Proinflammatory microenvironments within the intestine regulate the differentiation of tissue-resident CD8+ T cells responding to infection

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We report that oral infection with Yersinia pseudotuberculosis results in the development of two distinct populations of pathogen-specific CD8+ tissue-resident memory T cells (T_{RM} cells) in the lamina propria. CD103+ T cells did not require transforming growth factor-β (TGF-β) signaling but were true resident memory cells. Unlike CD103+CD8+ T cells, which were TGF-β dependent and were scattered in the tissue, CD103-CD8+ T cells clustered with CD4+ T cells and CX3CR1+ macrophages and/or dendritic cells around areas of bacterial infection. CXCR3-dependent recruitment of cells to inflamed areas was critical for development of the CD103+ population and pathogen clearance. Our studies have identified the 'preferential' development of CD103- T_{RM} cells in inflammatory microenvironments within the lamina propria and suggest that this subset has a critical role in controlling infection.

Effectors CD8+ T cells generated during infection with bacterial or viral pathogens undergo an activation phase that allows their entry into a variety of peripheral tissues, including the intestines. During this phase, surface expression of the integrin α4β7 and the chemokine receptor CCR9 facilitates the entry of CD8+ T cells into the intestinal tissue. Once they enter the tissue, CD8+ T cells acquire a tissue-resident memory T cell (T_{RM} cell) phenotype characterized by expression of the activation marker CD69 and the integrin CD103, as well as enhanced effector function, including constitutive expression of granzyme B.

Intestinal CD8+ T_{RM} cells are not able to reenter the circulation, and their long-term maintenance within the tissue provides immediate local protection against subsequent infections. T_{RM} cells have a transcriptional profile different from that of other memory T cell subsets, and data suggest that a core transcriptional program defines T_{RM} cells from disparate tissues; however, tissue- and pathogen-specific features might also regulate the phenotype and function of T_{RM} cells.

The signals within intestinal tissue that direct the development of T_{RM} cells, particularly in the context of local infection, are poorly understood. CD8+ T cells traffic to the intestine and remain there after infections with limited intestinal involvement. However, there is evidence that intestinal CD8+ T_{RM} cells that develop in the absence of local infection have lower expression of CD69 and CD103 than do those generated during local tissue colonization, which suggests that signals received during local infection lead to distinctive or more effective populations of T_{RM} cells. Development of the intestinal T_{RM} population is influenced by the local cytokine environment; transforming growth factor-β (TGF-β) is ubiquitously expressed in the intestine and is critical for the formation of T_{RM} cells in response to both local and systemic infection. Inflammatory signals induced during intestinal infection may also influence T_{RM} cell development, and culture of effector CD8+ cells ex vivo suggests that several inflammatory cytokines potentially influence markers of tissue residence and functionality. The presence of antigen in the intestinal tissue is not required for the development of CD8+ T_{RM} cells, but it is not known whether antigen-dependent signals affect the phenotype of T_{RM} cell populations in the context of tissue-specific infection.

Yersinia pseudotuberculosis (Yptb) is a Gram-negative bacterial pathogen that causes disease characterized by gastroenteritis and mesenteric lymphadenitis, and it can spread to the liver and spleen and cause fatal disease. CD8+ T cells responding to Yptb infection are a critical component of protection in subsequent challenges. Yptb is able to colonize the intestinal tissue and stimulate a robust antigen-specific CD8+ T cell response; however, the CD8+ T cell response to this oral pathogen in intestinal tissue has not been characterized. Yptb and other intestinal pathogens can spread to the liver and spleen directly from the intestine and do not rely on the creation of a bacterial pool in Peyer’s patches and mesenteric lymph nodes to access the bloodstream; therefore, an antimicrobial CD8+ T cell response directed to local areas of infection in the intestinal wall may be critical for controlling bacterial dissemination and clearance.

Here we used Yptb infection via the natural route as a model with which to study the CD8+ T cell response to a pathogen that causes robust intestinal disease. Our data indicated that Yptb induced a strong intestinal CD8+ T cell response and resulted in the development of a heterogeneous population of intestinal CD8+ T_{RM} cells. A portion of antigen-specific CD8+ T cells was dependent on signaling via the TGF-β receptor (TGF-βR), expressed CD103 and were evenly distributed in the intestine, whereas another portion of CD8+ T cells did not require these signals and were scattered in the tissue.
distributed throughout the intestinal epithelium and lamina propria; the remainder were CD103+ and initially clustered around areas of Yptb infection in the lamina propria, where they controlled bacteri­al replication. The CD103−CD8+ T cells in the lamina propria were developmentally distinct from their CD103+ counterparts, as they did not require TGF-βR signaling but instead required access to areas of bacterial infection and inflammation for their differentiation. Our findings indicate that the localization of CD8+ T RM cells to distinct microenvironments within the infected intestine regulates both their phenotype and their function.

RESULTS

Yptb generates a robust intestinal CD8+ T cell response
Infection of B6 mice with Yersinia species results in a strong CD8+ T cell response to a peptide from YopE, an effector protein that translocates into the cytosol of infected host cells. To develop a more tractable system that would allow us to monitor antigen-specific CD8+ T cell responses during intestinal infection, we generated a strain of Yptb expressing the model antigen ovalbumin (OVA). Fusion of a fragment of OVA to YopE has been shown to prime OT-1 T cells, which have transgenic expression of a major histocompatibility complex (MHC) class I–restricted T cell receptor specific for the OVA peptide SIINFEKL18. Published studies have used the integration of multiple MHC I–restricted T cell receptor specific for the OVA peptide SIINFEKL18. Published studies have used the integration of multiple genes into the Yersinia virulence plasmid, but we found that a strain constructed in this manner was attenuated during oral infection (data not shown). Therefore, we generated a YopE-OVA fusion construct and replaced the coding sequence for the translocated effector YopO on the plB1 virulence plasmid of Yptb (Yptb-OVA) (Supplementary Fig. 1a,b). Notably, YopO-deficient Yersinia strains do not show decreased virulence in vivo19, and use of the endogenous promoter of the gene encoding YopO allows the production of physiological amounts of the fusion protein, thereby preventing competition with other translocated virulence proteins.

