Original Paper

IL10-Deficiency in CD4⁺ T Cells Exacerbates the IFN γ and IL17 Response During Bacteria Induced Colitis

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Key Words

Citrobacter rodentium • Interleukin 10 • T helper cell 17 • Colitis

Abstract

Background/Aims: IL10 is a key inhibitor of effector T cell activation and a mediator of intestinal homeostasis. In addition, IL10 has emerged as a key immunoregulator during infection with various pathogens, ameliorating the excessive T-cell responses that are responsible for much of the immunopathology associated with the infection. Because IL10 plays an important role in both intestinal homeostasis and infection, we studied the function of IL10 in infection-associated intestinal inflammation. **Methods:** Wildtype mice and mice deficient in CD4⁺ T cell-derived or regulatory T cells-derived IL10 were infected with the enteric pathogen *Citrobacter (C.) rodentium* and analyzed for the specific immune response and pathogly in the colon. **Results:** We found that IL10 expression is upregulated in colonic tissue after infection with *C. rodentium*, especially in CD4⁺ T cells, macrophages and dendritic cells. Whereas the deletion of IL10 in regulatory T cells had no effect on *C. rodentium* induced colitis, infection of mice deficient in CD4⁺ T cell-derived IL10 exhibited faster clearance of the bacterial burden but worse colitis, crypt hyperplasia, and pathology than did WT mice. In addition, the depletion of CD4⁺ T cell-derived IL10 in infected animals was accompanied by an accelerated IFN γ and IL17 response in the colon. **Conclusion:** Thus, we conclude that CD4⁺ T cell-derived IL10 is strongly involved in the control of *C. rodentium*-induced colitis. Interference with this network could have implications for the treatment of infection-associated intestinal inflammation.

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Introduction

Inflammatory bowel diseases (IBDs) are chronic remitting or progressive inflammatory disorders of the gastrointestinal tract that affect several million persons worldwide. Although

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the etiology of these diseases is not fully understood, accumulating evidence indicates that the chronic inflammatory responses exhibited by IBD patients occur in genetically susceptible persons after abnormal exposure of the mucosal immune system to enteric bacteria [1, 2]. However, both the virulence mechanisms by which bacterial pathogens colonize the intestinal tract of their hosts and the specific host responses that defend against such infections are poorly understood. Therefore, models of infectious colitis are crucial for defining the potentially pathogenic host responses to enteric bacteria. Infection with *Citrobacter (C.) rodentium*, a noninvasive mouse pathogen that belongs to the group of attaching and effacing bacteria, is one of the few available models of infectious colitis [3, 4]. In addition to producing the typical signs of bacterially induced inflammation, including crypt hyperplasia and thickening of the mucosa, infection with *C. rodentium* causes robust TH1 and TH17 responses in C57BL/6 mice [5-8]. Cells of the adaptive immune system, such as CD4⁺ T cells and B cells, contribute to protection against *C. rodentium* infection and mice depleted of either cell type have an impaired ability to clear the infection [9-12]. Therefore, the immune response is crucially involved in the clearance of *C. rodentium* and in limiting inflammatory damage, but little is known about the mechanisms or molecules involved.

One of the key molecules participating in immunomodulation and regulation of intestinal homeostasis is IL10. The association between IL10 and intestinal homeostasis became clear with the discovery that intestinal inflammation develops spontaneously in mice deficient in IL10 and IL10 receptor (IL10R) with age [13, 14]. Similarly, severe enterocolitis develops within the first months of life in infants with deleterious mutations in IL10, IL10RA, or IL10RB [15]. However, although the therapeutic potential of IL10 has been investigated in animal models and in IBD patients [16, 17], its administration has not been associated with a beneficial response during inflammation [18]. In addition to its fundamental function during intestinal homeostasis, IL10 also plays a crucial role during infection. IL10 regulates the immune responses induced by various pathogens and their products, thereby preventing damage to host tissues [13]. However, with some infections IL10 impedes the ability of the host immune response to eliminate the pathogen, thereby contributing to chronic infection [19-21]. In contrast the ablation of IL10 signaling during infection increases pathogen clearance but is accompanied by an exaggerated immunopathology [21-23]. These effects demonstrate the dichotomy of IL10 activity during infection.

Because IL10 plays a substantial role in intestinal homeostasis and infection, the aim of the present study was to elucidate the relative importance of IL10 in the control of CD4⁺ T cell responses during *C. rodentium*-associated colitis. Understanding the mechanisms that contribute to the resolution of bacterially induced inflammation would support the design of new strategies for immune intervention.

