CCR2-dependent intraepithelial lymphocytes mediate inflammatory gut pathology during *Toxoplasma gondii* infection

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Mice of the C57BL/6 strain develop acute ileal inflammation after infection with the protozoan parasite *Toxoplasma gondii*. This pathology resembles many key features of human Crohn's disease, including a Th1 cytokine profile with high levels of interferon γ (IFN- γ), interleukin 12 (IL)-12, and tumor necrosis factor α (TNF)- α , presence of pathogenic CD4⁺ T cells, and infiltration of gut flora into inflamed tissue. Using CCR2^{-/-} mice, we identify a role for this chemokine receptor in the pathogenesis of inflammatory pathology during *T. gondii* infection. Lack of chemokine (C-C motif) receptor 2 (CCR2) was associated with low levels of CD103⁺ T lymphocytes in the intraepithelial compartment, Peyer's patch, and lamina propria relative to wild-type animals. Adoptive transfer of wild-type, but not IFN- γ ^{-/-}, intraepithelial T lymphocytes converted CCR2 knockout mice from a resistant to susceptible phenotype with respect to parasite-triggered inflammatory gut pathology. These results for the first time show a role for intraepithelial T lymphocytes in pathogenesis of ileitis triggered by a microbial pathogen.

INTRODUCTION

Certain inbred mice, most notably the C57BL/6 strain, develop severe small intestinal pathology in the ileum after oral infection with the opportunistic protozoan pathogen *Toxoplasma gondii*.¹ Antibody-mediated depletion and gene knockout studies indicate that the disease involves excess production of interferon γ (IFN- γ) and tumor necrosis factor α (TNF- α), and the activity of CD4⁺ T-cell effectors.^{1,2} The ileal inflammation induced after *T. gondii* infection shares a number of features in common with inflammatory bowel disease (IBD), and in particular with Crohn's disease (CD), which is associated with a hyperinflammatory Th1 cytokine profile and tissue necrosis in the ileum.^{2,3}

Dysregulated immune responses to microbial gut flora are implicated in disease pathogenesis during both *Toxoplasma* infection and IBD. Invasive Enterobacteriaceae can be found at inflamed sites of the small intestine after inoculation with *T. gondii*. Broad-spectrum antibiotic treatment to eliminate gut flora also prevents pathological consequences of oral infection.^{4,5} Toll-like receptor 4 (TLR4)-mediated sensing of commensal bacteria has been linked to murine ileitis triggered by *Toxoplasma* infection.⁵ Thus, an emerging view

is that *Toxoplasma* infection serves as a trigger for inflammatory pathology caused by intestinal bacteria rather than the parasite itself. In this regard, it is notable that invasive Enterobacteriaceae are also found at inflamed sites during human CD, and a dysregulated balance between commensal flora and pathogenic bacteria species is believed to have a role in the emergence of CD.^{6–8}

A key event in initiation of inflammation in the gut is thought to be activation of pathogenic T cells. In CD, it is believed that lamina propria Th1 and possibly Th17 T lymphocytes contribute to proinflammatory cytokine production.³ In CD patients and in experimental models of CD, upregulation of the Th1 transcription factor, T-bet, is associated with the disease.⁹ During *Toxoplasma* infection, pathology fails to develop in the absence of major histocompatibility complex class II-restricted CD4⁺ T cells.¹ The parasite surface protein SAG-1 (p30) has been identified as an antigen recognized by pathogenic CD4⁺ T cells.¹⁰ Other lines of evidence indicate that lamina propria T cells are a source of IFN- γ that mediates pathology, and that CD8⁺ intraepithelial lymphocytes (IELs) can downregulate this activity through transforming growth factor- β production.^{11,12}

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Figure 1 Absence of chemokine (C–C motif) receptor 2 (CCR2) protects against *T. gondii*-mediated intestinal pathology. Wild-type (WT) (a) and *CCR2*^{−/−} (b) mice were orally infected with 100 ME49 cysts, and tissues were collected for hematoxylin and eosin staining 8 days later. Sections of ileum from day 8-infected WT (c) and *CCR2*^{−/−} (d) mice were hybridized with *Eub338* (Cy3; red) and non-EUB338 (FITC (fluorescein isothiocyanate); green) to detect localization of intestinal flora. Sections were counterstained with DAPI (4′,6-diamidino-2-phenylindole) (blue) and examined by fluorescence microscopy. Bacteria appear red/orange (yellow arrows). Autofluorescence and nonspecific hybridization of the probe appear as yellow/green. Bars = 50 μ m.

