CX₃CR1⁺ macrophages support IL-22 production by innate lymphoid cells during infection with Citrobacter rodentium

C Manta¹, E Heupel², K Radulovic¹, V Rossini¹, N Garbi³, CU Riedel² and JH Niess¹

Innate immune cells, such as intestinal epithelial cells, dendritic cells (DCs), macrophages, granulocytes, and innate lymphoid cells provide a first line of defence to enteric pathogens. To study the role of CX₃CR1⁺ DCs and macrophages in host defence, we infected CX₃CR1-GFP animals with Citrobacter rodentium. When transgenic CX₃CR1-GFP animals are infected with the natural mouse pathogen C. rodentium, CX₃CR1⁻/⁻ animals showed a delayed clearance of C. rodentium as compared with (age- and sex-matched) wild-type B6 animals. The delayed clearance of C. rodentium is associated with reduced interleukin (IL)-22 expression. In C. rodentium-infected CX₃CR1-GFP animals, IL-22 producing lymphoid-tissue inducer cells (LTI cells) were selectively reduced in the absence of CX₃CR1. The reduced IL-22 expression correlates with decreased expression of the antimicrobial peptides RegIIIβ and RegIIIγ. The depletion of CX₃CR1⁺ cells by diphtheria toxin injection in CX₃CR1-GFP×CD11c.DOG animals confirmed the role of CX₃CR1⁺ phagocytes in establishing IL-22 production, supporting the clearance of a C. rodentium infection.

INTRODUCTION

Innate immune cells, such as intestinal epithelial cells, dendritic cells (DCs), macrophages, granulocytes, and innate lymphoid cells (ILC) provide a first line of defence to insults in the gut, which is exposed to a complex intestinal microflora and, occasionally, pathogens. Defective immune responses to the commensal microflora may mediate intestinal diseases such as Crohn’s disease and ulcerative colitis. Infiltration of DCs and macrophages is a characteristic of intestinal inflammation. The majority of F4/80(high) CX₃CR1⁺ macrophages produce interleukin (IL)-10 in a CX₃CR1-dependent manner in the steady state, whereas a small population of F4/80(low) CX₃CR1⁺ DCs secrete IL-12, IL-23, and tumor necrosis factor (TNF)-α, and are able to activate T cells. CX₃CR1 has been implicated in host defence to Salmonella and bacterial peritonitis, but its role in host defence in the large intestine has not been studied in detail.

Citrobacter rodentium is a natural extracellular enteric mouse pathogen that serves as a mouse model of human infections with enteropathogenic Escherichia coli. C. rodentium colonizes the cecum and colon of mice after infection. C. rodentium targets and infects intestinal epithelial cells by characteristic attaching and effacing lesions. It represents an excellent model system to study innate host immune responses in the gut. Clearance of C. rodentium by the hosts partially depends on IL-22, a member of the extended IL-10 cytokine family, as shown in studies with IL-22⁻/⁻ animals. IL-22 induces expression of intestinal antimicrobial peptides (RegIIIβ and RegIIIγ, β-defensin-2, and β-defensin-3) and facilitates host responses by modulating the expression of various chemokines, including CXCL1, CXCL5, and CXCL9. IL-23 promotes IL-22 expression, as IL-23⁻/⁻ animals succumb to C. rodentium infection. IL-1β, the γc cytokines IL-2, IL-7, and IL-15, and lymphotoxin controls the production of IL-22 by related orphan receptor gamma-t (RO1γt)⁺ ILCs. Several ILCs have been recently described that are characterized by signature cytokines. IL-22 production is a characteristic of ILC22 cells. These cells share characteristics of lymphoid tissue inducer (LTI) and natural killer (NK) cells. ILC17 and LTI cells both produce IL-17A and IL-22. All ILC share an Id2-dependent precursor cell for their development. ILC22, ILC17, and LTI all express the transcription factor RO1γt.
DCs and macrophages have been suggested to support the production of IL-22 by ILCs. DCs support IL-22 production by ILCs in a lymphotxin-dependent pathway. In addition, DC-derived IL-23 and macrophage-derived IL-1β facilitates IL-22 production by ILCs. Addition of IL-25 to cultures of IL-17BR+CD11c+ DCs/macrophages is able to suppress IL-22 production by RORγt+ ILCs. In addition, Toll-like receptor agonists were shown to induce IL-22 expression by ILCs in vitro via stimulation of DCs and macrophages. However, the subset of DC or macrophages promoting ILC IL-22 expression is not yet identified.