We transferred 1 × 104 naive OT-I T cells that expressed green fluorescent protein under the control of the ubiquitin promoter (GFP+) into mice and infected the mice orally with Yptb-OVA. This resulted in expansion of the population of OT-1 T cells in the spleen, whereas infection with a Ypt strain that does not produce YopE did not prime OT-I T cells (Supplementary Fig. 1c). Both Yptb-OVA and control Yptb strains were able to prime a very strong endogenous CD8+ T cell response to an epitope consisting of amino acids 69–77 of YopE (YopE(69–77)), as shown by tetramer staining14,15 (Supplementary Fig. 1c). YopE-OVA production was sufficient to allow OVA to act as a protective antigen. Mice that had been previously immunized with vesicular stomatitis virus expressing OVA were protected more from challenge with Yptb-OVA than were immune mice challenged with control Yptb not expressing OVA (Supplementary Fig. 1d).

To more thoroughly examine the population expansion and persistence of CD8+ T cells, we transferred naive GFP+ OT-I cells into mice and infected the mice 1 d later with Yptb-OVA. We then determined the percentage of OT-I cells and YopE tetramer–positive cells among total CD8αβ+ T cells in the intestinal epithelium (IEL), lamina propria, mesenteric lymph nodes (MLNs) and spleen (Fig. 1a) at various time points after infection. The population expansion of OT-I T cells mirrored that of CD8+ T cells specific for the endogenous YopE epitope. There were about fourfold to fivefold more YopE tetramer-positive CD8+ T cells than OT-I cells both during acute infection (day 9) and after bacterial clearance at day 45 (Fig. 1a and Supplementary Fig. 2). Such considerable population expansion of YopE-specific cells was probably due to the greater production of YopE protein than of YopO protein20. The number of OT-I cells peaked in the MLNs 6 d after infection and at day 9 in all other organs (Fig. 1b). The OT-I T cell population contracted, and a stable memory population formed in the lamina propria and lymphoid organs (Fig. 1b) that was able to respond to rechallenge (data not shown). We saw a loss of OT-I T cells in the IEL compartment over time (Fig. 1b and data not shown). This is unlike what is seen after systemic viral infection, where IEL T cell numbers remain stable23; however, endogenous YopE-specific cells were also lost from the IEL (data not shown), which suggested that this is a phenomenon associated with Yptb infection and not an artifact observed only in OT-I T cells. Overall, these data indicated that the combination of OT-I T cell transfer and Yptb-OVA infection represented a useful model for examining the intestinal CD8+ T cell response to a pathogen that causes robust infection of the gut.

Development of population of CD103− T RM cells in the LP
Yptb-specific CD8+ T cell responses were most abundant in intestinal tissue, specifically the lamina propria, a site of Yersinia colonization. The phenotype and function of lamina propria CD8+ T cells have not been examined extensively, especially in the context of infection by oral pathogens. CD8+ T cells that enter the intestine undergo phenotypic changes characteristic of TRM cells, including upregulation of the expression of CD103, CD69 and granzyme B.25 As expected, OT-I T cells primed during Yptb-OVA infection upregulated their expression of CD103 soon after entry into the IEL compartment, and the percentage of CD103− cells remained stable after day 9 (Fig. 2a). In contrast, the lamina propria contained a more heterogeneous population of OT-1 cells; only about 50% of these cells became CD103+ (Fig. 2a). Both CD103+ and CD103− cells in the lamina propria expressed other markers of tissue residence, including CD69 and granzyme B (Fig. 2b and data not shown), which indicated that CD103− intestinal OT-I cells had undergone some of the phenotypic changes associated

Figure 1 Oral Yptb-OVA infection generates a robust intestinal CD8+ T cell response. (a) Flow cytometry of CD8+ T cells in the IEL, lamina propria (LP), MLNs and spleen (SP) at 9 and 45 d after infection of mice with Yptb-OVA. Numbers adjacent to outlined areas indicate percent OT-I T cells (right) or YopE(69–77) tetramer–positive cells (left) of total CD8αβ+ T cells. (b) Quantification of the total cells in the tissues in a at various time points after infection. Data are representative of three independent experiments (a) or are pooled from two experiments with at least three mice per time point (b; mean and s.d.).
with T<sub>RM</sub> cells. Expression of the antiapoptotic factor Bcl-2 was also upregulated in both CD103<sup>+</sup>CD8<sup>+</sup> and CD103<sup>-</sup>CD8<sup>+</sup> T cells in lamina propria (Fig. 2c), in support of the proposal of their long-term persistence<sup>8</sup>. Although CD103 has a role in the development and/or persistence of T<sub>RM</sub> cells in the brain<sup>21</sup>, skin<sup>8</sup> and IEL compartment<sup>7,10</sup>, it is not required for the maintenance of CD8<sup>+</sup> T cells in the lamina propria<sup>8,11</sup>. Downregulation of expression of the transcription factor KLF2 is observed in T<sub>RM</sub> cells and is thought to promote the establishment of this population via repression of Sip1, which encodes SIP1, the receptor for sphingosine 1-phosphate<sup>12</sup>. We assessed expression of Klf2 mRNA in sorted intestinal OT-I cells by quantitative RT-PCR. CD69<sup>+</sup>CD103<sup>-</sup>CD8<sup>+</sup> T cells in lamina propria failed to downregulate Klf2 to the extent observed for CD69<sup>+</sup>CD103<sup>+</sup>CD8<sup>+</sup> T cells (Fig. 2d). In fact, the expression of Klf2 mRNA in lamina propria CD103<sup>-</sup>CD8<sup>+</sup> T cells was not much different from that in splenic CD8<sup>+</sup> T cells (Fig. 2d). In support of an increase in functional KLF2, the expression of Klf2 mRNA was also higher in the CD69<sup>+</sup>CD103<sup>-</sup> lamina propria T cell population than in CD69<sup>+</sup>CD103<sup>+</sup> lamina propria and IEL OT-I cells (Supplementary Fig. 3). This raised the possibility that CD103<sup>-</sup> T cells might represent a population that is newly recruited from the circulation<sup>8,12</sup>. In line with that, a CD103<sup>-</sup> population of CD4<sup>+</sup> T cells that can transiently reside in the skin has been reported<sup>8</sup>. We hypothesized that if CD103<sup>-</sup> cells were transients in the lamina propria, they would be sensitive to treatment with a CD8<sup>-</sup>depleting antibody. To test this, we treated mice with antibody to CD8 (anti-CD8) 28 d after challenge with Yptb-OVA and analyzed intestinal OT-I populations 7 d after treatment. We noted no difference in the ratio of CD103<sup>-</sup> lamina propria populations to CD103<sup>-</sup> lamina propria populations between treated and untreated mice (Fig. 2e), which indicated that the lamina propria CD103<sup>-</sup>CD8<sup>+</sup> population represented true resident cells. In confirmation of the effectiveness of antibody depletion, there were 500-fold fewer CD8<sup>+</sup> T cells in the spleens of treated mice than in those of untreated mice (data not shown). These data indicated that infection resulted in a long-lived subset of T<sub>RM</sub> cells that failed to express CD103 and had increased amounts of Klf2 mRNA but upregulated the expression of other molecules associated with tissue residence, including CD69, granzyme B and Bcl-2.

