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

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Published Version</td>
<td>doi:10.1038/ncomms9726</td>
</tr>
<tr>
<td>Citable link</td>
<td><a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:24983923">http://nrs.harvard.edu/urn-3:HUL.InstRepos:24983923</a></td>
</tr>
<tr>
<td>Terms of Use</td>
<td>This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at <a href="http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA">http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA</a></td>
</tr>
</tbody>
</table>
Identification and characterization of latency-associated peptide-expressing γδ T cells

Rafael M. Rezende¹, Andre P. da Cunha¹, Chantal Kuhn¹, Stephen Rubino¹, Hanane M’Hamdi¹,², Galina Gabriely¹, Tyler Vandeventer¹, Shirong Liu¹, Ron Cialic¹, Natalia Pinheiro-Rosa³, Rafael P. Oliveira³, Jellert T. Gaublomme⁴,⁵, Nikolaus Obholzer⁶, James Kozubek⁶, Nathalie Pochet⁶,⁷, Ana M.C. Faria³ & Howard L. Weiner¹

γδ T cells are a subset of lymphocytes specialized in protecting the host against pathogens and tumours. Here we describe a subset of regulatory γδ T cells that express the latency-associated peptide (LAP), a membrane-bound TGF-β1. Thymic CD27⁺ IFN-γ⁺ CCR9⁺ α₄β₇⁺ TCRγδ⁺ cells migrate to the periphery, particularly to Peyer’s patches and small intestine lamina propria, where they upregulate LAP, downregulate IFN-γ via ATF-3 expression and acquire a regulatory phenotype. TCRγδ⁺ LAP⁺ cells express antigen presentation molecules and function as antigen presenting cells that induce CD4⁺ Foxp3⁺ regulatory T cells, although TCRγδ⁺ LAP⁺ cells do not themselves express Foxp3. Identification of TCRγδ⁺ 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+ or 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 2,6-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 anti-microbial 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. Forkhead 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 humans 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). 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. 1a). To confirm this, we found that 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 Repetitions 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 naive mice. Consistent with this, we found that γδ T cells from PPs and spleen of naive 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 Tonegawa). In summary, our results identify a subpopulation of γδ T cells in mice that express LAP on their surface.

TCRγδ+LAP+ cells induce Tregs and ameliorate colitis. As LAP expression confers regulatory function to CD4 and CD8 T cells, we asked whether TCRγδ+LAP+ cells had in vivo regulatory activity. Two models of colitis were used to address this question: the T-cell model of colitis induced by oral administration of the chemical compound DSS and the innate immune-mediated model of colitis induced by oral administration of the chemical compound DSS (Supplementary Fig. 6a). RAG-1−/− mice transferred with either CD4+ or CD45RBhigh cells alone (control group) or together with TCRγδ+LAP− cells began to exhibit signs of colitis as measured by body weight loss at 5 weeks after transfer. This was not observed when animals received TCRγδ+LAP+ cells (Fig. 2b). The experiment was terminated at 7 weeks at which time mice had lost 15% of their body weight. Consistent with the weight reduction, histological analyses showed more severe colonic and small intestine tissue ulceration and inflammatory cell infiltrate in control or TCRγδ+LAP− mice than TCRγδ+LAP+ cell-treated mice (Fig. 2c). Furthermore, mice transferred with TCRγδ+LAP+ cells had less IL-6, TNF-α, and IL-17 expression as measured by mRNA expression (Fig. 2d).
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-transfected animals as compared with TCRγδ + LAP − cell-transfused 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 infiltration 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 TCR\(\gamma\delta\)+LAP+ cells prevent CD4+ CD45RB\(\text{high}\) cell transfer-induced colitis in mice, we sorted naive CD4+ T cells from Foxp3-GFP mice and stimulated them with anti-CD3e in the presence of Foxp3+ cells from WT mice plus antigen presenting cells (APCs). As a control, nTregs were tested. We found that neither TCR\(\gamma\delta\)+LAP+ nor TCR\(\gamma\delta\)+LAP− cells were suppressive \textit{in vitro} as compared with nTreg cells. Instead, TCR\(\gamma\delta\)+LAP+ cells induced higher proliferation than control or TCR\(\gamma\delta\)+LAP− cells even at a 1:8 responder:TCR\(\gamma\delta\)+LAP+ cell ratio (Supplementary Fig. 7a,b). Furthermore, no Foxp3 induction in responder cells was observed when either TCR\(\gamma\delta\)+LAP+ or TCR\(\gamma\delta\)+LAP− cells were added to the culture as compared with the Foxp3 induction by nTreg cells (Supplementary Fig. 7c,d). Consistent with this, TCR\(\gamma\delta\)+LAP+ cells stimulated \textit{in vitro} with plate-bound anti-CD3e and anti-CD28 acquired a pro-inflammatory profile with less TGF-\(\beta\)1 and more TNF-\(\alpha\) mRNA expression (Supplementary Fig. 8a,b). To further investigate whether TCR\(\gamma\delta\)+LAP+ cells had suppressive properties \textit{in vitro} and to determine whether the activation of TCR\(\gamma\delta\)+LAP+ cells by anti-CD3e was associated with their inability to induce CD4+ Foxp3+ cells \textit{in vitro}, we sorted naive CD4 T cells from 2D2xFoxp3-GFP mice (2D2 are MOG\textsubscript{35-55}-specific TCR transgenic animals) and stimulated them with MOG\textsubscript{35-55} peptide in the presence of TCR\(\gamma\delta\)+LAP− or TCR\(\gamma\delta\)+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 TCR\(\gamma\delta\)+LAP+ without stimulating \(\gamma\delta\) T cells with anti-CD3e as we did above.