Given the high abundance of CX3CR1+ cells in the colonic lamina propria (cLP), we hypothesized that CX3CR1+ phagocytes may have a role in regulating IL-22 production by ILCs in response to intestinal bacterial pathogens, and thus be critical for protection. Using a murine model of innate-mediated

Figure 1  Delayed clearance of Citrobacter rodentium in CX3CR1-GFP animals. (a) C. rodentium counts in fecal samples from wt B6 animals and (age- and sex-matched) heterozygous and homozygous CX3CR1-GFP animals were determined by collecting fecal pellets from each animal every 2–3 days over the course of the infection. Pellets were weighted and resuspended in 1 ml of phosphate-buffered saline (PBS), plated in serial dilutions, and bacterial load was calculated as cfu g−1 feces. P-values were calculated with a nonparametric Student’s t-test; P<0.05 was considered statistically significant. (b) Histopathological scores of colon sections taken from control or infected B6, CX3CR1GFP/+ and CX3CR1GFP/GFP. In the nonparametric Student’s t-test, P<0.05 was considered statistically significant. (c) At the end of the experiment, colons were removed and representative colons of the indicated groups are shown. (d) The colon weight and length was determined and expressed as colon weight/length ratios. In the nonparametric Student’s t-test, P<0.05 was considered statistically significant. (e) Colony-forming units (CFU) from plates spotted with homogenates from liver, spleen, and mesenteric lymph nodes (MLN) of the indicated groups were determined. The numbers of animals per group of each experiment is given within the figure. In the nonparametric Student’s t-test, P<0.05 was considered statistically significant.
protection against *C. rodentium*, we show that clearance of *C. rodentium* is delayed in the absence of CX3CR1. CX3CR1-GFP animals on a recombination-activating gene (RAG)−/− background are highly susceptible to *C. rodentium* infection. Absence of CX3CR1 resulted in reduced IL-22 expression and reduced numbers of CD3−CD4+ROTY+CD127+CD117+ ILC cells. In addition, depletion of CX3CR1+CD11c+ cells further indicated a role of CX3CR1+CD11c+ macrophages in facilitating IL-22 production, supporting host defence against *C. rodentium* infection.

**RESULTS**

**Delayed *C. rodentium* clearance in absence of CX3CR1 in CX3CR1GFP/GFP animals**

The fractalkine receptor CX3CR1 is required for the clearance of *Salmonella* in the small intestine8,26 and in a peritoneal sepsis model,9 although the cellular and molecular mechanisms are not yet fully understood. Increased susceptibility of CX3CR1GFP/GFP animals, in which the green fluorescent protein (GFP) is inserted into two alleles of the CX3CR1 locus and are hence CX3CR1-deficient, to *Salmonella* can be in part

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**Figure 2** *Citrobacter rodentium* can be located in CX3CR1+ macrophages. (a) Homozygous CX3CR1-GFP animals were infected with *C. rodentium* mutants expressing the red fluorescent protein mRuby. Twelve days post infection, living intestinal tissues from the proximal colon was analyzed by ex vivo microscopy. (b) CX3CR1+CD11c+ cells were isolated from homzygous CX3CR1-GFP animals infected with the *C. rodentium* mutant ICC 169 expressing the red fluorescent protein mRuby. Colonic lamina propria isolates were stained for CD11c and analyzed by flow cytometry. Histograms were obtained by gating on CX3CR1-GFP+CD11c+ cells. Gray areas represent isolates obtained from animals infected with *C. rodentium* ICC 169; open histograms represent isolates obtained from animals infected with mRuby expressing *C. rodentium* mutants. (c) CX3CR1+ cells were defined as area of interest and scatter diagrams obtained. The percentage of *C. rodentium* located in CX3CR1+ macrophages was determined. (d) Mean (±s.e.m.) percentage of *C. rodentium* located in CX3CR1+ macrophages from the indicated mice is shown. *P*-values were calculated with a nonparametric Student’s *t*-test; *P*<0.05 was considered statistically significant.
explained by the reduced production of TNF-α, interferon-γ, IL-6, and inducible nitric oxide synthase. The infection of B6 and CX3CR1GFP/+ and CX3CR1GFP/GFP animals with C. rodentium is associated with an increased colonic fractalkine (CX3CL1, the ligand of CX3CR1) expression at day 12 and 20 post infection (p.i.; Supplementary Figure S1 online). To determine the contribution of CX3CR1 to host defence to C. rodentium, we infected wild-type (wt, littermate controls) heterozygous CX3CR1GFP/+ and homozygous CX3CR1GFP/GFP animals by oral gavage, and monitored the number of pathogenic bacteria in the feces for 27 days. In wt B6 animals C. rodentium burden in feces peaked between day 10–15, declined over time, and was not detectable at day 21, consistent with previous work from our group (Figure 1a). Although the initial kinetics were comparable to control mice, C. rodentium burden in feces of CX3CR1GFP/+ and CX3CR1GFP/GFP animals remained >2 logs higher than in age- and sex-matched B6 animals on days 20 and 22 p.i., and complete C. rodentium clearance was delayed until day 27 p.i. for about 1 week (Figure 1a). The delayed clearance of C. rodentium in CX3CR1GFP/+ and CX3CR1GFP/GFP animals is associated with increased histopathological and macroscopic signs of C. rodentium-induced colitis (Figure 1b and c), increased colon weight/length ratios (Figure 1d), and reduced anti-C. rodentium immunoglobulin G (IgG) titers (Supplementary Figure S2 online). C. rodentium could not be detected in homogenates of mesenteric lymph nodes (MLNs), liver, and spleen of B6 animals, confirming previous reports (Figure 1e). By contrast, on day 12 p.i., C. rodentium could be cultured from homogenates of MLNs, liver, and spleen of CX3CR1GFP/+ and CX3CR1GFP/GFP animals (Figure 1e). C. rodentium counts were higher in CX3CR1GFP/GFP animals compared with CX3CR1GFP/+ animals in all organs tested, but this effect was not statistically significant. Thus, C. rodentium translocates across the intestinal epithelium to MLN and liver if CX3CR1 levels are reduced or absent. Overall, these data indicated that CX3CR1 is involved in the clearance of the C. rodentium infection, and deficiency in CX3CR1 is associated with a significant increase in the severity of the C. rodentium infection.