Figure 2 CD103<sup>-</sup>CD8<sup>+</sup> populations develop in the lamina propria and have features of resident memory cells. (a) CD103 expression on OT-I T cells in the IEL and lamina propria at various times (horizontal axis) after infection of mice with Yptb-OVA. (b,c) Expression of CD103 and CD69 (b) or Bcl-2 (c) by OT-I T cells at 14 d (b) and 28 d (c) after infection of mice with Yptb-OVA. Numbers in quadrants indicate percent CD69<sup>+</sup>CD103<sup>+</sup> cells (top left) or CD69<sup>+</sup>CD103<sup>-</sup> cells (top right) (b) or percent Bcl-2<sup>+</sup>CD103<sup>+</sup> cells (top left) or Bcl-2<sup>-</sup>CD103<sup>-</sup> cells (top right) (c). (d) Quantitative RT-PCR analysis of Klf2 expression by CD8<sup>+</sup> populations sorted from mice >28 d after infection with Yptb-OVA; results are presented relative to expression of the control gene Actb. NS, not significant; *P < 0.01 and **P < 0.001 compared with splenic CD8<sup>+</sup> cells (paired t-test). (e) CD103 expression by lamina propria OT-I T cells from untreated mice (Ctrl) or mice treated intraperitoneally with 150 μg anti-CD8<sup>+</sup>depleting antibody (α-CD8) 28 d after infection with Yptb-OVA, assessed 7 d after antibody treatment. Data are pooled from at least two independent experiments (a,e), mean and s.d. of three or more mice; d, mean and s.d. of two independent biological replicates) or are representative of at least two experiments with three or more mice per group (b,c).

Figure 3 TGF-β signaling is not required for development of the lamina propria CD103<sup>-</sup>CD8<sup>+</sup> T<sub>RM</sub> cell population. (a) Ratio of wild-type OT-I cells to Tgfbr2-deficient OT-I cells (WT/KO) in various tissues of C57BL/6 host mice given wild-type (CD45.1<sup>+</sup>) OT-I cells and Tgfbr2-deficient (CD45.1<sup>+</sup>CD45.2<sup>-</sup>) OT-I cells (5 × 10<sup>6</sup> each) and infected with Yptb-OVA 1 d later, followed by analysis of OT-I cells 9–28 d after infection. (b) Expression of CD103 and CD69 on total OT-I cells from the lamina propria 28 d after infection. Numbers in quadrants indicate percent CD69<sup>+</sup>CD103<sup>-</sup> cells (top left) or CD69<sup>+</sup>CD103<sup>+</sup> cells (top right) (Far left plot) or percent CD45.1<sup>+</sup>CD45.2<sup>-</sup> (wild-type) cells or CD45.1<sup>−</sup>CD45.2<sup>−</sup> (Tgfbr2-deficient) cells (middle and right plots). (c) Ratio of OT-I cells in the CD103<sup>+</sup>CD69<sup>+</sup> and CD103<sup>-</sup>CD69<sup>-</sup> lamina propria populations of mice. (d) Quantitative RT-PCR analysis of Klf2 expression in OT-I populations sorted from the lamina propria more than 21 d after infection as in b; results were normalized to Actb expression and are presented relative to those of wild-type OT-I splenocytes, set as 1. *P < 0.001 (unpaired (a,c) or paired (d) t-test). Each symbol (a,c) represents an individual mouse; small horizontal lines indicate the mean (± s.d.). Data are pooled from two independent experiments with three or four mice per group.
Figure 4  Yptb-OVA infection stimulates the formation of CD103−CD8+ T cell clusters in the lamina propria. (a) Immunohistochemistry analyzing the distribution of OT-I T cells (green) in the ileum of C57BL/6 host mice given 1 × 10^4 GFP+ OT-I T cells, then orally infected with Yptb-OVA the next day, followed by isolation of the terminal ileum 9 d after infection. Arrows indicate lamina propria clusters near the crypts; open arrowheads indicate those in the upper part of the villi; epithelial cells are stained with anti-EpCam (red). Scale bar, 100 µm. (b) Tissue sections from mice as in a, stained with anti-CD103 (red) to assess CD103 expression on villous (left) and clustered (right) OT-I T cells (green); open arrowhead indicates CD103+ OT-I T cells; nuclei were stained with the DNA-binding dye DAPI (blue). Scale bars, 25 µm. (c) Positioning of clusters of OT-I cells (green) relative to areas of Yptb-OVA infection (labeled with anti-Yptb (red) in a section as in a; nuclei, blue. Scale bars, 25 µm. Data are representative of two experiments with five mice (a) or three mice (b, c).

OT-I cells were completely absent from the CD103+CD69+ population (Fig. 3b). However, a sizable population of Tgfbr2-deficient OT-I T cells developed into CD103−CD69+ T RM cells (Fig. 3b), and this population was more stable than the lamina propria OT-I population overall (Fig. 3c). We also assessed the expression of Klf2 mRNA in sorted wild-type and Tgfbr2-deficient lamina propria populations. CD103+ wild-type OT-I cells had less Klf2 mRNA than did CD103− wild-type OT-I cells. Klf2 expression in Tgfbr2-deficient OT-I cells was identical to that observed in CD103+ wild-type OT-I cells (Fig. 3d). These data indicated that TGF-β signaling was not required for the development of CD103− T RM cells in the lamina propria, in contrast to other intestinal populations, which required TGF-β to form long-lived T RM cells (Fig. 3a).

