+ cells from MHC-II−/- mice, pulsed them with OVA323–339 peptide, and co-cultured them with CellTrace Violet-labeled naïve CD4+ T cells from OT-IxFoxp3-GFP. We found that MHC-II−/- but not MHC-II+/+ TCRγδ+ LAP+ cells pulsed with OVA323–339 peptide induced proliferation and Foxp3 expression in CD4 T cells (Fig. 4e,f). Thus, TCRγδ+ LAP+ cells have MHC-II dependent APC properties.

To investigate whether TCRγδ+ LAP+ cells could promote proliferation and Foxp3 induction in CD4 T cells in vivo, we co-transferred CellTrace Violet-labeled naïve CD4+ T cells from OT-IxFoxp3-GFP mice with either TCRγδ+ LAP+ or TCRγδ+ LAP− cells pulsed with OVA323–339 peptide to WT recipient mice and measured proliferation and Foxp3 expression in transferred CD4+ T cells 5 days later in the spleen. We found that both OVA323–339 peptide-pulsed TCRγδ+ LAP+ and TCRγδ+ LAP− cells induced CD4+ T cell proliferation in vivo, with greater proliferative activity induced by TCRγδ+ LAP+ cells (Supplementary Fig. 9a). In addition, TCRγδ+ LAP+ cells induced more Foxp3 than their LAP− counterparts in vivo (Supplementary Fig. 9b). Thus, LAP-expressing γδ T cells can function as APCs and induce CD4+ Foxp3+ cells in vivo.

**Table 1 | Transcriptional signatures of TCRγδ+ LAP+ and TCRγδ+ LAP− cells.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>LAP−</th>
<th>LAP+</th>
<th>LAP+ /LAP− (fold change)</th>
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<td>Itgae</td>
<td>57.640</td>
<td>150.950</td>
<td>2.638</td>
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<td>36.723</td>
<td>96.125</td>
<td>2.681</td>
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<td>3.938</td>
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<td>9.530</td>
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<td>336.200</td>
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<td>Gzma</td>
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RNA-Seq expression analyses of upregulated genes in TCRγδ+ LAP− and TCRγδ+ LAP+ cells with P < 0.05.

TCRγδ+ LAP+ cells arise from thymic γδT1 cells. It has been shown that thymic γδ T cells can be divided into two subpopulations: γδT1 cells, characterized by the expression of CD27 and the production of IFN-γ (ref. 7); and γδT17 cells, which are CD27− (ref. 7), express CCR6 and secrete IL-17 (ref. 6). Both subtypes are considered non-canonical γδ T cells and express Vγ1 and Vγ4 TCR chains7, which is consistent with what we observed in TCRγδ+ LAP+ and TCRγδ+ LAP− cells (Fig. 1g,h; Supplementary Fig. 4). To determine which subset gives rise to TCRγδ+ LAP+ cells, we examined γδ T cells from PPs, the site where TCRγδ+ LAP+ cells are in the greatest abundance. We found the majority of γδ T cells in PPs were positive for CD27, but negative for CCR6 (Fig. 5a). Thus, most of TCRγδ+ LAP+ (as well as TCRγδ+ LAP−) cells were γδT1 cells (Fig. 5a). Of note, 6–10% of thymic γδ T cells were γδT17 cells as they were negative for CD27 and expressed CCR6 (Fig. 5b). When we examined LAP expression on γδ T cells from CCR6−/− mice, there was no difference compared with WT mice (Fig. 5c), suggesting that TCRγδ+ LAP+ cells arise from thymic γδT1 cells. The expression of surface LAP on γδ T cells most likely occurs in the periphery because neither thymic γδT1 nor γδT17 cells expressed LAP on the surface (Fig. 5d). Intracellular LAP expression was detected in 15% of thymic γδT1, but not in γδT17 cells (Fig. 5e), indicating that LAP is intrathymically induced but only expressed on the cell surface in the periphery. As previously reported, thymic γδT1 cells expressed IFN-γ, which increased after phorbol myristate acetate (PMA) and ionomycin (ION) stimulation, but virtually no IL17A (ref. 7). Because TCRγδ+ LAP+ cells stimulated with PMA/ION downregulated LAP (Fig. 6a), we performed IFN-γ and IL17A intracellular staining from fresh ex vivo γδ T cells from both thymus and PPs. Thymic γδT1 cells produced IFN-γ, but not IL17A (Fig. 6b). TCRγδ+ LAP+ cells expressed less IFN-γ, at both protein and mRNA levels than either thymic γδT1 or TCRγδ+ LAP− cells (Fig. 6c), suggesting that LAP-expressing γδ T cells downregulate IFN-γ. IL17A protein and mRNA expression, however, was not observed in either TCRγδ+ LAP+ or TCRγδ+ LAP− γδ T cells (Fig. 6d). Taken together, these data indicate that thymic γδT1 cells acquire surface LAP in the periphery where IFN-γ is downregulated.