Materials and Methods

Mice

All animals used in this study were 8- to 12-week-old, bred and housed under specific pathogen-free conditions in the Laboratory Animal Facility of the University Hospital Essen. WT BALB/c mice were obtained from Harlan Winkelmann GmbH (Borchen, Germany) or bred in house. IL-10^{flox/flox} mice, CD4^{Cre} (BALB/c), and FIC mice (expressing Cre under the control of the forkhead box P3 [Foxp3] promoter) were established as described [24, 25]. Crossing CD4-Cre or FIC-Cre mice with IL-10^{flox/flox} mice resulted in mice specifically deficient in IL-10 in either CD4⁺ cells (termed CD4-IL10ko mice) or CD4⁺FoxP3⁺ T cells (termed FoxP3-IL10ko mice). IL-10/eGFP knock-in tiger mice (termed *IL10*-GFP mice), which express both IL-10 and GFP under the endogenous regulatory sequence of the IL-10 locus, were generated as described [26]. All mice used were generated on the BALB/c background. CD4-IL10ko mice, FoxP3-IL10ko mice, and *IL10*-GFP mice were subjected to the *C. rodentium* infection protocol.

All animal experiments were performed in strict accordance with the guidelines of the German Animal Protection Law and were approved by the state authorities for Ethics in Animal Experiments of North-Rhine Westphalia, Germany.

C. rodentium infection model

The nalidixic acid-resistant *C. rodentium* strain ICC169 used in this study shows the same infectivity as the WT strain ICC168 [27]. *C. rodentium* were cultured overnight in Luria-Bertani at 37°C, centrifuged at 3000×g for 10 min, and washed with PBS. Mice were infected by oral gavage with 200 µl PBS containing approximately 2×10⁹ CFUs of *C. rodentium*. After gavage, an aliquot of the remaining suspension was plated in serial dilutions on MacConkey agar to control the infective dose. Bacterial numbers in stool were determined by collecting fecal pellets at various time points after infection, weighing them, and homogenizing them in 1 ml of PBS. Serial dilutions of the homogenates were plated on MacConkey agar, and the numbers of CFUs were determined after overnight incubation at 37°C. Mice were analyzed at various time points after infection (p.i.), and the spleens, mesenteric lymph nodes (MLNs), and colon were removed and prepared for analysis as described below. Spleen weight was determined after the spleen was removed.

Isolation of splenocytes and mesenteric lymph node cells

Spleens were rinsed with an erythrocyte lysis buffer (containing 0.15 M NH₄Cl, 10 mM KHCO₃, and 0.5 M EDTA), meshed through a 100-µ cell strainer, and washed with PBS containing 2 mM EDTA and 2% fetal calf serum. Mesenteric lymph nodes were meshed through a 100-µ cell strainer and washed with PBS containing 2 mM EDTA and 2% FCS.

Isolation of lamina propria lymphocytes from the colon

Lamina propria (LP) lymphocytes were isolated as described previously [28], with minor modifications. Colons were flushed with PBS to remove feces, opened longitudinally, and cut into 1-cm pieces. Tissue pieces were washed twice in PBS containing 3 mM EDTA for 10 min at 37°C with rotation. EDTA was removed by washing colon pieces twice in Roswell Park Memorial Institute (RPMI) medium containing 1% FCS, 1 mM EGTA, and 1.5 mM MgCl₂ for 15 min at 37°C with rotation. Colon pieces were then subjected to intense vortexing, washed with PBS, and digested in RPMI containing 20% FCS and 100 U/mL collagenase (*Clostridium histolyticum*; Sigma-Aldrich, St. Louis, MO) for 90 min at 37°C. Remaining tissue was separated from cells by passing the cell suspension through a 40-µ cell strainer and washing it with culture medium.

Macroscopic and histopathologic assessment of colitis

Macroscopic colonic damage was assessed on the day of euthanasia. Assessment was based on two main characteristics of the pathologic state: colon length shortening and colon weight gain. We then determined the colon weight-to-length ratio, which is considered to be a reliable and sensitive indicator of the severity and extent of the inflammatory response in colitis [29].