Differences in the localization of intestinal T RM cells

Little is known about the distribution of antigen-specific CD8+ T cells after colonization with an intestinal pathogen, and we considered that the phenotypic differences we observed might have been due to different microenvironments created by Yptb infection within the intestine. We investigated the localization of GFP+ OT-I T cells in the intestine of Yptb-OVA–infected mice 9 d after infection. The OT-I cells in the ileum of the small intestine could be separated into two groups on the basis of their distribution: those that were present singly and evenly distributed throughout the tissue, and those that formed clusters (Fig. 4a). These CD8+ T cell clusters were present mainly under the crypts; however, they were occasionally present in the villus lamina propria (Fig. 4a). We then assessed the surface expression of CD103 on OT-I T cells within the intestine and found that whereas the majority of the uniformly distributed OT-I cells in the IEL and lamina propria expressed CD103 (Fig. 4b), OT-I T cells in clusters near the crypts were mostly CD103− (Fig. 4b and Supplementary Fig. 4a). Tissue sections were also stained with Yptb-specific antibodies (Fig. 4c), which indicated that OT-I T cells clustered around areas of bacterial infection. Similar CD8+ T cell clusters were also present in the cecum and colon but not the duodenum (Supplementary Fig. 4b–d). This is consistent with CD8+ T cell clusters’ indicating areas of lamina propria infection, as Yptb infected the distal part of the small intestine, cecum and colon but not the proximal small intestine (Supplementary Fig. 4e). These data suggested that recruitment to these intestinal aggregates during infection affected the phenotype and function of lamina propria CD8+ T cells, with clustered cells expressing the resident memory markers CD69 and granzyme B but not CD103.

Clusters contain CD4+ T cells and CX3CR1+ cells

The intestinal distribution of CD103+ and CD103− OT-I T cells during Yptb-OVA infection was distinct, and we sought to define the local immunological environment in clusters that contained CD103+ CD8+ T cells. Isolated lymphoid follicles (ILFs) in the gut are generated soon after birth in response to microbial colonization, and mature ILFs contain B cells, CD11c+ dendritic cells (DCs) and a small number of lymphoid tissue–inducer cells23. The M cells that overlie these structures are a major point for the entry of Yptb into the intestinal tissue24,25, and pathogens such as Salmonella have been shown to preferentially invade ILFs26. Therefore, we hypothesized that ILFs might support the invasion and/or replication of Yptb and CD8+ T cell clustering. To investigate this, we stained intestinal tissue sections from Yptb-OVA–infected mice with anti-B220 to identify B cells...
and assessed colocalization with OT-I cells. We occasionally noted a small number of OT-I T cells associated with clustered B220+ cells characteristic of ILFs (Fig. 5a), but OT-I T cell clusters contained few, if any, B220+ cells (Supplementary Fig. 5). These data indicated that OT-I T cell clusters were not formed in or around ILFs during Yptb-OVA infection, although OT-I T cells were not excluded from these structures.

OT-I T cell clusters in the lamina propria always contained endogenous CD8αβ+ T cells (Fig. 5b), consistent with the presence of large numbers of YopE-specific cells in the intestine. In addition, CD8+ T cell clusters always contained CD4+ T cells (Fig. 5b). We suggest that these CD4+ T cells are also Yptb-specific, as CD4+ T cell responses are generated during Yersinia infection and are associated with protection.27–29.

The presence of antigen-specific T cells and bacteria within the clusters suggested that they might also contain inflammatory antigen-presenting cells. The intestinal lamina propria contains a diverse population of macrophages and DCs, and we sought to define these populations in naive and Yptb-OVA–infected mice by flow cytometry. We used Cx3cr1gfp/+ mice (which have a sequence encoding GFP knocked into the Cx3cr1 locus; called ‘CX3CR1-GFP mice’ here) to aid in the identification of macrophage and DC populations.30 We gated on MHC class II+–positive cells and used expression of CD11c, CD11b, CD103 and CX3CR1-GFP to sort lamina propria macrophages and DCs into four populations (Fig. 5c). Small populations of conventional DCs (population 1: CD11c+CD11b+CD103−) and migratory DCs (population 2: CD11b+CD11cpos−loCD103+CX3CR1−) were present in naive mice, and these two populations remained stable after infection (Fig. 5d). In contrast, the CX3CR1+ expressing subset of antigen-presenting cells made up nearly 30% of MHC class II+–positive cells in naive mice, and the size of this population increased during Yptb-OVA infection. These CX3CR1+ cells could be further subsorted into those that had been recently recruited to the intestine (population 3: CD11b+CD11cpos−loCD103+CX3CR1int) and resident macrophages (population 4: CD11b+CD11cpos−hiCD103−CX3CR1hi) on the basis of the degree of CX3CR1-GFP expression (Fig. 5c). We noted that the recently recruited CX3CR1int macrophage and DC populations increased by approximately fourfold during infection, whereas the resident CX3CR1hi population was unchanged (Fig. 5d). The CX3CR1int population has been shown to be recruited to the colon during colitis and is derived from inflammatory monocytes.30 Consistent with that, during infection with Yptb-OVA, CX3CR1int cells retained monocytic markers, including CCR2 and Ly6C (Supplementary Fig. 6).

We hypothesized that these populations of CX3CR1+ macrophages and DCs would be recruited to areas of bacterial colonization with OT-I cells, and close interactions between clustered OT-I cells and CX3CR1+CD11cpos−lo cells were apparent (Fig. 5e). In contrast, CX3CR1+CD11chi cells were rarely associated with OT-I cells (Fig. 5e). These data suggested that the T cell–containing clusters represented structures formed in the intestine during local infection that contained antigen-specific CD8+ T cells, and T cells and resident and recruited CX3CR1+ antigen-presenting cells and that these sites of infection might constitute a unique microenvironment that affects the phenotype and function of lamina propria CD8+ T RM cells. OT-I cells and endogenous CD8+ cells remained clustered at 30 d after infection, when Yptb-OVA was no longer detectable in the intestinal tissue (Supplementary Fig. 7a–c). The clusters had dispersed by 120 d after infection (Supplementary Fig. 7b,d); however, the percentage of CD103− cells in the lamina propria remained stable (Fig. 2a), which suggested that any effect localization had on CD8+ T cells was ‘imprinted’ relatively early after infection. These results suggested that clusters of antigen-presenting cells, CD4+ T cells and CD8+ T cells formed at sites of Yptb infection.