TCRγδ+ LAP+ cells downregulate IFN-γ through ATF-3. Our RNA-Seq data demonstrated that activating transcription factor 3 (ATF-3) was upregulated in TCRγδ+ LAP+ cells (Table 1; Supplementary Data 1 and Supplementary Data 2). We focused on ATF-3 because it relates directly to a potential mechanism by which IFN-γ is downregulated in TCRγδ+ LAP+ cells. ATF-3 is a member of the ATF/CREB family of transcription factors that recognize the sequence 5′-AGGTCA-3′ and increase transcription of genes that contain this motif.

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whether thymic T cells are imprinted with gut-homing molecules. Because the highest percentage of TCRγδ T cells expressed the CCL25 chemokine receptor CCR9 as well as the integrin α4β7, which are considered gut-homing molecules. We found that expression of CCR9 and α4β7 was primarily detected on γδ T cells with lower expression on γδT17 cells (Fig. 7a). To further investigate the role of CCR9 and α4β7 on homing of γδ T cells to the gut, CCR9−/− and β7−/− mice were used and we found reduced frequency/absolute number of γδT1 and TCRγδ+LAP+ cells in the PP (Fig. 7b). Because γδT1 cells correspond to the majority of γδ T cells in the PP (Fig. 5a; Fig. 6b), the absolute number of total γδ T cells was also decreased (Fig. 7b). In association with the smaller γδT1 and TCRγδ+LAP+ cell compartments in the gut of CCR9−/− and β7−/− mice, we found these γδ T cell populations increased in the spleen of both CCR9−/− and β7−/− mice (Supplementary Fig. 10a). Consistent with the fact that CCR9 and α4β7 are expressed less on γδT17 than γδT1 cells, neither frequency nor absolute number of γδT17 cells from CCR9−/− and β7−/− mice were altered in PPs, though number, but not percentage of these cells were increased in the spleen of CCR9−/− but not β7−/− mice (Fig. 7b; Supplementary Fig. 10a). To further confirm the gut-homing ability of γδT1 cells, we transferred sorted thymic TCRγδ+CD27+ cells from WT CD45.2 C57BL/6 mice to WT congenic CD45.1 mice. We then tracked the CD45.2+ cells 36 h later and found higher frequency of these cells in PPs (0.5%) than