Chemokines and their receptors have a dominant role in orchestrating the activity of T cells in IBD, and in particular, there is evidence that chemokine (C–C motif) receptor 2 (CCR2) and its ligands are involved in human CD.¹³ Immunohistochemical staining of gut biopsy samples from CD patients reveals infiltrating CD4⁺ T cells that are uniformly positive for CCR2.¹⁴ Furthermore, disease phenotypes of CD patients have been linked to polymorphisms in CCL2 and CCR2 genes.^{15,16} Although these studies indicate that CCR2 and its ligands are associated with proinflammatory damage in the intestine, their role in the etio-pathogenesis of mucosal inflammation is unknown.

Here, we show a requirement for CCR2 in *Toxoplasma*-triggered intestinal pathology. We identify a population of CD103⁺ IELs that is expanded depending on the presence or absence of this chemokine receptor. Most importantly, wild-type (WT), but not *IFN- γ* ^{−/−}, IELs induce severe intestinal damage on transfer into infected *CCR2*^{−/−} mice. Our data for the first time show that microbial infection can trigger pathogenic IELs that cause inflammation and necrosis of the small intestine depending on their ability to produce IFN- γ .

RESULTS

CCR2^{−/−} mice display increased susceptibility during oral infection with *T. gondii*

We infected WT and *CCR2*^{−/−} mice with the type II ME49 *Toxoplasma* strain to examine the role of the CCR2 chemokine

receptor in resistance to the parasite. As reported by others^{17,18}, during low-dose infection (20 cysts), absence of CCR2 led to increased mortality (**Supplementary Figure S1A** online). Cyst numbers were elevated in the brains of surviving animals compared with WT controls (**Supplementary Figure S1B** online), consistent with a defect in microbicidal effector activity. When we raised the infectious dose to 100 cysts, both WT and *CCR2*^{−/−} animals succumbed to infection with the same kinetics (**Supplementary Figure S1C** online).

Mice deficient in CCR2 are resistant to *Toxoplasma*-induced damage to the intestinal mucosa

To understand the role of CCR2 in development of intestinal inflammation during microbial infection, we used the ME49 parasite strain to examine pathological consequences of oral infection in WT and *CCR2*^{−/−} mice. In agreement with previously published data, WT mice developed severe intestinal pathology by day 8 post-infection¹ (**Figure 1a**). Inflammation was localized to the ileum and consisted of extensive cellular infiltration into the lamina propria and submucosa, hemorrhage, villus blunting, and areas of epithelial layer destruction. This pathology has previously been established to be partly dependent on CD4⁺ T cells and IFN- γ .^{19,20} In sharp contrast to the pathology induced in WT animals, inflammation in *CCR2*^{−/−} mice was mild (**Figure 1b**; **Supplementary Figure S2** online), characterized by low-level cellular

Figure 2 Absence of chemokine (C–C motif) receptor 2 (*CCR2*) results in diminished recruitment of CD103⁺CD11c⁺ and CD103⁺CD11c⁻ cells to sites of infection. CD11c and CD103 expression was examined in Peyer's patches (a and b) lamina propria (c and d), and the intraepithelial lymphocyte compartment (e and f) of day 4-infected wild-type (WT) (a, c, e) and *CCR2*^{-/-} (b, d, f) mice. The numbers in each rectangle indicate the relative percentage of cells out of the total population. Panel g shows mean values obtained from the Peyer's patches (PP), lamina propria (LP), and intraepithelial lymphocyte (IEL) compartment in a representative experiment using three mice per strain (**P* < 0.05). KO, knockout.

infiltration into the lamina propria, and modest thickening of the villi and submucosa.

To further substantiate the contribution of *CCR2* to pathology after infection, we used fluorescence *in situ* hybridization (FISH) employing oligonucleotide probes recognizing the 16S ribosomal RNA subunit to identify bacteria present in mucosal tissue. Previous studies have shown that in areas of *Toxoplasma*-induced intestinal damage, gut flora penetrate mucosal tissue, further enhancing inflammation.^{4,21} In agreement with our pathology data, WT mucosa was colonized with high numbers of translocated bacteria (Figure 1c). In Supplementary Figure S3A online, an enlarged view of the region indicated by the arrow in

Figure 1c is shown. Supplementary Figure S4 online shows an area of translocated bacteria that appear to have been internalized by resident cells, possibly macrophages. Supplementary Figure S5A online shows a low-power image of infected WT small intestine. Contrasting with these results, bacteria were strictly confined to the lumen of the gut in *CCR2*^{-/-} mice (Figure 1d). The yellow arrow in this figure shows luminal bacteria. This area is enlarged in Supplementary Figure S3B online, and a low-power image of the infected *CCR2*^{-/-} intestine is shown in Supplementary Figure S5B online. Thus, in the absence of *CCR2*, mice are protected from inflammatory pathology and accompanying bacterial translocation in the gut.