C. rodentium localizes within CX3CR1+ phagocytes
The C. rodentium infection is associated with an infiltration of CX3CR1+ cells (Supplementary Figure S3 online). Significant differences between heterozygous and homozygous CX3CR1-GFP animals were not observed (Supplementary Figure S3E and F online). Multi-color flow cytometry demonstrated that F4/80+CD11c+ macrophages and F4/80(low)CD11c+ DCs expressed CX3CR1 (Supplementary Figure S3B and C online). In C. rodentium-infected animals, an increase of the CX3CR1+F4/80+CD11c+ macrophages was observed (Supplementary Figure S3B online). To examine C. rodentium infections in CX3CR1GFP/+ and CX3CR1GFP/GFP by fluorescence microscopy, we generated a red fluorescent C. rodentium strain (RF-C. rodentium) constitutively expressing the protein mRuby from a single copy of a derivative of plasmid p16Slux28 integrated into the bacterial chromosome (Supplementary Figure S4 online). Intestinal tissues of infected animals were analyzed by ex vivo confocal imaging 12 days p.i. (peak infection). RF-C. rodentium had translocated into the cLP of the colon at peak infection (Figure 2a). RF-C. rodentium is closely associated with CX3CR1+ cells and can be located within CX3CR1+ cells (Figure 2a). RF-C. rodentium seems thus to be phagocyted by CX3CR1+ macrophages in the cLP. Flow cytometry confirmed the presence of RF-C. rodentium within CX3CR1+ cells (Figure 2b). To further analyze the percentage of RF-C. rodentium located within CX3CR1+ DCs and macrophages in heterozygous and homozygous CX3CR1-GFP animals, CX3CR1+ cells were defined as a region, for which colocalization of red and green fluorescent signals were analyzed by generating scatter diagrams (Figure 2c and d). In CX3CR1GFP/+ and CX3CR1GFP/GFP animals, C. rodentium is found within the

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**Figure 3** Interleukin (IL)-22 expression is reduced in *Citrobacter rodentium*-infected CX3CR1GFP/GFP animals. (a) From the proximal colon of noninfected and *Citrobacter rodentium*-infected CX3CR1GFP/GFP and wild-type littermate controls mRNA was isolated. cDNA was prepared by reverse transcription and quantitative real-time PCR was performed with specific and the indicated primers. β-actin was used as a housekeeping gene to normalize cDNA input between samples. Normalized ct-values of the untreated samples (baseline) are set to 1 and values are plotted as fold expression of the baseline. The assays were carried out in triplicates. As per indicated, group 4 animals were analyzed. (b) IL-22 expression by colonic lamina propria (cLP) cell isolates from noninfected and *Citrobacter rodentium*-infected B6, (age- and sex-matched) and CX3CR1GFP/+ and CX3CR1GFP/GFP animals was analyzed by multicolor flow cytometry. Numbers indicate the percentage of IL-22-positive cells. (c) cLP IL22+ cells from noninfected and *Citrobacter rodentium*-infected B6, (age- and sex-matched), and CX3CR1GFP/+ and CX3CR1GFP/GFP animals were stained for CD3ε and CD4. Numbers indicate the percentage of CD3ε+, CD3ε+CD4+, CD4+, and CD3ε+CD4− cells within the IL-22+ cell population. Data from an individual representative mouse per group (of five to seven individual mice analyzed per group) are shown. (d) cLP IL22+ CD3ε−CD4+ cells were (intracellular) stained for related orphan receptor gamma-t (RORγt) or surface stained for CD25, CD127, and CD117, and analyzed by multi-color flow cytometry. Opened squares represent the respective negative controls. Seven mice were analyzed and the data from a representative individual mouse are presented. Numbers represent the percentage of cells that are positive for the indicated antigen. (e) Mean (±s.e.m.) percentage of IL-22+ cells isolated from the cLP of *Citrobacter rodentium*-infected B6, (age- and sex-matched) and CX3CR1GFP/+ and CX3CR1GFP/GFP animals is shown. In the nonparametric Student's t-test, P<0.05 was considered statistically significant (F<0.05). (f) Mean (±s.e.m.) percentage of CD3ε−CD4+ lymphoid-tissue inducer cells within the IL-22+ cell population. Data from an individual representative mouse per group (of five to seven individual mice analyzed per group) are shown. (g) Total RNA isolated from the colonic tissues of noninfected and *Citrobacter rodentium*-infected B6, (age- and sex-matched), and CX3CR1GFP/+ and CX3CR1GFP/GFP animals were reverse transcribed to cDNA and RegIIIγ expression was analyzed by qRT-PCR. The number of animals per group of each experiment is given within the figure. In the nonparametric Student's t-test, P<0.05 was considered statistically significant.
CX3CR1+ macrophages/DC at peak infection. CX3CR1 seems to somewhat increase the uptake of C. rodentium (4.8 vs. 1.6%). Hence, C. rodentium is phagocytosed by CX3CR1+ cells at peak infection.