Basic leucine zipper transcription factors that has been shown to negatively modulate IFN-γ either indirectly by reducing cytokine production, including IL-12 (ref. 35), or directly by interacting with a cis-regulatory element of the IFN-γ gene. We confirmed increased expression of ATF3 in TCRγδ+LAP+ cells by RT-PCR (Fig. 6e). Consistent with this, we found that PP from ATF-3−/− mice had twice as many IFN-γ-producing TCRγδ+LAP+ cells than WT mice (Fig. 6f), suggesting that ATF-3 plays an important role in down-modulating IFN-γ in TCRγδ+LAP+ cells.
**Figure 4** | TCRγδ + LAP+ cells function as APCs and induce Foxp3 in CD4 T cells. (a) Soluble ovalbumin (OVA) coupled to Alexa Fluor 488 (OVA-AF488) endocytosis by CD3 + TCRγδ + or CD3 + TCRγδ + LAP+ cells after 3 h of culture in vitro at 37 °C (n = pooled cells from 10 mice per experiment). (b,c) Proliferation (b) and Foxp3 induction (c) in CellTrace Violet-stained naive CD4 T cells from OT-IixFoxp3-GFP mice co-cultured with OVA323–339-loaded CD3 + TCRγδ + LAP+, CD3 + TCRγδ + LAP−, CD103−CD11c+ or CD103 + CD11c+ cells from WT C57BL/6 mice for 4 days at 37 °C (n = pooled cells from 10 mice per experiment). (d) Foxp3 induction in CellTrace Violet-stained naive CD4 T cells from OT-IixFoxp3-GFP mice co-cultured with OVA323–339-loaded TCRγδ+ LAP+ cells from WT C57BL/6 mice in the presence or absence of 30 μg ml−1 of anti-LAP mAb for 4 days at 37 °C (n = pooled cells from 10 mice per experiment). (e,f) Proliferation (e) and Foxp3 induction (f) in CellTrace Violet-stained naive CD4 T cells from OT-IixFoxp3-GFP mice co-cultured with OVA323–339-loaded (or not) CD3 + TCRγδ + or CD3 + TCRγδ + LAP+ cells from either WT C57BL/6 or MHCII−/− mice for 4 days at 37 °C (n = pooled cells from 10 mice per experiment). These data are representative of at least three independent experiments. One-way analysis of variance followed by Tukey multiple comparisons (b,e,f) and Student’s t-test (a,c,d) were used. **P<0.01, ***P<0.001, ****P<0.0001.

**Figure 5** | TCRγδ + LAP+ cells are originated from thymic γδT1 cells. (a) CD27 and CCR6 expression on γδ T cells (CD3 + TCRγδ+) as well as expression of LAP on CD3+ TCRγδ+ CD27+ and CD3+ TCRγδ+ CCR6+ cells from PPs of naive C57BL/6 mice (n = 9). (b) CD27 and CCR6 expression on thymic γδ T cells (CD3 + TCRγδ+) from naive C57BL/6 mice (n = 9). (c) LAP expression on γδ T cells (CD3 + TCRγδ+) from WT and CCR6−/− mice (n = 6 per group). (d) Surface LAP expression on thymic CD3+ TCRγδ+ and CD3+ TCRγδ+ LAP+ cells from naive C57BL/6 mice (n = 9). (e) Intracellular LAP expression in thymic CD3+ TCRγδ+ and CD3+ TCRγδ+ LAP+ cells from naive C57BL/6 mice. Cells were first incubated with purified anti-LAP (clone TW7-16B4) to block surface LAP and then fixed/permeabilized and labeled with PE-anti-LAP antibody (n = 6). These data are representative of at least three independent experiments. Data are shown as mean ± s.e.m. Student’s t-test was used. **P<0.01, ***P<0.001, ****P<0.0001.
in spleen (0.15%; Fig. 7c). Moreover, transferred CD45.2-\(\gamma\delta\) T cells found in PPs expressed more LAP than splenic CD45.2-\(\gamma\delta\) T cells (Fig. 7d). Consistent with our observation that TCR\(\gamma\delta\) + LAP + cells downregulate IFN-\(\gamma\) (Fig. 6b), transferred CD45.2 + TCR\(\gamma\delta\) + LAP + cells had significantly less IFN-\(\gamma\) than their LAP - counterpart (Supplementary Fig. 10b). Neither CD45.2 + TCR\(\gamma\delta\) + LAP - nor CD45.2 + TCR\(\gamma\delta\) + LAP + cells expressed IL-17A (Supplementary Fig. 10c). PMA + ION was not used to stimulate these cells, since, as shown in Fig. 5f, LAP cannot be detected under these conditions. Thus, gut-homing \(\gamma\delta\) T1 cells migrate to the periphery with preferential accumulation in the gut.