CD103+ T_RM cells develop independently of local antigen

The role of antigens in the development of different tissue-resident memory populations is somewhat unclear. Continual antigenic stimulation by self peptides has been shown to prevent CD103 expression on intestinal CD8+ T cells.10 In the skin, CD103 is expressed regardless of whether antigen is present,31 whereas in the brain, antigen recognition is required for upregulation of CD103 expression.21. The localization of CD103+ lamina propria T cells to areas containing bacterial colonization and recruited inflammatory monocytes suggests that this subset of lamina propria T_RM cells might be exposed to unique signals during their development that lead to this altered phenotype. CX3CR1int and, to a lesser extent, CX3CR1hi intestinal macrophages and DCs are able to present antigens and produce inflammatory cytokines in the normally immunosuppressive lamina propria environment.30,32 We hypothesized that the presentation of Yptb-OVA antigens within CD8+ T cell aggregates might affect CD103 expression on clustered wild-type cells. To assess the presentation of Yptb-OVA antigens by
CX3CR1-expressing populations, we sorted CX3CR1<sub>int</sub> and CX3CR1<sub>hi</sub> populations from the lamina propria of Yptb-OVA–infected mice on day 6 after infection. We mixed those cells with CD4<sup>+</sup>CD8<sup>+</sup> T cells from Yptb-OVA–immune mice (including both YopE-specific CD8<sup>+</sup> T cells and OVA-specific CD8<sup>+</sup> T cells) labeled with the cytosolic dye CFSE and assessed proliferation after 3 d. We found that the CX3CR1<sub>int</sub> population from infected mice stimulated more proliferation of Yptb-OVA CD8<sup>+</sup> memory T cells than did the CX3CR1<sub>hi</sub> population from naive mice (Fig. 6a,b). The CX3CR1<sub>hi</sub> population from infected mice stimulated some proliferation of Yptb-OVA CD8<sup>+</sup> memory T cells, but the frequency of division was not significantly greater than that seen in the same population from uninfected mice (Fig. 6b). Together these findings indicated that CD8<sup>+</sup> T cells in clusters during Versinia infection were exposed to their cognate antigen.

To more definitively determine the role of intestinal antigen presentation in regulating CD103 expression on lamina propria CD8<sup>+</sup> T cells, we transferred OT-1 T cells into mice and then infected the mice with Yptb-OVA. Six days after infection, we transferred CD8<sup>+</sup> T cells from the spleen and MLNs into congenically marked recipient mice infected with Yptb that did not express ovalbumin. We assessed the frequency of OT-I cells from each group in the intestine and systemic tissues 9 d after infection. The proportion of donor cells that did not receive antigen stimulation in the second host (OT-1 cells) has ratios of CD103<sup>+</sup> cells to CD103<sup>−</sup> cells similar to those of Yptb-specific donor cell populations (Fig. 6c,d). These data suggested that inflammatory signals in CD8<sup>+</sup> T cell clusters, not antigen presentation, caused development of CD103<sup>−</sup> T<sub>Rm</sub> cells in the lamina propria.

**Formation of CD8<sup>+</sup> T cell clusters requires CXCR3**

CD8<sup>+</sup> T cell clusters within the intestine may be exposed to a specific array of inflammatory cytokines produced by CX3CR1<sub>int</sub> cells at sites of local infection, and this may redirect their phenotype to CD103<sup>−</sup> T<sub>Rm</sub>. CXCR3 has been identified as important for the localization of CD8<sup>+</sup> T cells to areas of infection in nonlymphoid tissue, including the brain, lung and female reproductive tract<sup>33–36</sup>, and we hypothesized that CXCR3 might be involved in targeting CD8<sup>+</sup> T cells to areas of intestinal inflammation during Yptb-OVA infection. We used RT-PCR to assess the expression of mRNA encoding the CXCR3 ligands CCL9 and CCL10 (ref. 37) in intestinal macrophage and DC populations sorted as described above (Fig. 7c). We found that expression of Ccl9 mRNA was uniform in all populations and was not upregulated during Yptb-OVA infection (Fig. 7a); however, Ccl10 mRNA was expressed most in CX3CR1-expressing populations in naive mice, and during infection, expression of Ccl10 mRNA was significantly increased in both resident CX3CR1<sub>hi</sub> populations and recruited CX3CR1<sub>int</sub> populations (Fig. 7a). Production of the CXCR3 ligand CCL10 suggested that CXCR3 might be important for localization to areas of inflammation within the intestine.

To investigate the role of CXCR3 in intestinal entry and localization, we transferred equal numbers of naive CXCR3-sufficient (wild-type) OT-I T cells and CXCR3-deficient (Cxcr3-deficient) OT-I T cells into mice and then infected the mice with Yptb-OVA. We then assessed the frequency of OT-I cells from each group in the intestine and systemic tissues 9 d after infection. Wild-type and Cxcr3-deficient OT-I T cells were present in similar numbers in the MLNs and spleens of infected mice (Fig. 7b). In the gut, we found no difference in the frequency of wild-type and Cxcr3-deficient cells in the IEL population, but in the lamina propria there were twofold fewer Cxcr3-deficient OT-I T cells than wild-type OT-I T cells (Fig. 7b). We assessed the distribution of OT-I cells by histology and found that CD8<sup>+</sup> T cell clusters in the ileum contained wild-type cells but few, if any, Cxcr3-deficient T cells (Fig. 7c). In areas of the intestine without clusters of OT-I T cells, both populations were present in a similar number and distribution (Fig. 7c). We quantified T cell clustering by determining the number of OT-I T cells in a single villus, including all underlying tissue (Fig. 7d), and presented these data as the frequency of villi containing a given number of OT-I T cells (Fig. 7e). We found that 13.7% of villi contained more than ten wild-type OT-I cells; in contrast, we did not observe any villi with more than ten Cxcr3-deficient OT-I cells (Fig. 7e). These data indicated that CXCR3 was required for the
migration of CD8+ T cells to foci of infection but not for migration to other areas of the intestine.