We found that TCR\(\gamma\delta\)+LAP+ but not TCR\(\gamma\delta\)+LAP− cells induced both proliferation and Foxp3 expression in CD4 T cells (Supplementary Fig. 8c,d). Thus, when TCR\(\gamma\delta\)+LAP+ cells are not stimulated by anti-CD3e \textit{in vitro}, they are able to induce CD4+ Foxp3+ Treg cells as they do \textit{in vivo}. Moreover, because we did not add APCs to the co-culture, these results suggest that TCR\(\gamma\delta\)+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 \textit{vivo} TCR\(\gamma\delta\)+LAP+ cells ameliorate colitis by promoting the induction of Foxp3 Treg cells. In \textit{vivo} experiments demonstrate that they do not have direct regulatory function, but indirectly induce Tregs through their APC properties.

**Antigen presenting cell function of TCR\(\gamma\delta\)+LAP+ cells.** To further characterize TCR\(\gamma\delta\)+LAP+ cells, we performed RNA-Seq of both TCR\(\gamma\delta\)+LAP+ and TCR\(\gamma\delta\)+LAP− cells (Table 1; Supplementary Data 1 and Supplementary Data 2). We identified a signature of 407 genes that were enriched in TCR\(\gamma\delta\)+LAP+ versus TCR\(\gamma\delta\)+LAP− cells with \(P<0.05\). Among the upregulated genes, we found increased expression of genes related to antigen presentation, including MHC class II molecules (H2-Aa, H2-Ab1, H2-Eb1 and H2-Eb2), CD40 and CD86. We confirmed the expression of these APC-associated molecules on TCR\(\gamma\delta\)+LAP+ cells by flow cytometry (Fig. 3). Thymic \(\gamma\delta\)T1 cells and TCR\(\gamma\delta\)+LAP− cells from PPs expressed MHC-II, to a lesser extent CD86, but did not express CD40 (Fig. 3a–d). TCR\(\gamma\delta\)+LAP−