Colons were prepared as Swiss rolls, and stored in 4% paraformaldehyde until the tissue was embedded in paraffin for histologic scoring. Tissue sections (4 µm) were prepared from paraffin-embedded tissue blocks, H&E stained and evaluated histopathologically in a blinded manner. The colon was divided into 3 equal portions (oral, middle, and rectal) and assessed for inflammatory cell infiltrates, epithelial damage, goblet cell depletion, neutrophil infiltration, crypt abscesses, and crypt hyperplasia, each in a 0 to 3 scoring system (0=no change; 1=mild change; 2=moderate change; 3=profound change) (Klopfleisch, 2013). All evaluated parameters together gave rise to an overall inflammatory score (0-54) for oral (0-18), middle (0-18), and rectal (0-18) colon regions. Crypt heights were measured by micrometry; 30 measurements were taken in the distal colon for each mouse. Only well-oriented crypts were measured.

Antibodies and flow cytometry

Splenocytes, MLN cells, and LP lymphocytes were stained with fluorochrome-labeled anti-mouse CD4 (RM4-5), CD8 (53-6.7), CD11c (HL3), CD45 (30-F11), CD25 (7D4), F4/80 (BM8), I-A/I-E (M5/114.15.2), CD19 (1D3), B220 (RA3-6B2) and CD69 (H1.2F3) antibodies. All antibodies used in this study were obtained from either BD Biosciences (Heidelberg, Germany) or eBioscience (Frankfurt, Germany). For analysis of intracellular cytokines, lymphocytes (0.5×10⁶ cells per well) isolated from spleens, MLNs, and LPLs were stimulated for 4-h with 10 ng/mL PMA and 1 µg/mL ionomycin in the presence of 5 µg/mL Brefeldin A (all from Sigma-Aldrich) or 5 µg/mL Monensin (e-bioscience). After staining cell surface antigens, the cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% NP-40, and stained for intracellular cytokines (anti-IL-17A [TC11-18H10.1], anti-IFN-γ [XMG1.2], anti-IL10 [JES5-16E3] and anti-IL22 [Poly5164] antibodies). Where indicated, splenocytes and MLN cells were stimulated for 3 days with 1 µg/ml anti-CD3

(145-2C11) antibody before intracellular cytokine staining. Cells were analyzed by flow cytometry on an LSR II instrument using DIVA software (BD Biosciences).

Cytokine detection

Spleen and MLN cells were isolated and stimulated with anti-CD3 antibody as described. The levels of IFN- γ and IL-17 in cell culture supernatants were quantified with the Procarta Cytokine assay kit (Panomics, Fremont, CA) according to the manufacturer's recommendations. The assay was performed on a Luminex 200 instrument, and levels of cytokines were calculated with the Luminex IS software (Luminex Corporation, Austin, TX).

Quantitative reverse transcriptase PCR

RNA was obtained from distal colon biopsy samples with the RNeasy Fibrous Tissue Kit (Qiagen). After DNase digestion (Qiagen), cDNA was synthesized with M-MLV Reverse Transcriptase (Promega, Mannheim, Germany) and Oligo-dT mixed with Random Hexamer primers (Invitrogen, Karlsruhe, Germany) on an ABI PRISM cyclor (Applied Biosystems, Life Technologies, Darmstadt, Germany). Real-time RT-PCR was performed with the SYBR Green PCR kit and specific primers for IL-10 (5'-CTG GAC AAC ATA CTG CTA ACC GAC TC-3' and 5'-ATT TCT GGG CCA TGC TTC TCT GC-3'), IL10R α (5'-TCA TGG TGA CAT TCC AGG GC-3' and 5'-TGG AGG CCA AGC CAA ATC AT-3'), IL-17 (5'-GCT CCA GAA GGC CCT CAG ACT ACC-3' and 5'-TTC CCT CCG CAT TGA CAC AGC-3'), IFN γ (5'-AGG AAC TGG CAA AAG GAT GGT GA-3' and 5'-TGT TGC TGA TGG CCT GAT TGT CTT-3'), IL-22 (5'-CTC CCC CAG TCA GAC AGG TTC C-3' and 5'-ACA GCA GGT CCA GTT CCC CAA TC-3'), IL-1 β (5'-ACT ACA GGC TCC GAG ATG AAC AAC-3' and 5'-CCC AAG GCC ACA GGT ATT TT-3'), FoxP3 (5'-CTG GCG AAG GGC TCG GTA GTC CT-3' and 5'-CTC CCA GAG CCC ATG GCA GAA GT-3'), and ribosomal protein S9 (RPS9) (5'-CTG GAC GAG GGC AAG ATG AAG C-3' and 5'-TGA CGT TGG CGG ATG AGC ACA-3'). Relative RNA levels were determined with included standard curves for each individual gene and further normalization to the housekeeping gene *RPS9*.