**Discussion**

Gamma-delta (\(\gamma\delta\)) T cells are a unique subset of lymphocytes which originate in the thymus after recombination activating gene (RAG)-mediated V(D)J rearrangement\(^{38}\). \(\gamma\delta\) T cells are important in the immune response against pathogens and tumours\(^{39}\) and are enriched in the skin and mucosal tissues.\(^{40}\) In addition to their cytotoxic characteristics, regulatory functions of \(\gamma\delta\) T cells have been described, although they are not completely understood\(^{12,16,18-20,41}\). Of note, we found increased expression of GzmA and B in TCR\(\gamma\delta\) + LAP + cells suggesting that they may have cytotoxic properties, though this was not measured in our study. Foxp3 expression occurs in \(\gamma\delta\) T cells stimulated *in vitro* and a subset of IL-10-producing \(\gamma\delta\) T cells that protect mice liver from Listeria-elicited, CD8 T-mediated injury has been described\(^{42}\). Nonetheless, conflicting data have been reported in the literature regarding effector versus regulatory function of \(\gamma\delta\) T cells in models of disease in mice\(^{10,20,42}\). Here we describe a subset of regulatory \(\gamma\delta\) T cells in mice that are Foxp3 negative and express LAP. We also observed TCR\(\gamma\delta\) + LAP + cells in human peripheral blood.

**Figure 6** | IFN-\(\gamma\) is downregulated in TCR\(\gamma\delta\) + LAP + cells. (a) LAP expression on \(\gamma\delta\) T cells (CD3 + TCR\(\gamma\delta\) + ) from PPs with and without PMA + ION stimulation (\(n = 6\)). (b-d) IFN-\(\gamma\) and IL17A expression (protein and quantitative RT-PCR) in non-stimulated CD3 + TCR\(\gamma\delta\) + CD27 + cells from thymus (b) as well as CD3 + TCR\(\gamma\delta\) + LAP - and CD3 + TCR\(\gamma\delta\) + LAP + cells from PPs (c,d; \(n = 9\); ND = non-detected). These data are representative of at least three independent experiments. (e) Quantitative RT-PCR analysis of Atf3 mRNA from CD3 + TCR\(\gamma\delta\) + LAP - and CD3 + TCR\(\gamma\delta\) + LAP + cells (\(n = 3\) pooled cells from 10 mice per experiment). (f) FACS plots and frequency of IFN-\(\gamma\) expression in non-stimulated CD3 + TCR\(\gamma\delta\) + LAP + cells from C57BL/6 WT or ATF-3 –/– mice (\(n = 9\) per group). These data are representative of at least three independent experiments. Data are shown as mean ± s.e.m. Student's t-test was used. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.0001\).