**Absence of CX3CR1 is associated with reduced IL-22 expression**

As IL-22 has an important role for the clearance of the C. rodentium infection,29 we screened B6 and heterozygous and homozygous CX3CR1-GFP animals for IL-22 expression. IL-22 mRNA expression was first determined by quantitative real-time PCR. IL-22 mRNA expression levels peaked at early infection (day 8 p.i.) and declined in the further course of the infection. Previous reports have already indicated that highest IL-22 expression can be found at early infection.29,30 Infected CX3CR1<sup>GFP</sup> animals produced significantly less IL-22 mRNA as compared with littermate controls (Figure 3a). IL-22 expression was >4-fold increased in C. rodentium-infected B6 mice at peak infection compared with noninfected controls. Significant differences between noninfected B6 and (age- and sex-matched) heterozygous and homozygous CX3CR1-GFP animals were not observed (Figure 3b). In contrast, C. rodentium-infected
CX$_3$CR1$^\text{GFP/GFP}$ produced less IL-22 as compared with B6 animals (Figure 3b and e). At peak infection, differences in IL-10 production were not observed between heterozygous and homozygous CX$_3$CR1-GFP animals (data not shown), but IL-1$\beta$, IL12p40, and not IL-12p19 transcripts were reduced in these animals as compared with the B6 control mice (Supplementary Figure S5 online). Our results show that normal CX$_3$CR1 expression is required for optimal colonic

**Figure 4**   Recombination-activating gene (RAG$^{-/-}$) mice lacking CX$_3$CR1 develop a rapid *Citrobacter rodentium* infection.  
(a) RAG$^{-/-}$×CX$_3$CR1$^\text{GFP/GFP}$ and RAG$^{-/-}$ mice were infected with $2 \times 10^9$ *C. rodentium*. Mean ± s.e.m. loss of body weight (%) per group is shown for the indicated animals. *P*-values were calculated with a nonparametric Student's *t*-test; *P* < 0.05 was considered statistically significant. (b) Survival of RAG$^{-/-}$×CX$_3$CR1$^\text{GFP/GFP}$ and RAG$^{-/-}$ infected with $2 \times 10^9$ *C. rodentium* is shown. (c) Colons were removed at the end of the experiment and representative colons of the indicated groups are shown. (d) The colon weight and length was determined and expressed as colon weight/length ratios. In the nonparametric Student's *t*-test, *P* < 0.05 was considered statistically significant. (e) Interleukin (IL)-22 expression in the proximal colon of infected RAG$^{-/-}$ and RAG$^{-/-}$×CX$_3$CR1$^\text{GFP/GFP}$ animals was analyzed by quantitative real-time PCR. $\beta$-actin was used as housekeeping gene to normalize cDNA input between samples. Normalized ct-values of the noninfected samples (baseline) are set to 1 and values are plotted as fold expression of the baseline. (f) IL-22 expression by colonic lamina propria (cLP) cell isolates from *C. rodentium*-infected RAG$^{-/-}$, (age- and sex-matched) and RAG$^{-/-}$×CX$_3$CR1$^\text{GFP/GFP}$ animals was analyzed by multicolor flow cytometry. Numbers indicate the percentage of IL-22-positive cells. (g) Mean (±s.e.m.) percentage of IL-22$^+$ cells isolated from the cLP of *C. rodentium*-infected B6, (age- and sex-matched), and CX$_3$CR1$^\text{GFP/GFP}$ and CX$_3$CR1$^\text{GFP/GFP}$ animals is shown. In the nonparametric Student's *t*-test, *P* < 0.05 was considered statistically significant (* *P* < 0.05).
IL-22 production in infected animals (Figure 3). Several cell population in the colon produce IL-22, including Th17, γδ T cells, ILC17, ILC22, and LTI cells as major producers. We carried out multi-color flow cytometry to investigate whether there was a specific cell population whose IL-22 production was particularly affected in mice with reduced CX3CR1 expression. For this, cLP cell isolates from noninfected and C. rodentium-infected B6, heterozygous, and homozygous CX3CR1-GFP animals were stained for CD45, CD3e, and CD4 (Figure 3c). In B6 mice, the population of CD4+CD3−IL-22+ cells increased from about 6% to over 20% upon infection with C. rodentium (Figure 3c,f). This population was significantly reduced in infected CX3CR1-GFP/GFP mice compared with B6 animals (Figure 3e,f). The CD4−CD3−IL-22+ cells could be CD4−NKp46+RORγt and CD4−NKp46−RORγt ILCs. Further analysis of the CD4+CD3−IL-22+ population revealed that these cells are characterized by RORγt, CD25, CD127, and CD117 expression, indicating that CD4+CD3−IL-22-producing ILCs are LTi cells (Figure 3d). The reduced IL-22 expression in C. rodentium-infected CX3CR1-GFP/GFP animals is associated with reduced RegIIIγ and RegIIβ expression in the colon of infected CX3CR1-GFP/GFP mice (Figure 3g,h), two secreted lectins involved in defence to pathogens in the intestinal tract. Our data indicated that reduced CX3CR1 levels result in impaired IL-22 expression, specifically by CD4+CD3−LTi cells in the colon of C. rodentium-infected animals, to a similar extent in CX3CR1 heterozygous and knock-out mice. IL-22 has been shown to be critical for clearance of C. rodentium. Thus, the reduced expression of IL-22 by colonic LTi in infected mice with reduced CX3CR1 described herein is a likely explanation of impaired C. rodentium clearance in these mice (Figure 1).