---

**Figure 2 | TCR\(\gamma\delta\)+LAP+ cells prevent CD4+ CD45RB\(\text{high}\) cell transfer-induced colitis in mice.** (a) Schematic protocol of CD4+ CD45RB\(\text{high}\) cell transfer-induced colitis and \(\gamma\delta\) T cell adoptive transfers. (b) Body weight (% of initial weight) was measured throughout the experiment. Graph shows the mean ± s.e.m. of naive, CD4+ CD45RB\(\text{high}\) cell-treated only (Control) or together with CD3+ TCR\(\gamma\delta\)+LAP− or CD3+ TCR\(\gamma\delta\)+LAP+ cells groups. (c) COL3A1, COL4A1 and COL5A1 were detected at week 7, and 5-micron serial sections were stained with haematoxylin-eosin. Magnification × 40. Scale bars, 600 \(\mu\)m. (d) Quantitative RT-PCR analysis of pro-inflammatory and anti-inflammatory cytokine mRNAs from SI-LP of cell transfer-induced colitis mice. These data are representative of three independent experiments. (e,f) FACS plots, frequency and absolute number of Foxp3 expression in transferred CD4 T cells (e) and frequency and absolute number of total transferred CD4 T cells as well as total transferred CD3+CD4+ (f) in SI-LP of cell transfer-induced colitis mice. These data are representative of three independent experiments. Data are shown as mean ± s.e.m. (n = 9 for naive; n = 15 for control and TCR\(\gamma\delta\)+LAP− groups; n = 9 for TCR\(\gamma\delta\)+LAP+ group). Two-way analysis of variance (ANOVA) (b) and one-way ANOVA followed by Tukey multiple comparisons (d–f) were used. *, statistically different from control group; #, statistically different from both control and TCR\(\gamma\delta\)+LAP+ groups (\(P<0.05\)). **P<0.01, ***P<0.001, ****P<0.0001.
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 CD4+ DCs is consistent with their well-known tolerogenic properties measured Foxp3 expression in naive CD4+ T cells (Fig. 4b). 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-1×Foxp3-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-1×Foxp3-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-1×Foxp3-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− log2 (fold change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Itgae</td>
<td>57.64</td>
<td>150.95</td>
<td>1.389</td>
</tr>
<tr>
<td>Aif3</td>
<td>9.12</td>
<td>29.45</td>
<td>1.691</td>
</tr>
<tr>
<td>Cd81</td>
<td>5.27</td>
<td>17.70</td>
<td>1.748</td>
</tr>
<tr>
<td>Cd86</td>
<td>2.13</td>
<td>7.19</td>
<td>1.754</td>
</tr>
<tr>
<td>Cd244</td>
<td>9.62</td>
<td>33.83</td>
<td>3.185</td>
</tr>
<tr>
<td>Lag3</td>
<td>6.36</td>
<td>30.39</td>
<td>2.257</td>
</tr>
<tr>
<td>H-2Eb2</td>
<td>0.70</td>
<td>3.57</td>
<td>2.350</td>
</tr>
<tr>
<td>Cd83</td>
<td>2.93</td>
<td>15.28</td>
<td>2.379</td>
</tr>
<tr>
<td>H-2-Dmb1</td>
<td>1.14</td>
<td>7.79</td>
<td>2.769</td>
</tr>
<tr>
<td>H-2-Dmb2</td>
<td>1.39</td>
<td>9.62</td>
<td>2.783</td>
</tr>
<tr>
<td>Cd74</td>
<td>32.30</td>
<td>293.34</td>
<td>3.182</td>
</tr>
<tr>
<td>H-2-Eb1</td>
<td>8.27</td>
<td>78.55</td>
<td>3.247</td>
</tr>
<tr>
<td>H-2-Aa</td>
<td>8.33</td>
<td>80.86</td>
<td>3.278</td>
</tr>
<tr>
<td>Apoe</td>
<td>5.60</td>
<td>108.03</td>
<td>3.645</td>
</tr>
<tr>
<td>GzmB</td>
<td>25.39</td>
<td>336.20</td>
<td>3.727</td>
</tr>
<tr>
<td>Gzma</td>
<td>100.62</td>
<td>1416.50</td>
<td>3.815</td>
</tr>
</tbody>
</table>