CCR2 mediates recruitment of CD103⁺ T cells into mucosal sites

We next investigated the immune cell composition of Peyer's patches, lamina propria, and IEL compartments after infection of WT and *CCR2*^{-/-} mice, in particular assessing expression of CD103, an integrin associated with homing to the intestine.²² In all mucosal compartments, WT mice accumulated more CD103⁺ cells than their *CCR2*^{-/-} counterparts (Figure 2a–f; averaged results of three animals per strain are shown in Figure 3g). Approximately half of these cells also expressed CD11c, a marker expressed by dendritic cells and, importantly, by activated T cells resident in the mucosal epithelium.²³ The difference between *CCR2*^{+/+} and *CCR2*^{-/-} strains was most striking in the IEL compartment in which there were up to six-fold more CD103⁺CD11c⁺ cells, and more than double the number of CD103⁺CD11c⁻ cells in the WT IEL population (Figure 2e) compared with *CCR2*^{-/-} IELs (Figure 2f). The difference in cell number in WT vs. knockout strains likely reflects differences in recruitment or retention of cells in response to infection. This is because the proportion of CD103⁺ cells was equivalent in noninfected animals in the presence or absence of *CCR2* (data not shown).

We further examined the phenotype of the CD103⁺ cell populations that were recruited depending on presence or absence of *CCR2* (Figure 3). The CD11c⁺ cell population was uniformly positive for CD8, and was approximately equally divided between $\alpha\beta$ - and $\gamma\delta$ -TCR (T-cell receptor) expression. Both populations also expressed the T-cell molecule CD3 (Supplementary Figure S6A and B online). The elevated percentage of $\alpha\beta$ -T cells may be the result of *Toxoplasma*-driven recruitment of these cells to the site of infection. The cells expressed a partially activated phenotype, in that they were CD25 and CD62L negative, but positive for CD69. The CD103⁺CD11c⁺ cell population also expressed high levels of *CCR5* and *CCR7*. The CD103⁺ cells that lacked expression of CD11c possessed similar phenotypic characteristics, although the distribution of T-cell markers differed. Thus, amongst CD103⁺CD11c⁻ cells, 70% were positive for the $\alpha\beta$ -TCR, and ~20% were CD4 positive. We then determined levels of *CCR2* on the CD103⁺ cell populations. In cells co-expressing CD11c, approximately half expressed *CCR2*. In the CD103⁺CD11c⁻ cell population, ~16% stained positive for *CCR2* (Figure 4). Lastly, we examined the phenotype of the CD103⁻CD11c⁻ cell population. A subpopulation

Figure 3 CD11c⁺ and CD11c⁻ cell populations of CD103⁺ cells express T cell lineage markers. Wild-type (WT) animals were orally infected with *T. gondii* and then with intraepithelial lymphocytes (IELs) prepared 4 days later. Four-color staining was used to identify cell surface molecules. Cells were stained for CD11c and CD103, and then for either T-cell receptor (TCR)- $\alpha\beta$ and TCR- $\gamma\delta$, or CD4 and CD8. The activation status of the cells was determined by staining with CD25, CD44, CD62L, and CD69, and chemokine receptor expression was examined by staining for CCR5 and CCR7.

Figure 4 Expression of chemokine (C–C motif) receptor 2 (CCR2) by intraepithelial lymphocytes (IELs) in the small intestine. IELs isolated from wild-type (WT) ilea on day 4 post-infection were triple-stained for CD103, CD11c, and CCR2. (a) CD11c vs. CD103 staining profile of isolated IELs. CCR2 surface expression is shown on double-positive (CD11c⁺CD103⁺) IELs in panel b, and single positive (CD11c⁻CD103⁺) CCR2 levels are shown in panel c.

(17%) of these cells expressed CD3 (Supplementary Figure S6C online), but the majority stained positive for the epithelial marker EpCAM (Supplementary Figure S6D online). We conclude that most of these cells are of epithelial origin.