**Figure 7** | Expression of gut-homing molecules on \(\gamma\delta\) T cells. (a) CCR9 and \(\alpha_{4}\beta_{1}\) expression on thymic \(\gamma\delta\) T cells (CD3 + TCR\(\gamma\delta\) + CD27 + and CD3 + TCR\(\gamma\delta\) + CCR6 + ) from naive C57BL/6 mice (\(n = 6\)). (b) Frequency and absolute number of total \(\gamma\delta\) T cells, CD3 + TCR\(\gamma\delta\) + CD27 + , CD3 + TCR\(\gamma\delta\) + LAP + and CD3 + TCR\(\gamma\delta\) + CCR6 + cells from C57BL/6 WT, CCR9 –/– and \(\alpha_{4}\beta_{1}\) –/– mice in the PPs (\(n = 5\) per group). (c) FACS plot and frequency of transferred CD45.2 + TCR\(\gamma\delta\) + CD27 + cells in the spleen and PPs of congenic CD45.1 mice 36 h after transfer (\(n = 3\)). (d) Histogram and frequency of CD45.2 + TCR\(\gamma\delta\) + LAP + cells in the spleen and PPs of congenic CD45.1 mice 36 h after transfer (\(n = 3\)). Data are shown as mean ± s.e.m. One-way analysis of variance followed by Tukey multiple comparisons (b) and Student’s t-test (a,c,d) were used. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.0001\), ****\(P < 0.00001\).
We found TCRγδ + LAP + cells throughout the immune system with highest expression in PPs and SI-LP. Both sites play an important role in defense against pathogens and the induction of immunological tolerance. Because γδ T cells have been shown to respond quickly to microbial and non-microbial tissue perturbation, which is particularly important in highly antigen-exposed sites, TCRγδ + LAP + cells may play a crucial role in gut homeostasis by providing a rapid regulatory response after encountering antigen. This is supported by the in vivo regulatory properties of TCRγδ + LAP + cells in the CD4 + CD45RBhigh cell transfer model of colitis. In this model, TCRγδ + LAP + cells decreased the inflammatory response caused by transferred CD4 T cells through reduction of pro-inflammatory cytokines such as IFN-γ, IL-17A, IL-6, TNF-α, CCL2 and CXCL10 and increase of the anti-inflammatory cytokines IL-10 and TGF-β1 mainly in the SI-LP. TCRγδ + LAP + cells induced proliferation and Foxp3 expression in the transferred CD4 T cells in the SI-LP and spleen, but not in the LI-LP, suggesting that TCRγδ + LAP + cells preferentially migrate to the SI-LP, where they control colitis by increasing the Foxp3 + Treg cell compartment and by switching the intestinal milieu from an inflammatory one to a regulatory one. Splenic TCRγδ + LAP + cells appear to play an important role in inducing CD4 + Foxp3 + cells and controlling colitis because, although there is a lower frequency of TCRγδ + LAP + cells in the spleen, the absolute number of TCRγδ + LAP + cells is 5-fold more than in SI-LP. In the DSS model of colitis, transfer of TCRγδ + LAP + cells ameliorated disease. How TCRγδ + LAP + cells exerted their regulatory activity in this model is not yet clear. In mice that were killed during the recovery phase of the colitis, we found differences in anti-inflammatory cytokines, such as IL-10 and TGF-β1 as well as IL-22, an important interleukin involved in the promotion of antimicrobial immunity, inflammation and tissue repair at barrier surfaces. Of note, the mechanism by which DSS induces intestinal inflammation is believed to result from damage to the epithelial monolayer lining in the large intestine allowing the dissemination of pro-inflammatory intestinal contents (such as bacteria and their products) into underlying tissue. Thus, TCRγδ + LAP + cells may control DSS-induced colitis by protecting gut epithelium. Furthermore, mice given TCRγδ + LAP + cells had higher frequency and absolute cell number of CD4 + Foxp3 + cells in the spleen, but no difference was seen in the LI-LP, the DSS target site. It is possible that analysis of CD4 + Foxp3 + cells at earlier stages in the DSS-induced colitis model would show Treg cell expansion induced by TCRγδ + LAP + cell treatment in the colonic lamina propria. Taken together, these data indicate that the regulatory effects induced by TCRγδ + LAP + cells in DSS colitis is related to an increase of the Foxp3 + Treg cell compartment as well as production of anti-inflammatory and epithelium protective cytokines.

Suppressive activity of TCRγδ + LAP + cells was not observed in a conventional in vitro suppression assay in which responder naïve CD4 T cells were stimulated with anti-CD3ε in the presence of APCs. Under these conditions TCRγδ + LAP + cells induced proliferation of responder cells to a greater extent than TCRγδ + LAP − cells or controls. Furthermore, contrary to what we observed in vivo, under these in vitro conditions, Foxp3 expression was not induced in responder cells, suggesting that the in vivo regulatory function of TCRγδ + LAP + cells involves more complex cell-cell interactions than in vitro. These data also suggest that TCRγδ + LAP + cells acquire a pro-inflammatory phenotype following anti-CD3ε stimulation in vitro. Consistent with this, we found that TCRγδ + LAP + cells stimulated in vitro with plate-bound anti-CD3ε/anti-CD28 produced less TGF-β1 and more TNF-α mRNA than freshly isolated TCRγδ + LAP + cells. Accordingly, Foxp3 was induced when MOG specific CD4 + TCR Tg cells were cultured with freshly isolated TCRγδ + LAP + cells. Thus, stimulation of TCRγδ + LAP + cells with anti-CD3ε impairs their ability to induce CD4 + Foxp3 + cells, but does not affect their ability to induce proliferation of CD4 + T cells. Foxp3 induction by TCRγδ + LAP + cells was reversed by anti-LAP blocking antibody, indicating that induction of Foxp3 is mediated by LAP/TGF-β1, analogous to the infectious tolerance induced by CD4 + Foxp3 + Treg cells which also relies on LAP/TGF-β1 expression.