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 sub-populations: γδT1 cells, characterized by the expression of CD27 and the production of IFN-γ (ref. 7); and γδT17 cells, which are CD27− and express CCR6 and secrete IL-17 (ref. 6). Both subtypes are considered non-canonical γδ T cells and express Vγ1 and Vγ4 TCR chains5, which is consistent with what we observed in TCRγδ+ LAP+ and TCRγδ+ LAP− cells (Fig. 1g; 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 γδT1 cells as they were negative for CD27 and expressed CCR6 (Fig. 5b). When we examined LAM 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 LAM on γδ T cells most likely occurs in the periphery because neither thymic γδT1 nor γδT17 cells expressed LAM on the surface (Fig. 5d). Intracellular LAM expression was detected in 15% of thymic γδT1, but not in γδT17 cells (Fig. 5e), indicating that LAM 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 LAM 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
whether thymic 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 is primarily detected on γδT1 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

**Figure 3** | Expression of antigen presentation-related molecules in γδ T cells. (a,b) MHC-II, MHC-I, CD86 and CD40 expression on γδ T cells from thymus (a, CD3 + TCRγδ + CD27 +) and PPs (b, CD3 + TCRγδ + LAP +) (n = 9). (c,d) Frequency of MHC-II, MHC-I, CD86 and CD40 on γδ T cells from thymus (c) and PPs (d). These data are representative of at least five independent experiments. Student’s t-test was used. ****P < 0.0001.
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+ LAP− 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-IICxFoxp3-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-IIγδxFoxp3-GFP mice co-cultured with OVA323-339-loaded TCRγδ+ LAP+ cells from WT C57BL/6 mice for 4 days at 37 °C (n = pooled cells from 10 mice per experiment). (e) Proliferation (e) and Foxp3 induction (f) in CellTrace Violet-stained naive CD4 T cells from OT-IIγδxFoxp3-GFP mice co-cultured with OVA323-339-loaded (or not) CD3+ TCRγδ+ LAP− or CD3+ TCRγδ+ LAP+ cells from either WT C57BL/6 or MHC-II−/− 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+ cells from naive C57BL/6 mice (n = 9). (e) Intracellular LAP expression in thymic CD3+ TCRγδ+ and CD3+ TCRγδ+ 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<0.0001.
which originate in the thymus after recombination activating gene (RAG)-mediated V(D)J rearrangement\textsuperscript{38}. \(\gamma\delta\) T cells are important in the immune response against pathogens and tumours\textsuperscript{39} and are enriched in the skin and mucosal tissues\textsuperscript{40}. In addition to their cytotoxic characteristics, regulatory functions of \(\gamma\delta\) T cells have been described, although they are not completely understood\textsuperscript{12,16,18–20,41}. Of note, we found increased TCR\(\gamma\delta\)+ LAP cells in human peripheral blood.

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 \(\gamma\delta\) T 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
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 T 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 internal 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 vivo. 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 were lost when TCRγδ + LAP + cells from MHC-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 in turn would increase the basal expression 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 (CD27 + 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 zββ, 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 IFN-γ an effect that may be mediated by ATF-3. ATF-3 is an adaptive-response gene and may modulate IFN-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 IFN-γ gene (at least in NK cells). We found that ATF-3+/− mice had higher expression of IFN-γ in TCRγδ + LAP + cells, suggesting that ATF-3 may have a direct effect on IFN-γ transcription in TCRγδ + LAP + cells, as IL-12 is not required for IFN-γ expression in γδ T cells. Our data suggest that thymic γδT1 cells expressing CCR9 and zββ, integrin migrate to the gut, upregulate GARP and LAP, downregulate IFN-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. 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.
Methods

**Mice.** Male and female, 8–10-week-old and on a B6 genetic background mice were used in this study. C57BL/6 wild type, congenic CD45.1, RAG-1 −/−, CCR6 −/−, MHCI–II −/− and β2 −/− mice were purchased from the Jackson Laboratory. CCR9 −/− mice were kindly provided by Dr Jesus Rivera-Nieves (University of California at San Diego—UCSD). ATF3 −/−, Foxp3-3-GFP, OT-I Foxp3-GFP and 2D2xFoxp3-GFP mice were housed in a conventional specific pathogen-free facility at the Harvard Institutes of Medicine according to the animal protocol with the full knowledge and permission of the Standing Committee on Animals at Harvard Medical School.