Accelerated lethality in RAG−/− mice lacking CX3CR1

To investigate the specific effect of LTi cells without the contribution of γδ lymphocyte-produced IL-22, we next analyzed C. rodentium infections in RAG−/− and RAG−/−×CX3CR1-GFP/GFP animals. We measured the body weight and collected fecal samples to determine the viable counts of C. rodentium. The experiment was ended when > 15% body weight loss occurred or when infected animals displayed serious signs of colitis such as rectal prolapses. Unlike wt B6 animals, RAG−/− animals did not clear the infection, confirming previous reports, and had to be killed by day 25 of infection (Figure 4a,b). The absence of CX3CR1 in RAG−/−×CX3CR1-GFP/GFP animals resulted in an accelerated body weight loss and lethality as compared with RAG−/− animals (Figure 4a,b). Also, C. rodentium infection in RAG−/−×CX3CR1-GFP/GFP is associated with increased macroscopic signs of C. rodentium-induced colitis (Figure 4c) and increased colon weight/colon length ratios (Figure 4d). Moreover, C. rodentium infection in RAG−/−×CX3CR1-GFP/GFP resulted in a strong reduction of IL-22-producing cells in the cLP. Reduced IL-22 mRNA expression was most evident at early infection (day 4 p. i.; Figure 4e). In addition, IL-22-expressing cells were reduced in RAG−/−×CX3CR1-GFP/GFP animals (Figure 4f and g). The CX3CR1-dependent IL-22 production by LTi cells promotes innate immunity to enteric pathogens in the large intestine.

Depletion of CX3CR1+ CD11c+ cells result in severe pathology

As CX3CR1 is expressed by macrophages/DCs and NK cells (Supplementary Figure S3A online), we crossed CX3CR1-GFP animals with CD11c.DOG animals to further examine the role of CX3CR1+CD11c+ cells for the induction of IL-22 production by innate lymphocytes without the possible contribution of CX3CR1+ NK cells. Efficient depletion of CD11c+ cells can be achieved by diphtheria toxin injection over prolonged times. CX3CR1+CD11c+ phagocytes can be depleted for more than 14 days as indicated by flow cytometry analysis of isolates obtained from spleen, MLN, and cLP (Figure 5a). Fluorescence microscopy confirmed the efficient depletion of CX3CR1+CD11c+ in CX3CR1-GFP×CD11c.DOG animals (Figure 5b). We infected CD11c.DOG, CD11c.DOG×CX3CR1-GFP/+ and CD11c.DOG×CX3CR1-GFP/GFP animals with C. rodentium. In infected CD11c.DOG, CD11c.DOG×CX3CR1-GFP/+ and CD11c.DOG×CX3CR1-GFP/GFP animals, CD11c+ cells were depleted by diphtheria toxin injection. The depletion of CX3CR1+CD11c+ cells resulted in an accelerated body weight loss (Figure 5c), increased lethality (Figure 5d), and increased C. rodentium load in MLN, spleen, and liver (Figure 5h). Significant differences between depleted CD11c.DOG, CD11c.DOG×CX3CR1-GFP/+ and CD11c.DOG×CX3CR1-GFP/GFP animals were not observed (Figure 5c, d and h). Further analysis revealed that IL-22 expression is reduced after the depletion of CX3CR1+CD11c+ phagocytes (Figure 5e). Multi-color flow cytometry demonstrated that the CD4+CD3−IL-22+ innate cell population is reduced after the depletion of CX3CR1+CD11c+ phagocytes (Figure 5e–g). CX3CR1+CD11c+ DCs and macrophages hence seem to facilitate IL-22 production by ILCs during a C. rodentium infection.