**Cxcr3-deficient T cells fail to control bacterial replication**

Our data indicated that Yptb-OVA infection created distinct micro-environments within the intestine. Wild-type OT-I cells present in CD8+ T cell clusters failed to upregulate CD103 expression (Fig. 4b), and we hypothesized that the exclusion of CXCR3-deficient cells from these structures might result in an altered phenotype compared with that of wild-type OT-I cells. To investigate this, we analyzed the expression of CD103 on intestinal wild-type and Cxcr3-deficient OT-I cells after Yptb-OVA infection. Yptb-OVA–primed wild-type cells in the IEL compartment showed upregulation of CD103, and an even greater percentage of Cxcr3-deficient IEL cells were CD103+ compared to wild-type IELs (Fig. 8a,b). This phenotype was even more pronounced in the lamina propria, with a significantly greater percentage of Cxcr3-deficient OT-I cells expressing CD103 compared to wild-type lamina propria cells (Fig. 8a,b). The CXCR3-deficient CD8+ T cell population included a greater number of cells with a memory precursor phenotype (KLRG1 −CD127+) than did the wild-type population, due to altered exposure to inflammatory cytokines during priming. It has been suggested that CD8+ T cells with a memory precursor phenotype ‘preferentially’ develop into CD103+ T cells. However, in vitro activation of wild-type and Cxcr3-deficient OT-I T cells allows similar access to inflammatory cytokines, and when we transferred in vitro–activated OT-I cells into Yptb-OVA–infected mice, we observed higher CD103 expression on intestinal Cxcr3-deficient OT-I cells than on wild-type OT-I cells (Supplementary Fig. 8b). It was conceivable that exposure of recruited CD8+ T cells to CXCL10 in the tissue might directly modulate their expression of CD103. To investigate this, we stimulated wild-type and Cxcr3-deficient effector OT-I cells with TGF-β ex vivo, which resulted in upregulation of CD103 expression in both wild-type OT-I cells and Cxcr3-deficient OT-I cells (Supplementary Fig. 8c). The addition of CXCL10 to the culture did not prevent the upregulation of CD103 expression in response to TGF-β (Supplementary Fig. 8c), which indicated that CXCR3 signaling did not directly regulate CD103 expression. Together these data suggested that during intestinal infection, localization to these lymphoid clusters altered the expression of a subset of T RM cell markers, including CD103, and indicated a critical role for CXCR3-mediated trafficking in establishing this population.

Cxcr3-deficient OT-I cells in the lamina propria failed to localize to lymphoid clusters and developed almost exclusively into the CD103−CD69+ subset; we used this characteristic to analyze the contribution of this population to the control of bacterial replication in the lamina propria. We depleted CD90.1+ mice of T cells and infected them with Yptb-OVA. Two days later, mice received CD4+ T cells from Yptb-infected CD90.2+ wild-type mice and CD8+ T cells from either CD90.2+ wild-type or Cxcr3-deficient mice. Three days after T cell transfer, we quantified Yptb-OVA in the intestine and spleen. We found a significant decrease in the bacterial burden in the ileum of mice receiving wild-type T cells compared to mice receiving no T cells, but no difference was observed when we compared mice receiving Cxcr3-deficient T cells to those receiving no T cells (Fig. 8c). Wild-type and Cxcr3-deficient CD8+ T cell transfers did reduce the abundance of Yptb-OVA in the spleens (Fig. 8c), although this did not reach statistical significance. Together these data suggested that Cxcr3 regulated the differentiation of CD103+CD69+CD8+ T cells in lamina propria by altering their localization within the intestine, and that this T RM cell subset was superior at limiting bacterial replication in the gut.

**DISCUSSION**

CD103+ T RM cells have been reported in a variety of tissues; however, the abundance of this population varies by location, nature of infection and availability of antigen. It is often speculated that these cells are in a transitional phase and will eventually become CD103+ cells or that they are circulating cells that are present only transiently in the tissue. Here we have identified a stable population of lamina propria CD103+ T RM cells that developed after Yptb infection; these cells were distinct from CD103+ CD8+ intestinal T cells in both their phenotype and their requirements for inflammatory signals within the intestine. CD103+ T RM cells did not require TGF-β signaling, unlike CD103+ cells in both IEL and lamina propria compartments. CD103−CD8+ T cells in lamina propria clustered in areas of infection with other resident and recruited cells of the immune system, including inflammatory monocyte-derived antigen-presenting cells and antigen-specific T cells. CD103−CD8+ T cells localized to areas that are abundant in antigen, which can affect CD103 expression on CD8+ T cells. However, in the lamina propria, antigen did not alter the development of CD103+ T RM cells, which suggested that inflammatory signals are involved in this process. Cxcr3-deficient CD8+ T cells were not recruited to areas of infection and, accordingly, were skewed to the CD103+ phenotype, and they were unable to control bacterial replication. These data identify intestinal CD103+CD8+ lamina propria T cells as developmentally distinct from their CD103+ counterparts and indicate that T RM cells are heterogeneous and have distinct functions during infection.

The formation of intestinal CD8+ T RM cells is negatively affected by the loss of TGF-β signaling; however, cells in the lamina propria are not as sensitive to the loss of TGF-β-dependent signals as those in the IEL. The lamina propria population that persists in the absence of TGF-β signaling is analogous to the CD103−CD8+ immunoreactive population.
population that develops in a wild-type setting. It is possible that these cells fail to receive TGF-β signals; however, Yptb effector CD8+ T cells all responded to TGF-β stimulation ex vivo, the expression of Tgfβ was increased in CX3CR1+ cells during infection, and both CD103+ and CD103− T cell populations in the lamina propria had equivalent expression of Tgfb2 (data not shown). TGF-β is maintained in a latent form and requires activation by integrins or proteases40, and local production of these activating factors could be altered during infection and thereby regulate the availability of active TGF-β.

An alternative possibility is that CD8+ T cells that enter the intestine are responsive to and exposed to active TGF-β, but other signals change the differentiation pattern of these cells. Intestinal antigen has been suggested to alter CD103 expression in vivo10; however, antigen did not downmodulate CD103 expression in the context of Yptb infection. There is evidence that exposure of CD8+ effectors to TGF-β in the presence of inflammatory cytokines such as interleukin 33 (IL-33) or type I interferon prevents the upregulation of CD103 expression10. However, this would not explain the differences in the expression of Klf2, as both IL-33 and type I interferon act in synergy with TGF-β to stimulate Klf2 downregulation13. Further experiments are needed to determine whether signaling via the TGF-β receptor is activated in CD103+ populations and what specific inflammatory signals drive the development of these cells. The CX3CR1int DC population that is a major component of intestinal aggregates has a characteristic cytokine profile that is more inflammatory than that of resident intestinal macrophage populations, and during colitis this subset is able to produce IL-6 and IL-23 (ref. 30). In addition, both CX3CR1int and CX3CR1high populations have increased IL-1 expression30, and these cytokines have all been linked to the host response to Yersinia infection41–44. Determining the inflammatory cytokine profile of CX3CR1+ populations within CD8+ T cell clusters would aid in identifying which cytokines intestinal T cell populations are exposed to during infection.