We found that TCRγδ + LAP + cells upregulated antigen presentation-associated molecules including MHC-II, CD40 and CD86. Consistent with this, the APC-like function and Foxp3 induction capability of TCRγδ + LAP + cells was lost when TCRγδ + LAP + cells from MHCI-II−/− mice were used. Of note, although TCRγδ + LAP − cells did not induce proliferation in vitro, they did induce proliferation in vivo. This difference may be related to the activation of TCRγδ + LAP − cells in vivo, which would increase the breadth of antigen presentation molecules and enhance their APC function. However, because TCRγδ + LAP − cells do not express LAP, they do not have regulatory properties.

We found that thymic γδT1 (CD272 + INF-γ + ) cells gave rise to both TCRγδ + LAP + and TCRγδ + LAP − cells. This is consistent with our observation that TCRγδ + LAP + and TCRγδ + LAP − cells as well as γδT1 cells expressed Vγ1 and Vγ4 TCR chains, a characteristic of non-canonical γδ T cells. Intracellular LAP was detected in 15% of thymic γδT1 cells and LAP was further upregulated after γδT1 cells migrated from the thymus to the periphery, primarily to the gut (PPs and SI-LP). Because GARP, a glycoprotein known to bind and attach LAP to the cell surface was not detected on thymic γδT1 cells, this may explain why thymic γδT1 cells do not express surface LAP. Thymic γδT1 cells expressed the gut-homing imprint molecules CCR9, the CCL25 chemokine receptor, and α4β7 integrin, which binds to the mucosal addressin cell adhesion molecule-1 (MadCAM-1) expressed on the high endothelial venules of the PPs and gut lamina propria. When γδT1 cells become TCRγδ + LAP + cells, we observed downregulation of INF-γ an effect that may be mediated by ATF-3. ATF-3 is an adaptive-response gene and may modulate INF-γ production either indirectly by reducing the production of cytokines, such as IL-12 (ref. 35), or directly by targeting a cis-regulatory element in the INF-γ gene (at least in NK cells). We found that ATF-3−/− mice had higher expression of INF-γ in TCRγδ + LAP + cells, suggesting that ATF-3 may have a direct effect on INF-γ transcription in TCRγδ + LAP + cells, as IL-12 is not required for INF-γ expression in γδ T cells. Our data suggest that thymic γδT1 cells expressing CCR9 and α4β7 integrin migrate to the gut, upregulate GARP and LAP, downregulate INF-γ via ATF-3 and acquire their APC properties. When TCRγδ + LAP + cells present antigen to a CD4 T cell, TGF-β1 is released from LAP and induces Foxp3 in the CD4 T cell, rendering them regulatory. One of the major molecules that converts LAP to TGF-β1 is thrombospondin-1 (TSP-1). TSP-1 is expressed in both naïve and activated CD4 T cells and activates TGF-β1 both in vitro and in vivo. Thus, the induction of Tregs by TCRγδ + LAP + cells appears to be an important physiologic mechanism by which TCRγδ + LAP + cells contribute to gut homeostasis.