WT IELs are skewed to a proinflammatory phenotype after *T. gondii* infection

Toxoplasma-induced ileal pathology is dependent on proinflammatory mediators that include IFN- γ and TNF- α , but production of these mediators during infection in the small intestine

Figure 5 Intraepithelial lymphocytes (IELs) are heavily skewed to a proinflammatory cytokine and transcription factor profile after parasite infection. (a) Cytokine secretion from mucosal tissue was detected by cytometric bead array on supernatants from overnight culture of gut biopsy samples. (b) RNA extracted from IELs of both noninfected and infected wild-type (WT) mice was subjected to real-time PCR amplification and normalized to GAPDH (glyceraldehyde 3-phosphate dehydrogenase). The data are expressed relative to transcript levels in noninfected animals (defined as 1). Bars represent s.d. values from triplicate samples. Eomes, eomesodermin; IFN, interferon; IL, interleukin; MCP, monocyte chemotactic protein; TNF, tumor necrosis factor.

has not been examined. Here, we cultured gut biopsy samples from infected mice and examined cytokine release. Although this approach does not identify the specific cell source of the cytokines measured, it allows an assessment of the overall cytokine environment in the intestine of animals undergoing infection. As shown in **Figure 5a**, high levels of IFN- γ and TNF- α were produced. In addition, cultures contained high amounts of MCP-1 (monocyte chemotactic protein-1), the major chemokine ligand of CCR2. In contrast, only low amounts of IL-10 and IL-6 were produced.

To further characterize the functional phenotype of the IEL population, we performed real-time PCR on IELs isolated from noninfected and infected WT mice. Transcripts for signature T cell transcription factors were examined. The data in **Figure 5b** show a major increase in expression of the Th1 master regulator, T-bet, relative to IELs from noninfected mice. In comparison with IELs from noninfected mice, levels of the Th-17-associated transcription factor, ROR γ t, were downregulated after infection, but comesoderm, a regulator of CD8⁺ T-cell differentiation, was modestly elevated. In the same samples, upregulation of mRNA for IFN- γ , TNF- α , and IL-17 was detected. We conclude that IELs from infected mice express an overwhelmingly proinflammatory transcriptional program, associated, in particular, with increased expression of the transcription factor T-bet (**Figure 5**).

***T. gondii*-primed WT IELs induce IFN- γ -dependent pathological damage in *CCR2*^{-/-} mice**

Intraepithelial lymphocytes have been linked to protective immune responses in the gut during *T. gondii* infection,^{12,24,25} but here, we show an association between parasite-induced pathology and CCR2-dependent IEL recruitment. To establish a causal link between pathology and *T. gondii*-primed IELs, we determined whether adoptive transfer of WT IELs was sufficient to mediate intestinal damage in *CCR2*^{-/-} mice. First, we confirmed that primed IELs were capable of homing to the intestinal epithelium after transfer. Accordingly, IELs were isolated from day 4-infected CD45.1 congenic mice and adoptively transferred into day 4-infected C57BL/6 (CD45.2) mice. As shown in **Figure 6a**, the transferred CD45.1⁺ cells trafficked to the intestinal epithelium within 24 h of injection. Gating on this population revealed that both CD11c⁺ and CD11c⁻ cell populations of IELs were capable of migrating back to the IEL compartment (**Figure 6b**). Notably, there was minimal homing of CD103⁻ cells back to the intestine. This is consistent with the known role of CD103 as a mucosal addressin molecule.

To directly determine whether IELs mediate intestinal pathology, IELs from day 4-infected WT mice were transferred into day 4-infected *CCR2*^{-/-} recipients. Mice were killed 5 days post-transfer and pathology assessed. WT mice developed typical pathological changes in the small intestine (**Figure 6c**), whereas damage in the ileum of *CCR2*^{-/-} mice was mild (**Figure 6d**). Dramatically, *CCR2*^{-/-} mice receiving day 4 post-infection WT IELs, developed severe intestinal inflammation similar to that of WT mice (**Figure 6e**; **Supplementary Figure S2** online). To further substantiate this result, tissues were subjected to FISH

Figure 6 Wild-type (WT) CD103⁺ cells traffic to the intraepithelial lymphocytes (IELs) compartment and mediate interferon- γ (IFN- γ)-dependent ileal pathology in *CCR2*^{-/-} mice. IELs were isolated from day 4-infected C57BL/6 background CD45.1 congenic mice. Cells were stained for CD11c and CD103 to confirm IEL phenotype (**a** inset) then i.v. injected into day 4-infected C57BL/6 (CD45.2) recipient mice. IELs were isolated from recipients 24-h later, and CD45.1 cells tracked by flow cytometry, as shown in (**a**). Gating on CD45.1 IELs, and side scatter (SSC) then examining expression of CD11c and CD103 (**b**) determined the phenotype of the transferred cells. Pathological damage in the intestine was evaluated 5 days post-transfer. Wild-type (WT) (**c**) but not *CCR2*^{-/-} (**d**) mice display characteristic inflammatory pathology. *CCR2*^{-/-} mice reconstituted with IELs from day 4-infected WT mice develop WT-like pathology (**e**) with concomitant translocation of bacteria into the intestinal mucosa (**f**). In contrast, transfer of IELs from day 4-infected IFN- γ ^{-/-} animals into *CCR2*^{-/-} mice does not trigger pathological damage (**g**) and bacteria remain in the lumen of the intestine (**h**). Arrows in **f** and **h** point to bacteria, visible as yellow staining in the submucosa (**f**) and lumen (**h**). The bars in each panel indicate 50 μ m.