TH1 and TH17 cell-polarization

CD4⁺ CD25⁻ T cells were FACS-sorted and activated *in vitro* with plate-bound α -CD3 (TH1: 5 μ g/ml, TH17: 0.75 μ g/ml) and soluble α -CD28 (TH1 and TH17: 1 μ g/ml) (both BD Bioscience). For TH1 polarization cultures contained recombinant IL12 (20ng/ml) (R&D Systems) and anti-IL4 antibody (200 ng/ml) (eBioscience). For TH17 polarization media were supplemented with recombinant TGF β (2ng/ml), IL21 (100ng/ml), IL23 (20ng/ml) (all R&D Systems, Wiesbaden, Germany), IL1 β (20ng/ml) (eBioscience), IL6 (50ng/ml) (PreproTech), α -IL4 (200ng/ml), α IFN γ (200ng/ml) and α IL2 (200ng/ml) (eBioscience) antibodies. Where indicated, recombinant IL10 (250ng/ml) (eBioscience) or anti-IL10 antibody (5 μ g/ml) (BioXCell) was added.

Statistical analysis

All results were expressed as mean $\pm$ SEM. Differences were assessed by Student's *t*-tests, One-Way ANOVA or Mann Whitney *t*-tests. Data analysis was performed with Prism 5.0 software (GraphPad, La Jolla, CA). Statistical significance was set at the level of *P* < 0.05.

Results

C. rodentium infection in BALB/c mice induces IL10 expression in colonic tissue

The appropriate temporal and spatial expression of IL10 is believed to play a key role in sustaining the balance between inflammation and immunoregulation. Especially in the gut, IL10 is described as a mediator of intestinal homeostasis, but little is known about the function of IL10 during bacterially induced colitis. To assess the expression of IL10 during the course of intestinal infection, WT BALB/c mice were infected with *C. rodentium* and analyzed at several time points post infection. Quantification of the *C. rodentium* load in fecal samples from infected mice showed detectable *C. rodentium* colonization at day 3 post-infection (p.i.), maximal burden with 10⁸ CFUs/g of feces between days 7 and 14 p.i., and bacterial clearance by day 21 p.i. (Fig. 1A). The inflammatory score in the colon peaked between days 10 and 17

Fig. 1. *Citrobacter rodentium* infection induces production of colonic IL10. BALB/c mice were infected with *C. rodentium* (CR) or given vehicle control (PBS) by orogastric gavage (A-C). At various time points post infection (days post-infection, dpi) CR burdens in fecal samples (n=9) (A) and the inflammatory score (n=3) in oral (O), middle (M), and rectal (R) colon regions (B) were determined. IL10 mRNA expression in rectal colon tissue biopsy samples (n=5) was quantified at indicated time points by real-time PCR (C). Gene expression was normalized to the expression of ribosomal protein S9 (RPS9) and is shown as fold change in induction over naïve tissues (C). FACS analysis of IL10 expression by CD4⁺ T cells (gated on CD4⁺) (D), macrophages (gated on CD45⁺MHCII⁺) (E) and dendritic cells (gated on F4/80⁺/CD11c⁺ of CD45⁺MHCII⁺) (F) in spleen, MLNs and LPLs of CR-infected or PBS-treated *IL10-GFP* reporter mice (n=7-9) (D) or BALB/c mice (n=8-11) (E-F) was performed on day 10 after infection. Data shown in the graphs are given as mean ± SEM.

after infection (Fig. 1B). Quantitative PCR analysis of colonic IL10 mRNA showed a transient response, with a significant increase (3- to 4-fold) on days 7 and 10 after infection, followed by a reduction to control levels at day 21 (Fig. 1C). Therefore, there is an association between maximal IL10 expression and maximal *C. rodentium* burden on day 10 post-infection (Fig. 1A and 1C). To define the cellular source of IL10 production during *C. rodentium* infection, we infected *IL10-GFP* reporter mice (1D) or BALB/c mice (1E-F) with *C. rodentium* and used