In summary, our data identify TCRγδ + LAP + cells as a new subset of γδ T cells with regulatory properties. The identification of TCRγδ + LAP + cells provides a new avenue for understanding immune regulation and biologic processes linked to intestinal function and disease.
In some experiments, naïve cells from 2D2Foxp3-GFP mice were sorted and co-cultured with sorted TCRγδ+ L–P or TCRγδ+ L+ cells from WT mice in the absence of APCs and stimulated with myelin oligodendrocyte glycoprotein (MOG35-55 peptide, 20 μg/ml⁻¹) in 200 μl of IMDM medium supplemented with 10% FBS in 96-well round-bottom plates. Proliferation and Foxp3 expression were assessed 72 h later by flow cytometry, based upon the dilution of the CellTrace violet dye and GFP expression, respectively.

In vitro activation of TCRγδ+ L–P and/or TCRγδ+ L+ cells. Sorted TCRγδ+ L–P and/or TCRγδ+ L+ cells were incubated for 5 days at 37 °C in the presence of plate-bound anti-CD3 and anti-CD28 (1 μg/ml⁻¹ each). On the fourth day, RNA was extracted as described below in the real-time PCR section.

Uptake and presentation assays. C10D3+ CD11c+ L–P, C10D3+ CD11c+ L+ dendritic cells and TCRγδ+ L–P, TCRγδ+ L+ cells were first enriched using CD11c microbeads or TCRγδ isolation kit (all from Miltenyi Biotec) and gated. For uptake assay, TCRγδ+ L–P and/or TCRγδ+ L+ cells were incubated for 3 h at 37 °C with 50 μg/ml⁻¹ of ovalbumin (OVA) coupled to Alexa Fluor 488 (Invitrogen) in a 96-well round-bottom plate. After incubation, cells were collected, thoroughly washed and analysed by flow cytometry. For pre-presentation assay, sorted C10D3+ CD11c+ L–P, C10D3+ CD11c+ L+ dendritic cells and TCRγδ+ L–P, TCRγδ+ L+ cells (from WT or MHC-II–/– mice) were first incubated overnight at 37 °C with 30 μg/ml⁻¹ of OVA257–264, OVA323–339, OVA326–339, OVA328–340 (Invitrogen) or medium only (unloaded cells as control) in a 96-well round-bottom plate. Next day, the cells were thoroughly washed and incubated at 1:1 ratio with sorted naive (CD4+ CD262L+ CD44-Foxp3–) cells from OT-I/IlsxFoxp3-GFP mice previously stained with CellTrace Violet (Invitrogen) for 4 days. Proliferation and Foxp3 induction were then analysed by flow cytometry. In some experiments, purified anti-CD25 mAb (T7–16B4) was used to study the involvement of LAP in the Foxp3 induction by TCRγδ+ L–P at a concentration of 30 μg/ml⁻¹.

In vivo presentation and Foxp3 induction assays. For the in vivo presentation and Foxp3 induction study, sorted TCRγδ+ L–P or TCRγδ+ L+ cells from C57BL/6 mice were first loaded overnight with 50 μg/ml⁻¹ of OVA257–264, OVA323–339, OVA326–339, OVA328–340 peptide (Invitrogen) or medium only (unloaded cells as control), thoroughly washed, and 1 × 10⁵ cells per animal were intravenously transferred together with 2 × 10⁵ CellTrace Violet (Invitrogen)-stained naive CD4 T cells (CD4+ CD262L+ CD44-Foxp3– from OT-I/IlsxFoxp3-GFP mice) per animal in a volume of 100 μl. CellTrace violet staining was performed according to the manufacturers’ recommendation. The mice were killed 5 days later and the spleens removed for FACS analysis.

Dextran sodium sulfate-induced colitis model. TCRγδ+ L–P and/or TCRγδ+ L+ cells were sorted from C57BL/6 mice as described above and intraperitoneally transferred to syngeneic mice at 5 × 10⁵ cells per animal in 100 μl of PBS at days 0 and 2 (Supplementary Fig. 6a). Colitis was induced by 3% (w/v) dextran sodium sulfate (DSS; Sigma-Aldrich) in a 1% methyl cellulose solution (MCC, 600,000 μl⁻¹; LLC) added to the drinking water for 7 consecutive days. Mice were weighted daily until the end of the experiment (14 days). At day 14, the mice were killed and the colons were removed for length measurement, histological analysis, RT–PCR and FACS.