CD8+ TRM cells in the female reproductive tract and skin act as an alarm upon re-exposure to antigen, rapidly recruiting circulating memory CD8+ T cells and other cells of the immune system and inducing an antimicrobial state within the tissue36,45,46. The evenly distributed CD103+CD8+ T cells we noted in the intestine were ideally positioned to carry out this alarm function, as they were present throughout the tissue and developed independently of the nature of the pathogenic insult. In contrast, CD103−CD8+ T cells develop around sites of infection within the tissue and have an immediate effect during primary infection, but their role in combating reinfection is unclear. We propose that instead of affecting tissue residence, CD103 regulates migration within the intestinal tissue, and its absence allows CD8+ T cell clusters to move in identifying which cytokines intestinal T cell populations are exposed to during infection. CD8+ TRM cells in the female reproductive tract and skin act as an alarm upon re-exposure to antigen, rapidly recruiting circulating memory CD8+ T cells and other cells of the immune system and inducing an antimicrobial state within the tissue36,45,46. The evenly distributed CD103+CD8+ T cells we noted in the intestine were ideally positioned to carry out this alarm function, as they were present throughout the tissue and developed independently of the nature of the pathogenic insult. In contrast, CD103−CD8+ T cells develop around sites of infection within the tissue and have an immediate effect during primary infection, but their role in combating reinfection is unclear. We propose that instead of affecting tissue residence, CD103 regulates migration within the intestinal tissue, and its absence allows CD8+ T cell clusters to move in identifying which cytokines intestinal T cell populations are exposed to during infection.

Bacterial pathogens can seed systemic organs directly from the intestine16,17, and the formation of these isolating structures could prevent reintroduction into the lumen of the intestine, as well as access to the blood or lymph vessels that are abundant in the lamina propria.

Overall these data suggest that Yptb infection provides a useful model for examining the recruitment of CD8+ T cells to the intestine and how distinct microenvironments within this tissue can ‘instruct’ the development of these cells into distinctive intestinal CD8+ TRM cell subsets. Studies comparing the profiles of CD8+ cells from different tissues suggest that only a small number of genes represent the core transcriptional changes that dictate the formation of TRM cell subsets. Distinguishing between CD103+CD8+ and CD103−CD8+ populations may be important when analyzing transcriptional signatures, as these two populations are exposed to different signals within the tissue9.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

T.B. and M.J.B. designed experiments and wrote the manuscript; T.B. performed experiments and analyzed data.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Bacteria. Yersinia pseudotuberculosis YPIII was modified using allelic exchange to generate Yptb-OVA. We generated a YopE–OVA fusion construct by amplifying a fragment of ovalbumin from nucleotides 742–1,068 followed by a stop codon (5'-TCTAGAAGTGAGCTGAGCTCTGGAG-3'), 5'-GGATCCCTAGACGCTTGCAGATCCTGACTC-3') and inserting it into the XbaI and BamHI sites of pYopE–Cys849, which contained the first 390 nucleotides of YopE. This YopE–OVA fusion construct was amplified with 5'-AGATCTGTGAAATATCATCTAT-3', 5'-AGATCTAAGGGCTTGCAGCA-3') and cloned into the BglII site of the pYopOαI–2199 suicide vector plasmid19. Allelic exchange was carried out as described30. Replacement of the gene encoding YopO with YopE–OVA was confirmed by PCR and sequencing.

Mice and infections. C57BL/6, Cxcr3−/− and Cx3cr1Tg825Sp mice were obtained from Jackson Laboratories and housed in specific pathogen-free conditions at the University of Washington animal facilities. OT-I T cell receptor–transgenic mice were examined 9 d after transfer. naive OT-I T cells were isolated from the spleen and lymph nodes using a CD90.1 (19E12) intraperitoneally at the time of infection and an additional CD8+ T cells were depleted by intraperitoneal injection of 150 μg anti-CD8α antibody (2.4.3) 1 week before analysis.

For effector CD8+ T cell transfer experiments, CD45.2 mice were given CD45.1/2 OT-I T cells and infected with Yptb-OVA. Six days after infection, effector CD8+ T cells were cultured at 1 × 106 cells per well of a 96-well plate in RPMI 1640 cells and were infected as described above, and mesenteric lymph nodes were isolated and cultured on the final day of incubation. CFSE dilution was examined by flow cytometry, and a live/dead stain was used to exclude dead cells from the analysis.

For protection experiments, CD90.1 recipient mice were given 200 μg anti-CD90.1 (19E12) intraperitoneally at the time of infection and an additional 200 μg every other day. Wild-type and Cx3cr-deficient T cells (day 7 Yptb-OVA effectors) were sorted from CD90.1 donor mice and transferred into recipient mice on day 2. Recipient mouse received 7.5 × 105 wild-type CD T cells and 7.5 × 103 wild-type or Cxcr3-deficient CD8+ T cells. Wild-type and Cxcr3-deficient donor populations had equivalent numbers of YopE-specific cells and expressed similar amounts of Yptb-OVA. Three days after T cell transfer, tissues were isolated and homogenized in PBS. Tissue homogenates were plated on cefsulodin-novobiocin-irgasan (CIN) agar (BD Biosciences), and we determined the total number of colony-forming units (CFUs) per organ.

For in vitro activation experiments, OT-I T cells were activated with splenocytes pulsed with 1 μM SIINFEKL in the presence of 100 ng/ml LPS. 20 U/ml IL-2 was added daily beginning on day 2. Four days after activation, cells were purified using Histopaque 1083 (Sigma), and 1 × 106 to 2 × 106 cells were injected i.v. into day 5 Yptb-OVA–infected mice. Cells were analyzed 4 d after transfer.