**FACS and intracellular cytokine staining.** A pool of cells from spleen and PPs or thymus of Foxp3-GFP and C57BL/6 mice was first enriched using CD4 microbeads (Follox-3-GFP) or TCRβ isolation kit (C57BL/6; all from Miltenyi Biotec). C57BL/6 (CD4 + CD262L + CD44-Foxp3 +) and CD4 + Foxp3 + cells were sorted (FACS Aria II, BD Bioscience) with peridinin chlorophyll protein (PerCP)-conjugated anti-CD4 (RM4–5; 1:250), allophycocyanin (APC)-conjugated anti-CD62L (MEL-14; 1:250) and phycoerythrin (PE)-conjugated anti-CD44 (IM7; 1:500; all from BioLegend). CD4 + Foxp3 + cells were sorted on the basis of expression, TCRγδ + LAP + and TCRγδ + LAP − T cells were sorted with Alexa Fluor 700 (AF700)-conjugated anti-CD3e (eBio500A2; 1:100), APC-conjugated anti-TCRγδ (eBioG3.1; 1:100) and PE-conjugated anti-late-associating peptide (LAP)/TGR7 (T8–16B4; 1:50; all from eBioscience). For TCRγδ + cell sorting, dead cells were excluded on the basis of 7-AAD (1:25; BD Biosciences) staining. For intracellular cytokine staining, surface markers were stained for 25 min at 4°C with anti-TCRγδ (10C11; 1:100), PE-anti-IFN-γ (XMG1.2; 1:100) and Anti-CD45.2 (104; 1:100; all from eBioscience), Pacific Blue (PB)-anti-CD8α (53–6.7; 1:100), PE-anti-CD44-Foxp3 (RM4–5; 1:250), allophycocyanin (APC)-conjugated anti-CD4 (145–2C11; 1:50; all from BioLegend) and CD44-Foxp3 (2.11; 1:100) previously stained with CellTrace Violet dye (Invitrogen) for 4 days. Proliferation and Foxp3 induction study, sorted TCRγδ + LAP + and TCRγδ + LAP − cells were incubated for 3h 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 TCD3+D11c +, TCD10+D11c + dendritic cells and TCRγδ + LAP +, TCRγδ + LAP − cells were first enriched using CD11c microbeads or TCRβ isolation kit (all from Miltenyi Biotec) and stained. For uptake assay, TCRγδ + LAP + and TCRγδ + LAP − cells were incubated for 3h at 37°C with 50 μg/ml -1 of OVA25–33,35–55 peptide (OVA25–33,35–55) peptide or medium only (unloaded cells as control) in a 96-well round-bottom plate. Per 48 h, the cells were thoroughly washed and incubated at 1:1 ratio with sorted naive (CD4 + CD262L + CD44-Foxp3 −) cells from OT-I Foxp3-GFP mice previously stained with CellTrace Violet dye (Invitrogen) for 4 days. Proliferation and Foxp3 induction were then analysed by flow cytometry. In some experiments, purified anti-LAP mAb (T8–16B4) was used to show the involvement of LAP in the Foxp3 induction by TCRγδ + LAP + and cells at a concentration of 30 μg/ml -1.