DISCUSSION

In this study, we identified a role of CX3CR1+ phagocytes in clearance of a C. rodentium infection. Immunophenotyping by flow cytometry of CX3CR1+CD11c+ cells demonstrated that CX3CR1 is expressed by F4/80(high)CD11c+ and F4/80(low)CD11c− cells in the cLP. CX3CR1+CD11c+ cells encounter C. rodentium and support IL-22 production of ILCs in the cLP. In part, fractalkine/CX3CL1 facilitates IL-22 production of ILCs by regulating IL-1β and IL-23p19 expression in C. rodentium–infected animals. Uptake of C. rodentium by CX3CR1+ cells may serve as a defence mechanism to enteric pathogen and initiate innate immune responses by supporting IL-22 production by ILCs (Supplementary Figure S6 online).

We have not carried out our experiments with C. rodentium mutants lacking the structural components of a type III secretion system. Hence, we cannot rule out that C. rodentium actively supports its uptake by CX3CR1+ cells. Experiments with fluorescent noninvasive commensal E. coli strain have demonstrated...
that luminal bacteria are directly phagocytosed by CX3CR1+ macrophages residing beneath the epithelium in the cLP (data not shown). CX3CR1+ macrophages and DCs may hence serve as first sentinels after infection of the host with enteric pathogens and initiate rapid innate immune responses. Reduced IL-1β but not IL-23p19 transcripts were observed in infected CX3CR1GFP/GFP animals in absence of CX3CR1. We speculate that phagocytosed *C. rodentium* provide signals to CX3CR1+ phagocytes that help to facilitate IL-22 expression by ILCs. Reduced anti-*C. rodentium* IgG titers in CX3CR1GFP/GFP animals indicated that CX3CR1 (beside its effects on IL-22 expression) may also be involved in regulating adaptive immune responses required for the clearance of *C. rodentium*. Polymorphism of the CX3CR1 gene has been described to be associated with ileal Crohn’s disease. Our work indicates that the CX3CR1–IL-22 axis could regulate REG IIIγ and REG IIIβ expression.
In addition, IL-22 has epithelial regenerative properties, increasing intestinal epithelial cell proliferation and wound healing, which also has a role in the protection of the host. Malfunction of the CX3CR1–IL-22 axis may thus contribute to the development of Crohn’s disease.

The stimulation of isolated CX3CR1+ small intestine LP and cLP macrophages have dose-dependent effects on secretion of cytokines by macrophages. Low concentration of CX3CL1 decreased the production of TNF-α by lipopolysaccharide-stimulated macrophages. High CX3CL1 concentrations facilitate the production of pro-inflammatory cytokines, such as IL-23 and TNFα, in a peroxisome proliferator-activated receptor-γ dependent mechanism. In light of these observations, reduced IL-10 production by CX3CR1+ macrophages was observed in the steady state and tolerogenic conditions. Bacterial peritonitis and/or infection of Clostridium difficile toxin A is associated with exacerbated disease in CX3CR1−/− animals. In part, the increased susceptibility of CX3CR1−/− mice is mediated by impaired regulation of the heme oxygenase-1, TNF-α, IL-6, and inducible nitric oxide synthase production. In spite of those findings, CX3CR1 may provide environmental signals to macrophages that regulate their adaptation to microenvironmental cues in the intestine.

As CX3CR1 is expressed by DCs/macrophages and NK cells, we crossbred CX3CR1-GFP animals with CD11c.DOG animals to further examine the role of CX3CR1+ CD11c+ cells for the induction of IL-22 production by innate lymphocytes without the possible contribution of CX3CR1+ NK cells. The depletion of CD11c+ macrophages and DCs resulted in increased susceptibility of transgenic animals to the C. rodentium infection. The depletion of CD11c+CX3CR1+ macrophages was associated with reduced IL-22 production and reduced numbers of CD3−CD4− RORγt+ LTi cells. The CD11c+ cell depletion gives similar results in CD11c.DOG and CD11c.DOG×CX3CR1GFP/GFP animals (that lack CX3CR1). The lack of CX3CR1 did not result to an additive effect after depletion of CD11c+ cells in infected animals. Thus, we would suggest a model in which CX3CR1+ macrophages in the gut supports IL-22 production by ILCs in infected animals required for the clearance of C. rodentium.

**METHODS**

**Mice.** Littermate C57BL/6j (B6) mice, CX3CR1-GFP (B6.129P-Cx3cr1tm1Litt/j), CD11c.DOG, RAG−/− (Ragtm1Mom), and RAG−/− × CX3CR1−/− were bred and kept under specific pathogen-free conditions in the animal facility of the Ulm University (Ulm, Germany). CX3CR1-GFP animals were crossed with CD11c.DOG mice to obtain CX3CR1−/−CD11c.DOG animals. Female and male mice were used at 6–12 weeks of age. All animal experiments were performed with groups of age- and sex-matched animals, and carried out according to the guidelines of the local Animal Use and Care Committee and the National Animal Welfare Law.