analysis to determine the status of gut flora in intestinal tissues. **Figure 6f** shows that translocated bacteria are present in the lamina propria of *CCR2*^{-/-} mice reconstituted with *T. gondii*-elicited WT IELs. An expanded view of the area indicated by the yellow arrow that points out translocated bacteria is shown in **Supplementary Figure S7A** online.

As IELs from infected animals produce large amounts of IFN- γ , a cytokine implicated in *Toxoplasma*-induced

Figure 7 Both CD11c⁺ and CD11c⁻ intraepithelial lymphocytes (IELs) subsets contribute to intestinal inflammation. IELs were prepared from day 4-infected mice, then CD11c⁺ and CD11c⁻ cell populations were isolated employing anti-CD11c antibody and immunomagnetic beads. **(a)** Starting population of IEL; **(b)** CD103⁺ cell population; **(c)** CD103⁻ cell population. The cells shown in panels **b** and **c** were adoptively transferred into day 4-infected *CCR2*^{-/-} animals, and small intestine tissue was evaluated 5 days later by hematoxylin and eosin staining. Micrographs showing the outcome of transfers in mice receiving CD11c⁺CD103⁺ **(d)** or CD11c⁻CD103⁺ **(e)** IELs show that both cell subsets induce inflammatory pathology to a similar degree. Images are representative of five independent experiments.

immunopathology,^{1,2} we sought to determine whether IFN- γ production by CD103⁺ IELs accounted for their pathogenicity by repeating the transfer experiments using cells from *IFN- γ* ^{-/-} mice as donors. Strikingly, *CCR2*^{-/-} mice receiving IELs from *IFN- γ* ^{-/-} mice did not develop inflammatory pathology (**Figure 6g**; **Supplementary Figure S2** online). We subjected tissue sections to FISH analysis, and, as predicted, bacteria remained luminal in animals receiving *IFN- γ* ^{-/-} IELs, consistent with a lack of damage to the intestinal epithelium (**Figure 6h** and **Supplementary Figure S7B** online). We conclude that IFN- γ is necessary for the pathogenicity of *CCR2*-dependent IELs.

Finally, we used our adoptive transfer model to ask whether damage to the intestine was mediated by CD11c⁺ or CD11c⁻ IEL populations. IELs from day 4-infected WT mice (**Figure 7a**) were immunomagnetically separated into CD11c⁺ (**Figure 7b**) and CD11c⁻ (**Figure 7c**) fractions. These were adoptively transferred into day 4-infected *CCR2*^{-/-} mice and small intestines were collected for histopathological evaluation 5 days later. Mice receiving double positive cells (CD103⁺CD11c⁺) developed intestinal inflammation as characterized by extensive immune cell infiltrates, tissue hemorrhaging, and villus fusion (**Figure 7d**; **Supplementary Figure S2** online). A similar outcome was observed in the mice receiving single-positive (CD103⁺CD11c⁻) IELs (**Figure 7e**; **Supplementary Figure S2** online). Control day 9-infected *CCR2*^{-/-} mice not receiving WT IELs showed minimal pathology indistinguishable from that shown in **Figure 1b** (data not shown). We conclude that

both CD11c⁺ and CD11c⁻ IEL populations possess pathogenic activity in this model.

DISCUSSION

Here, we show an essential role for IELs in proinflammatory intestinal pathology, induced by infection with *T. gondii*. Damage to the intestinal mucosa was furthermore dependent on ability of the cells to produce IFN- γ . In addition, emergence of pathogenic IELs during infection required expression of *CCR2*. Consequently, mice lacking *CCR2* were protected from development of ileal inflammation. CD4⁺ T lymphocytes have previously been implicated in *Toxoplasma*-induced intestinal pathology, but this study now shows that the IEL compartment, mostly composed of CD8⁺ T cells, also possess pathogenic activity during infection.