**In vitro activation of TCRγδ + LAP + and TCRγδ + LAP − cells.** Sorted TCRγδ + LAP − and TCRγδ + LAP + cells were incubated for 3 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.** CD103+D11c +, CD103+D11c − dendritic cells and TCRγδ + LAP +, TCRγδ + LAP − cells were first enriched using CD11c microbeads or TCRβ isolation kit (all from Miltenyi Biotec) and stained. For uptake assay, TCRγδ + LAP + and TCRγδ + LAP − cells were incubated for 3h at 37°C with 50 μg/ml -1 of OVA25–33,35–55 peptide (OVA25–33,35–55) peptide or medium only (unloaded cells as control) in a 96-well round-bottom plate. Per 48 h, the cells were thoroughly washed and incubated at 1:1 ratio with sorted naive (CD4 + CD262L + CD44-Foxp3 −) cells from OT-I Foxp3-GFP mice previously stained with CellTrace Violet dye (Invitrogen) for 4 days. Proliferation and Foxp3 induction were then analysed by flow cytometry. In some experiments, purified anti-LAP mAb (T8–16B4) was used to show the involvement of LAP in the Foxp3 induction by TCRγδ + LAP + and cells 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 + LAP − or TCRγδ + LAP + cells from C57BL/6 mice were first loaded overnight with 50 μg/ml -1 of OVA25–33,35–55 peptide (Invitrogen) or medium only (unloaded cells as control), thoroughly washed, and 1 × 105 cells per animal were intravenously transferred together with 2 × 106 CellTrace Violet (Invitrogen)-stained naive CD4 T cells (CD4 + CD262L + CD44-Foxp3 −) from OT-I Foxp3-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 + LAP + and TCRγδ + LAP − 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; MP Biomedicals, Santa Barbara, CA, USA) added to the drinking water for 7 consecutive days. Mice were weighed and checked for image quality using visual inspection.

**Histopathology.** Colons and/or small intestines were excised from animals at the end of both colitis experiments, flushed with PBS, cut longitudinally, rolled into ‘Swiss rolls’ and immediately fixed in formaldehyde 4% for 48 h and kept in ethanol 70%. Samples were then embedded in paraffin and 5 μm and cut and stained with haematoxylin and eosin. Sections were evaluated for histopathological changes, such as tissue integrity and inflammatory cells infiltration after being loaded into an Aperio ScanScope XT (Aperio), scanned via the semi-automated method and checked for image quality using visual inspection.

**Real-time PCR.** Naive CD4 + CD262L + CD44-Foxp3 −, CD4 + Foxp3 +, TCRγδ + LAP + and TCRγδ + LAP − cells were sorted and RNA was extracted with a miRNeasy kit (Qagen), then was reverse-transcribed with a high capacity cDNA reverse transcription kit (Applied Biosystems) and analysed by quantitative
RT–PCR with a Viia 7 Real-time PCR system (Applied Biosystems) with the following primers and probes (from Applied Biosystems; identifier in parentheses): Tgfb1 (Mm00441242_m1), Il6 (Mm00443961_m1), Il1β (Mm00474393_m1), Aflt (Mm00476032_m1), Foxp3 (Mm00475166_m1), Tnfa (Mm00443325_m1), Il6 (Mm00446191_m1), Il10 (Mm00439616_m1), Il12 (Mm00444241_m1), Cd27 (Mm00441242_m1), Cd25 (01301247_m1) and Ccl10 (Mm00442352_m1). The comparative threshold cycle method and the internal control G6pdh (Mm9999915_g1) was used for normalization of the target genes.

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 TruSeq 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 v2.2.1. We aligned our reads to mouse genome version GRCm38 (mm10) with the Gencode GRCm38M2 gene set as annotation. Using Cuffdiff’s traditional FPKM with a pooled replicate model, we disregarded low expression genes that did not have an arbitrary minimum absolute difference. After read preprocessing and GC 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 v2.2.1. We aligned our reads to mouse genome version GRCm38 (mm10) with the Gencode GRCm38M2 gene set as annotation. Using Cuffdiff’s traditional FPKM with a pooled replicate model, we disregarded low expression genes that did not have an arbitrary minimum absolute 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.

References

Acknowledgements
This work was supported by the NIH (R01 AI43458 to H.L.W.) and Susan Furbacher Conroy Fellowship to A.P.C. We thank Deneen Kozoriz for her excellent technical support in cell sorting.

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.I. 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
Overexpression of the plasmid pCMV-NS-EGFP at high concentrations can lead to cell death in vitro. The authors declare no competing financial interests.

Reprints and permission information is available online at http://npg.nature.com/reprintsandpermissions/


This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material.
To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/