**C. rodentium infection.** The strain C. rodentium ICC169 used in this study is a spontaneous nalidixic-acid-resistant mutant of the wt C. rodentium ICC168, which shows the same infectivity as the wt. RF-C. rodentium was generated by integrating the plasmid p165 PT5mRuby into a 16S locus of the bacterial chromosome by homologous recombination (Supplementary Figure S3 online). To construct p165-mRuby, the vector p165Lux was cut with PstI to excise the luxABCDE genes and the P_hsp6 promoter. The fragment encoding mRuby, including the upstream T5 promoter, was amplified from the plasmid pQE-32_mRuby using KOD Hot Start DNA polymerase (Merck, Nottingham, UK) and primers PT5mRuby_Pst1_fwd (5′-AACAGAGGGCCCTTTCTGTTACCC-3′) and PT5mRuby_rev (5′-GCTCGTATTAGTGCGTCC-3′). The PCR product was cut with PstI and ligated to the p165 plasmid to yield p165_mRuby. To generate RF-C. rodentium, the vector p165_mRuby was transformed into C. rodentium ICC169 by electroporation, using standard protocols. Transformants were selected by plating cells on Luria–Bertani (LB) agar containing 300 µg/ml erythromycin, and resistant clones were tested for presence of p165_mRuby by mini-prep and restriction analysis. Positive clones were incubated aerobically in LB broth containing erythromycin at 30 °C overnight, diluted 1:1,000 into fresh medium containing erythromycin, and incubated –6 h at 30 °C. Thereafter, bacteria were incubated overnight at the nonpermissive temperature (42 °C). Dilutions were plated on LB agar containing erythromycin and incubated at 42 °C. Under these conditions, p165_mRuby is unable to replicate due to the absence of the RF-plasmid.
to the thermosensitive origin of replication, and thus is forced to integrate into a 16S locus of the bacterial chromosome by homologous recombination in the presence of selective concentrations of erythromycin. Erythromycin-resistant colonies were checked for red fluorescence, and the integration of p165_PTSmRuby was confirmed by PCR. *C. rodentium* was prepared by culturing bacteria aerobically overnight in LB broth containing nalidixic acid (50 μg ml⁻¹) at 37 °C and centrifuged at 3,000 g for 10 min as previously reported.²³ RF-*C. rodentium* displayed identical infectivity as the parental wt strain *C. rodentium* ICC169, both in terms of bacteria recovered from the feces at all timepoints of infection and the clinical symptoms (data not shown). Pelleted bacteria were washed and resuspended in phosphate-buffered saline (PBS). Mice (6–12 weeks) were inoculated orally with 2×10⁵ colony-forming units of either RF-*C. rodentium* ICC169 (wt) or RF-*C. rodentium*. The weight of infected mice and their clinical condition were monitored every second day. Tissue samples were frozen in liquid nitrogen, cryosections were used and fixed in acetone at 4 °C for 30 min, mounted on slides, and stained with hematoxylin and eosin. Histology of the large intestine was categorized as for the severity of epithelial injury (graded 0–3, from absent to mild including superficial epithelial injury, moderate including focal erosions, and severe including multifocal erosions), the extent of inflammatory cell infiltrate (graded 0–3, from absent to transmural), and goblet cell depletion (0–3) as previously published.⁴¹ In addition, the colon length and weight were determined, and colon length/weight ratios were calculated.

### Isolation of CX³CR¹⁺ cells or of ILCs from the cLP

Segments of the colon were washed with PBS to remove debris and mucous. The epithelium was removed by incubation at 37 °C for 30 min under gentle shaking with 1 mM dithiothreitol and 1 mM EDTA in Ca²⁺/Mg²⁺−free PBS supplemented with 1% fetal calf serum. The remaining tissue was washed in PBS to remove residual epithelial cells, and the supernatants were discarded. Denuded tissues were cut into two to three 2-mm pieces and digested with 0.5 mg ml⁻¹ collagenase type VIII (cat. no. C-2139; Sigma-Aldrich, St Louis, MO) and 5 U ml⁻¹ DNase (cat. no. 1284932; Roche, Basel, Switzerland) for 2 h at 37 °C in RPMI 1640/5% fetal calf serum. Supernatants were collected from which LP lymphocytes were pelleted. LP lymphocytes were resuspended in RPMI 1640 medium containing 40% Percoll (density 1.124 g ml⁻¹; cat. no. L-6145; Biochrome, Berlin, Germany). This cell suspension was overlaid onto 70% Percoll and centrifuged for 20 min at 750 g. Viable cells at the 40%/70% interface were collected and washed twice.

### Flow cytometry analyses

Cells were washed twice in PBS/0.3% w/v bovine serum albumin supplemented with 0.1% w/v sodium azide. Nonspecific binding of antibodies to Fc receptors was blocked by pre-incubation of cells with monoclonal antibody (mAb) 2.4G2 (cat. no. 01241D; BD Biosciences) directed against the FcγRII/III CD16/CD32 (1 μg/ml mAb per 10⁶ cells). Cells were washed and incubated with 0.5 μg per 10⁶ cells of the relevant mAb for 20 min at 4 °C. Four-color flow cytometry (FCM) analyses were performed using a FACSCalibur (BD Biosciences). The forward narrow-angle light scatter was used as an additional parameter to facilitate the exclusion of dead cells and aggregated cell clumps. Data were analyzed using FCS Express V3 software (De Novo Software, Los Angeles, CA).