Lack of *CCR2* in *Toxoplasma*-infected mice was recently associated with failure to recruit anti-microbe inflammatory monocytes and increased pathology in several tissues, including the intestine.¹⁷ In the present study, we also found that *CCR2*^{-/-} mice are more susceptible to infection using a low parasite inoculum, as measured by increased mortality and significantly higher parasite burdens in the surviving mice. However, after high-dose infection, we found that deletion of *CCR2* mediates protection against pathology in the small intestine. In agreement with us, another recent study found that *CCR2*^{-/-} mice are resistant to *Toxoplasma*-induced damage to the gut.¹⁸ Issues of parasite dose may underlie differences reported in these studies regarding parasite-induced pathology in *CCR2*^{-/-} mice. Low infection

inoculums employed by Dunay *et al.*¹⁷ may trigger CCR2-dependent inflammatory monocytes whose absence leads to uncontrolled parasite replication associated with tissue destruction at the site of infection. Under higher infection conditions, CCR2 appears to be important in recruitment and activation of IELs that cause proinflammatory pathology in the intestine.

The CCR2-dependent IELs identified in this study were a mixed population. Although the cells uniformly expressed CD103, approximately half were positive for CD11c, a molecule known to be expressed on activated T cells in the intestinal mucosa.²³ Our data show that both CD11c⁺ and CD11c⁻ IELs induce damage, but further studies are needed to determine whether this is an activity mediated by $\alpha\beta$ - or $\gamma\delta$ -T lymphocytes that are present in both populations.

The CD103 molecule, also known as αE , forms a heterodimer with the integrin $\beta 7$ subunit. Binding of CD103/ $\beta 7$ to epithelial cell E-cadherin is important in homing and retention of lymphocytes and dendritic cells to the intestinal mucosa.²² There is evidence that CD103 is involved in intestinal graft vs. host disease pathology and TNP-OVA-induced colitis in IL-2-deficient mice.^{26,27} Other studies suggest that CD103⁺ T cells regulate pathology in TNF-mediated experimental colitis, and there is evidence that CD103⁺ dendritic cells control disease in T-cell transfer models of colitis.^{28,29} Thus, whether CD103 is associated with inflammation or prevention of pathology most likely depends on the effector function of the cells expressing this intestinal homing molecule.

Although we found that IELs contribute to pathology in the gut during infection, this compartment has been linked to protection and maintenance of homeostasis during *T. gondii* infection.^{12,24,25} Although lamina propria CD4⁺ T cells were found to synergize with intestinal epithelial cells for proinflammatory cytokine production, IEL secretion of transforming growth factor- β downregulated the response, and *in vivo* transfer studies suggested a role for this anti-inflammatory cytokine in preventing parasite-induced ileitis.^{12,20,25} Furthermore, $\gamma\delta$ -T cells have been associated with epithelial barrier function during *Toxoplasma* infection.³⁰ Other studies have shown that adoptive transfer of IELs protects against infection and induces long-term immunity against *Toxoplasma*.^{24,31,32} Consistent with our studies, IEL effector activity in these cases was associated with production of IFN- γ .²⁴

The divergence between the protective function of the IEL compartment reported previously²⁴ and the clear pathological activity reported here may be a consequence of differences in parasite doses employed in each case. Whereas we isolated IELs from animals undergoing high-dose infection, earlier studies employed IELs from animals undergoing low-dose infection. We hypothesize that exposure to a low parasite dose elicits IEL activity that mediates protection against inflammatory pathology, and that high parasite doses trigger a switch to IELs that mediate inflammatory pathology in the intestine.

On the basis of previous studies in animal models, IBD pathogenesis has been attributed to the activity of lamina propria CD4⁺ cells responding to gut flora.³³ Here, we show that IELs induce similar pathology. As the overwhelming majority

of these cells express the CD8 molecule, it is likely that this IEL subset mediates disease, in particular, the CD11c⁺ IELs that are ~98% positive for CD8 transfer pathology on inoculation into CCR2^{-/-} mice. However, we cannot completely exclude a role for CD4⁺ T lymphocytes, because these cells comprise ~20% of the CD103⁺CD11c⁻ cell population that also mediates damage.