Isolation of intestinal cells. For isolation of intestine IEL and lamina propria cells, Peyer’s patches were removed, the intestine was cut open longitudinally, and intestinal contents and mucus were removed by gentle scraping. The intestine was cut into 1-cm pieces and incubated in HBSS buffer containing 1 mM dithiothreitol and 10% FBS at 37 °C with stirring for 20 min to isolate IELs. Intestinal tissue was transferred to HBSS containing 1 mM EDTA and stirred at 37 °C for 20 min to remove the epithelium. Intestinal pieces were then incubated in HBSS containing 5% FBS and 150 U/ml collagenase type 2 (Worthington) at 37 °C with stirring for 45 min to isolate lamina propria cells. IEL and lamina propria cells were further purified by gradient centrifugation with 44% and 67% Percoll.

Flow cytometry. Single cell suspensions from lymph nodes, spleen and intestines were fixed in the indicated time points after infection (see figures) and stained with antibodies for CD3 (H35-17.2), CD103 (2E7), CD90.1 (HI101), I-Aα (AF6-120.1), CD11c (N418), CD11b (M1/70), CD127 (A7R34), KLRG1 (2F1), CCR9 (CW-1.2), αβγδ (DATK32) and F4/80 (BM8) from eBioscience; antibodies for CD69 (H.12F3), Bcl2 (100), CD45.1 (A20), CD45.2 (104) and Ly6C (AL-21) from BD Biosciences; and YopE(69–77)–MHC class I tetramer (Fred Hutchinson Cancer Research Center Tetramer Core Facility). Cells were analyzed with a FACSComp (BD Biosciences) and analyzed with FlowJo software (TreeStar).

Immunohistochemistry and microscopy. Intestinal tissues were fixed in 4% paraformaldehyde, rehydrated in 20% sucrose and frozen in OCT media (Sakura). Tissues were cut into 7–8 μm sections and treated with ice-cold acetone. Sections were treated with biotin-avidin blocking reagent when necessary (Vector Labs) and stained with the following biotinylated or directly conjugated antibodies: CD8α (YTS156.7.7, Bioregen, CD4 (RM4-5, eBioscience), CD103 (M290, BD Biosciences), CD90.1 (H11.1, eBioscience), EpCam (G8.8, Bioregen), CD11c (HL3, BD Biosciences) and B220 (RA3-6B2, eBioscience). Rabbit anti-Yersinia pseudotuberculosis (ab26120, Abcam) and anti-rabbit Dylight 649 (ab96926, Abcam) were used to stain for bacterial antigens. Stained slides were mounted with Prolong Gold antifade reagent (Invitrogen), imaged using a Nikon 90i and analyzed using Adobe Photoshop software.

We determined the number of OT-I cells per villus by sectioning a ‘Swiss roll’ of the distal third of the small intestine. Five or more sections per mouse that were at least 400 μm apart were stained and imaged. A villus and the underlying submucosa and muscularis were considered a single villus, and the number of OT-I cells in each region was determined. The numbers of OT-I cells per villus were binned and plotted as the percentage of villi containing a given range of OT-I cells.

Quantitative RT-PCR. Cxcr3Tg9 mice were infected with 2 × 106 Yptb-OVA bacteria, and on day 7 after infection the lamina propria cells were isolated from the ileum. Cells were stained with antibodies for CD11c, CD11b, CD103 and 1-β. CD8+ T cells were isolated from tissues at various time points after infection and stained with antibodies for CD8α, CD69, CD103 and CD45.1/2. Cells were sorted using a FACSaria (BD Biosciences) to greater than 96% purity. IEL CD8+ populations were defined as CD69+CD103+ and lamina propria CD8+ cells were sorted into CD69+CD103+ and CD69−CD103+ populations. RNA was isolated using an RNeasy RNA isolation kit (Qiagen) and amplified using a SYBR one-step RT-PCR kit (Qiagen) with the described primers for Cxcl9/Cxcl10 (ref. 53) and Klf2/Ilipt1 (Actb expression was used as a normalization control (actbF, 5′-GGCTGATTCCCTCCATCG-3′; actbR, 5′-CCAGTTGGAACAATGGCAGTG-3′).

Ex vivo stimulation assays. Mice received wild-type and Cxcr3-deficient OT-I cells as described above, and mesenteric lymph nodes were isolated on day 5 after infection. Single cell suspensions of bulk lymph node cells were cultured at 1 × 106 cells per well of a 96-well plate in RPMI 1640 supplemented with 10% FBS, 5 mM L-glutamine, 55 μM β-mercaptoethanol, 1 mM sodium pyruvate, 10 mM HEPES, 1× MEM non-essential amino acids (Invitrogen), 100 μg/ml streptomycin, and 100 U/ml penicillin with or without 2.5 ng/ml TGF-β1 (R&D Systems) and 25 ng/ml CXCL10 (Peprotech) for 20 h. Cells were stained for surface markers in the presence of a fixable live/dead stain (Invitrogen) to exclude dead cells.

Ex vivo presentation assays. Cxcr3Tg9 mice were infected with 2 × 106 Yptb-OVA bacteria, and on day 6 after infection lamina propria cells were isolated from the ileum and cecum. Cells were stained with antibodies for CD11c, CD11b, I-Aβ and dump gate (CD103, IgA (DA3, LSBio), CD19 (1D3, eBioscience), TCRβ (H57-597, eBioscience), TCRβγ (GL5, eBioscience)) and sorted using a FACSAria (BD) to greater than 96% purity. Congenically marked CD8αCD44hi memory cells were sorted from Yptb-OVA memory mice and labeled with CFSE. 5 × 105 antigen-presenting cells and 5 × 103 memory T cells were mixed and incubated for 3 d with the addition of 5 U/ml IL-2 for the final day of incubation. CFSE dilution was examined by flow cytometry, and a live/dead stain was used to exclude dead cells from the analysis. Yptb-OVA memory cells were also incubated with antigen-presenting cells pulsed with SIINFEKL and YopE(69–77) peptides to determine the maximum
antigen-specific response, and 60%–70% of sorted Yptb-OVA memory cells divided in response to this peptide mixture.

**Statistical analysis.** Differences between experimental groups were analyzed using Student's t-test, and the \( \chi^2 \) test was used to analyze OT-I distribution within the tissue.

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