### Monoclonal antibodies

The following reagents and mAbs from eBioscience (Frankfurt, Germany) were used: antigen-presenting-cell-conjugated mAb binding CD11c N418 (cat. no. 17-0114-82), anti-CD3 145-2C11 (cat. no. 553060), anti-CD25 PL61.5 (cat. no. 13-0251-81), and phycoerythrin-conjugated mAb-binding CD117 (c-Kit) B8B (cat. no. 12-1171-81). From BD Biosciences, the following biotinylated mAbs were used: anti–CD103 M290 (cat. no. 557493) and anti-CD3 145-2C11 (cat. no. 553060).

### Intracellular cytokine staining

Cells (1×10⁶ per ml) from MLN or cLP were stimulated for 4 h at 37 °C with 50 ng ml⁻¹ PMA (cat. no. 79346; Sigma-Aldrich, St Louis, MO) and 500 ng ml⁻¹ Ionomycin (cat. no. 10634; Sigma-Aldrich) in the presence of 10 μg ml⁻¹ Brefeldin A (cat. no. ALX-350-019-M025; Alexis Biochemicals, Lorrach, Germany). Cells were harvested, washed, and stained with anti-CD3 145-2C11 (cat. no. 553060); eBioscience). Surface-stained cells were fixed (4% paraformaldehyde in PBS) and resuspended in permeabilization buffer (PBS, 0.5% bovine serum albumin, 0.5% saponin, 0.05% sodium azide). Permeabilized cells were incubated for 30 min at room temperature, dark with 0.25 μg/ml per 10⁶ cells of the following antibodies: phycoerythrin-conjugated anti-IL-10 JES5-16E3 (cat. no. 12-7101-81; eBioscience), anti-ROTYt AFKJS-9 (cat. no. 12-6988-82; eBioscience), anti-IL-22 (cat. no. IC582P; R&D, Wiesbaden, Germany), anti-IL-12/IL-23p40 C17.8 (cat. no. 12-7123-81); antigen-presenting cell-conjugated anti-IL-22 (cat. no. IL582A; R&D). Stained cells were washed twice in permeabilization buffer and resuspended in PBS supplemented with 0.3% w/v bovine serum albumin and 0.1% w/v sodium azide. The number of cytokine-expressing innate cells was determined by FCM.

### Cytokine detection by quantitative real-time PCR

RNA was prepared from frozen colon tissue using the RNAeasy mini kit (cat. no. 794904; Qiagen, Hilden, Germany). Contaminating genomic DNA was eliminated from samples by treatment with RNase-free DNase I (cat. no. 1010395; Qiagen). A total of 2 μg of RNA isolated from tissue or 200 ng of RNA isolated from tissues was reverse transcribed with SuperScript II Reverse Transcriptase (cat. no. 18064-014; Invitrogen, Paisley, Scotland) using random primers (cat. no. 48190-011; Invitrogen) according to the manufacturer's instructions. SYBR Green qPCR Master mix (cat. no. PA-012-12; SABiosciences, Valencia, CA) was used for amplification and detection. Real-time PCR reactions were performed using the 7500 Fast Real-Time PCR System (Applied Biosystems, Darmstadt, Germany) and the following conditions: 50 °C for 2 min, repeat 1; 95 °C for 10 min, repeat 1; 95 °C
for 15 s, 60 °C for 1 min, repeats 40; 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, 60 °C for 1 min. Repeat 1. β-actin PCR signals were used to equalize cDNA amounts between preparations. Following primers were used: β-actin (cat. no. PPM02945A; SABiosciences); REG IIIγ (cat. no. QT00147455; Qiagen); REG IIIβ (cat. no. QT00239302; Qiagen); Cx3cl1 (cat. no. PPM0967B; SABiosciences); IL-1β (forward) 5′-AGA GCA GTA G-3′; IL-22 (cat. no. MMP5481A; Qiagen); IL-12p40 (forward) 5′-ACT TGA TCA ACA AGA GGA CAT AAC ACA CAA A-3′; IL-12p19 (IL23a) (cat. no. QT01663613; Qiagen). Expected product length: 154 bp for β-actin, 93 bp for REG IIIγ, 141 bp for REG IIIβ, 113 bp for Cx3cl1, 28 bp for IL-12p40, 80 bp IL-12p19, 99 bp for IL-22, and 26 bp for IL-1β. β-actin PCR signals were used to equalize cDNA amounts between preparations.

Statistics. A one-way ANOVA test (nonparametric data) and a t-test for two unequal variances were used. P < 0.05 was considered statistically significant.

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

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DISCLOSURE

The authors declared no conflict of interest.

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