There is increasing evidence that CD8⁺ T cells may trigger pathogenic CD4⁺ responses during IBD, possibly by damaging the epithelium and allowing access of luminal bacteria or by releasing activating cytokines.³³ In a hapten-induced colitis model, hapten-sensitized CD8⁺ T cells were identified as the earliest initiators of infection.³⁰ In another study, transgenic CD8 T cells specific for influenza hemagglutinin A that had developed in the absence of cognate antigen were able to mediate severe intestinal destruction after transfer into transgenic mice expressing a hemagglutinin A transgene in the intestinal epithelial compartment.³¹ The results of the present study reinforce and extend the hypothesis that pathogenic CD8⁺ T cells are initiators of intestinal pathology. Importantly, our study provides evidence that IELs themselves mediate ileal damage triggered by microbial infection. Furthermore, our results reveal, for the first time, that chemokine receptor CCR2 is a key player in this pathology.

Unraveling the roles of IELs in the pathogenesis of intestinal inflammation is a complex task. The IEL compartment is diverse, consisting of T cells expressing $\alpha\beta$ - and $\gamma\delta$ -TCRs.³⁶ Most IELs express the CD8 molecule, yet, of these, some are generated independently of classical major histocompatibility complex class I molecules.^{37,38} Some IELs are generated in the thymus, but other subpopulations are believed to derive from cryptopatches in the intestinal mucosa.²² By examining expression of chemokine receptors and TCRs, as well as the need for major histocompatibility complex in generating the cells, it should be possible to elucidate requirements for local recruitment and expansion of protective or pathogenic IELs. In turn, this can be expected to shed light on pathogenesis of IBD.

METHODS

Mice. Female C57BL/6, Swiss Webster, IFN- γ ^{-/-}, and CCR2^{-/-} mice (6–8-week-old) were purchased from The Jackson Laboratory (Bar Harbor, ME). Breeding colonies of B6.SJL-*Ptprca*^a*Pep3*^b/BoyJ (CD45.1 congenic) and CCR2^{-/-} mice were established in the Transgenic Mouse Facility at the Cornell University College of Veterinary Medicine. Animals were housed under specific pathogen-free conditions at the Cornell University College of Veterinary Medicine animal facility, which is accredited by the American Association of Laboratory Animal Care.

Parasites and infections. Cysts of the type II low-virulence *T. gondii* strain, ME49, were obtained from chronically infected (>1 month) Swiss Webster mice by homogenizing brains in sterile phosphate-buffered saline. Homogenate was passaged through an 18-G needle and cysts were enumerated by phase contrast microscopy. Age-matched mice were infected with 100 cysts by oral gavage.

Cell isolation. Small intestines were removed, cleaned of mesentery and fat, and flushed with 37°C phosphate-buffered saline. Mesenteric lymph nodes were excised and the fat removed before cutting into small fragments and homogenizing through a 70- μ m filter (BD, Franklin Lakes, NJ). Peyer's patches were removed and passed through a 70- μ m filter to

yield a single cell suspension. Isolation of IELs was performed as previously described.³⁷ Briefly, intestines were cut into 5 cm lengths and opened longitudinally on a sterile plastic sheet. The mucosal layer, containing epithelial cells and IELs, was scraped off with a blunt scalpel. The cells were washed by centrifugation and resuspended in RPMI containing 10% fetal bovine serum and 10 mM dithioerythritol (Sigma, Saint Louis, MO) pre-warmed to 37°C. The cells were incubated for 20 min with magnetic stirring. Cells were filtered through a prewashed glass wool column to remove contaminating enterocytes and cell debris. The IELs eluted from the column were further purified by discontinuous Percoll gradient separation. Lamina propria leukocytes were isolated according to the standard techniques.⁴⁰ Briefly, intestinal tissue with the mucosal layer removed was cut into 5-mm fragments and cells were liberated from the tissues by digestion (37°C, 2 h) in RPMI, 10% fetal bovine serum, 100 U ml⁻¹ collagenase (Sigma), and 15 µg ml⁻¹ DNase (Sigma). Cells were separated from contaminating debris by discontinuous Percoll gradient separation.

Gut biopsy culture. Intestines were removed from infected WT mice at day 4 post-infection and flushed extensively with phosphate-buffered saline containing antibiotics. Intestines were opened longitudinally and a biopsy punch was used to collect uniform pieces of tissue. Intestinal sections were incubated overnight (37°C, 4% CO₂) in Dulbecco's modified Eagle's medium supplemented with 10% bovine growth serum (Hyclone), 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 0.05 mM β-mercaptoethanol, 100 U/ml penicillin, 100 µg/ml streptomycin, and 30 mM HEPES (reagents from Sigma). The supernatants were collected and levels of cytokine determined using the cytometric Bead Array, following the manufacturer's instructions (BD).