CD4+ CD245Rhigh cell transfer-induced colitis model. CD4+ CD25– CD4+ CD245Rhigh cells were sorted from C57BL/6 mice using CD245R (XMG1.2), PerCp-Cy5.5 anti-CD45RB (C363-16A), FITC anti-CD25 (PK13.1), FITC anti-CD3 (1452C11,1.2), PE anti-zeta (DATK32;1.000), eFluor450-anti-CCR9 (CW-1.2;1.000), PE anti-GARP (YGC86;1.000), PE anti-TCR Vγ2 (UC3-10A6;1.000), FITC anti-TCD7 (CG7.9P;1.000), PE anti-CD27 (LG.7F9;1.000), PE anti-CCR6 (6.7;1.000), FITC anti-TCRγδ L–P cells from WT mice were intravenously transferred to mice at 5 × 10⁵ cells per animal in 100 μl of PBS at days 0 and 2 (Supplementary Fig. 6a). Colitis was induced by 3% (w/v) dextran sodium sulfate (DSS; Sigma-Aldrich) in a 1% methyl cellulose solution (MCC, 600,000 μl⁻¹; LLC) added to the drinking water for 7 consecutive days. Mice were weighted daily until the end of the experiment (14 days). At day 14, the mice were killed and the colons were removed for length measurement, histological analysis, RT–PCR and FACS.
Expression analysis of TCRγδ + LAP + versus TCRγδ + LAP – cells using RNA-Seq. Total RNA samples were supplied to the Broad Institute’s Genomics Platform and were QC’d by Agilent Bioanalyzer for RNA Integrity Scores (RIN >6), and normalized by Nanodrop to a minimum of 5 ng/μl and 250 ng.

Libraries were constructed using Illumina’s TrueSeq kit with Poly A selection, pooled and sequenced on the Illumina HiSeq 2000 with 76 bp paired-end reads to a read coverage of 15 M reads per sample.

After read preprocessing and QC bias removal, we processed our sequencing data using the latest Tuxedo RNA-Seq pipeline28, in particular, TopHat v2.0,11, Bowtie v2,2.0.0, and Cufflinks v2.1. We aligned our reads to mouse genome version GRCm38.p2 (mm10) with the GeneCode GRCm38R2 genome as annotation. Using Cauffman’s traditional FPKM with a pooled replicate model, we generated signatures of differentially expressed genes for TCRγδ + LAP + versus TCRγδ + LAP – cells. In addition to Cauffman’s significance measure, we chose to disregard low expression genes that did not have an arbitrary minimum absolute difference between FPKM between expression values as cutoff to account for detection noise. Using these criteria, we identified a signature of 41 genes that were enriched specifically in TCRγδ + LAP + cell samples with q < 0.05, while 407 genes were enriched in TCRγδ + LAP + cells with P < 0.05. We used the likelihood function proposed by Trapnell et al.29 and Roberts et al.30 to calculate P and q values. To remove ‘infinity’ values of log2-fold change for plotting, we added a constant of 0.001 to all the expression values and then recalculated the log2-fold-expression difference.

Statistics. GraphPad Prism 6.0 was used for statistical analysis (unpaired, two-tailed Student’s t-test or one-way analysis of variance, followed by Tukey multiple comparisons). For weight loss experiments, two-way analysis of variance was used. Differences were considered statistically significant with a P value of less than 0.05.

References

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Author contributions

R.M.R. initiated the project, designed the experiments, carried out most of the experiments and wrote the manuscript. A.P.C. helped design and perform the experiments. H.M., G.G., T.V., S.L. and C.K. helped perform the experiments. N.P.R and R.P.O. initiated the project. R.C., J.T.G., N.O., J.K. and N.P. performed and analysed RNA-Seq experiments. A.M.C.F. provided input for the manuscript and H.L.W. supervised the experiments and the manuscript.

Additional information


Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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