Semi-quantitative real-time PCR. Semi-quantitative PCR was performed as described elsewhere.⁴¹ RNA was extracted from IELs using Trizol (Sigma) and reverse transcribed into cDNA according to standard protocols. PCR was performed on cDNA using primers specific for TNF-α, IFN-γ, T-bet, Foxp3, and eomesodermin. SYBR green 1 (Invitrogen, Carlsbad, CA) was employed to quantitate amplification. Fluorescence was measured using an Applied Biosystems 7500 sequence detector (Applied Biosystems, Warrington, UK). All samples were amplified in triplicate and normalized to GAPDH (glyceraldehyde 3-phosphate dehydrogenase).

Flow cytometry. Single cell suspensions obtained from lamina propria, Peyer's patch, and IEL compartments were incubated in ice-cold FACS buffer (phosphate-buffered saline, 1% bovine serum albumin, 0.01% NaN₃) containing 10% normal mouse serum to block Fc receptor binding (30 min, 4°C). Cells were pelleted by centrifugation and resuspended in optimal concentrations of fluorochrome-conjugated antibodies in ice-cold FACS buffer to stain surface molecules. The antibodies used in this study were anti-TCR-γδ, anti-CD3, anti-CD4, anti-CD8, anti-CD11c, and anti-EpCAM conjugated to fluorescein isothiocyanate; anti-CD62L, anti-CCR5, anti-CCR7, and anti-CD45.1 conjugated to phycoerythrin; anti-TCR-αβ, anti-CD69, and anti-CD25 conjugated to PerCP; and anti-CD11c and anti-CD44 conjugated to allophycocyanin. Antibodies were purchased from either Biolegend (San Diego, CA) or eBioscience (San Diego, CA). CCR2 was detected using rat anti-mouse CCR2 (clone MC-21) followed by anti-rat AF488 secondary antibody (Invitrogen) before staining with other antibodies. Antibodies were incubated with cells for 30 min at 4°C. After washing, at least 50,000 cells per sample were collected for analysis on a BD FACSCalibur flow cytometer. Data analysis was performed using FlowJo software (Ashland, OR).

Adoptive transfer. A total of 5×10⁶ IELs recovered from day 4-infected mice were transferred into infected CCR2^{-/-} recipients by intravenous retro-orbital injection under anesthesia. On day 9 post-infection, intestines were removed and fixed in 10% neutral-buffered formaldehyde. Some paraffin-embedded tissue sections were stained with hematoxylin and eosin and examined for pathological changes, and others were

subjected to FISH. In some experiments, whole IELs isolated from day 4-infected WT mice were incubated with anti-CD11c beads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Cells were separated into CD11c⁺ and CD11c⁻ subpopulations using an AutoMacs separator (Miltenyi Biotec). Aliquots of the unseparated population as well as the IEL subsets were stained with CD11c and CD103, and analyzed by flow cytometry to evaluate the efficiency of the magnetic separation. A total of 2×10⁶ IELs of each subset were transferred into day 4-infected CCR2^{-/-} mice. Ileae were collected 5 days after adoptive transfer, formalin fixed, and processed for histological evaluation.

Fluorescence *in situ* hybridization. Fluorescence *in situ* hybridization was performed as previously described.⁸ Paraffin-embedded sections were de-paraffinized and rehydrated by serial immersion in xylene and graded alcohols, then in finally water. Sections were incubated with FISH probes (EUB338, or non-EUB338) labeled on the 5' end with either FITC (fluorescein isothiocyanate) or Cy3 (Integrated DNA Technologies, Coralville, IA) at 5 µg ml⁻¹ in hybridization buffer (42°C, 14 h). Sections were washed in hybridization buffer to remove un-bound probe, rinsed in sterile water, air-dried, and mounted with Prolong Antifade Gold (Molecular Probes, Carlsbad, CA). Images were collected with a BX51 microscope (Olympus, Center Valley, PA), equipped with a DP70 camera using DP Controller Software (version 1.1.1.65; Olympus), and DP Manager software (version 1.1.1.71; Olympus).

Pathology scoring. Pathology was scored between 0 (not apparent) and 4 (severe) for 5 criteria. These were villus fusion/blunting, lamina propria inflammation, sloughing of epithelial tips, necrosis of villus tips, and transmural inflammation.⁴² Individual mice were assigned a cumulative score out of a maximum of 20. Data were graphed with each point representing an individual mouse and the bar as the mean of each group.

Statistical analyses. Student's *t*-test was used to analyze statistical differences between groups. Values for *P* < 0.05 were considered significant. All experiments were repeated a minimum of three times. Pathology scores were assessed for significance using one-way ANOVA (analysis of variance) test.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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DISCLOSURE

The authors declare no conflict of interest.

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