Fig. 2. Influence of *C. rodentium* infection on IL17, IFN γ and IL22 effector cytokine expression by CD4 $^{+}$ T cells. BALB/c mice (n=3-4/time point) were infected with *C. rodentium* (CR) or given vehelic control (PBS) by orogastric gavage (A-C). At various time points post infection (days post-infection, dpi) splenocytes, MLNs and LPLs were analyzed by fluorescence-activated cell sorting (FACS) for IL17 (A), IFN γ (B) and IL22 (C) effector cytokine expression by CD4 $^{+}$ T cells. Data shown in the graphs are given as mean $\pm$ SEM. *, $P < 0.05$; ***, $P < 0.001$.

flow cytometry to examine cells from the spleen, MLNs, and colonic lamina propria (LP) on day 10 after infection. We found that LP CD4 $^{+}$ T cells, macrophages, and dendritic cells (DCs) exhibit a significant increase in IL10 expression after *C. rodentium* infection (Fig. 1D-F). However, *C. rodentium* infection does not induce IL-10 in CD8 $^{+}$ T cells and CD19 $^{+}$ B220 $^{+}$ B cells (data not shown). To further define the inflammatory response of BALB/c mice after *C. rodentium* infection LP CD4 $^{+}$ T cells were characterized by flow cytometry for the expression of IFN γ , IL17 and TH17-associated IL22. As expected, the percentage of CD4 $^{+}$ IL17 $^{+}$, and CD4 $^{+}$ IL22 $^{+}$ -producing T cells increased on days 3 to 10 after infection and reduced to control levels at day 21 (Fig. 2A and C). However, there was no enhancement in the percentage of CD4 $^{+}$ IFN γ -producing TH1 cells (Fig. 2B) suggesting a difference in the immune response against *C. rodentium* infection in BALB/c mice and C57BL/6 mice.

Taken together, these results show that IL10 production is significantly increased in colonic tissue after *C. rodentium* infection and that this increase is correlated with effector cytokine production and disease progression.

Treg-derived IL10 is not sufficient to alter C. rodentium-induced colitis

IL10 is produced by several CD4 $^{+}$ T-cell subsets, such as regulatory T cells (Tregs) or TH2 cells. Especially in the intestine, FoxP3 $^{+}$ Tregs play a central role in the control of immune responses. Our results are consistent with this knowledge: biopsy of the distal colon showed that on day 10 after infection FoxP3 expression was significantly higher in *C. rodentium*-infected mice than in PBS-treated control mice (Fig. 3A). In addition, the expression of IL10 was enhanced in colonic Tregs in *C. rodentium*-infected mice (Fig. 3B). To determine whether

Fig. 3. Influence of Treg-derived IL10 on *C. rodentium*-induced colitis. BALB/c mice were infected with CR (n=4) or treated with PBS (n=5) by orogastric gavage. On day 10 post-infection *FoxP3* mRNA expression in rectal colon tissue biopsy samples was determined. Gene expression was normalized to the expression of RPS9 and is shown as fold change (A). FACS analysis of IL10 expression by CD4⁺CD25^{hi} T cells in spleen, MLNs and LPLs of CR-infected (n=9) or PBS-treated (n=7) *IL10-GFP* reporter mice was performed on day 10 after infection (B). *FoxP3*-IL10ko mice (n=8) or control littermates (WT, n=6) were orally infected with CR. Bacterial burdens in fecal pellets were quantified at the time points indicated (C). On day 10 post-infection spleen weight (D) and colon weight-to-length ratio (E) were measured, rectal crypt length (F) was determined by micrometry, and rectal inflammatory score (G) was determined by histologic analysis. Data shown in the graphs are given as mean ± SEM. *, *P*<0.05; ***, *P*<0.001.

regulatory T cell-derived IL10 plays a role in *C. rodentium*-induced colitis, we infected IL10^{fllox/fllox} × FoxP3-IRES-Cre (FIC) mice (referred to as FoxP3-IL10ko mice), which display a specific depletion of IL10 in FoxP3⁺ Tregs, and littermate control mice with *C. rodentium*. Surprisingly, we found no significant differences in bacterial load (Fig. 3C), spleen weight (Fig. 3D), colon weight-to-length ratio (Fig. 3E), crypt length (Fig. 3F) or inflammatory score (Fig. 3G) between the two types of mice. This finding indicates that the absence of Foxp3⁺ Treg-derived IL10 alone is not sufficient to alter *C. rodentium*-induced pathology. Therefore, IL10 production by Foxp3⁺ regulatory T cells appears to be a negligible factor in controlling *C. rodentium*-associated colitis.

CD4⁺ T cell-derived IL10 controls the immunopathology of *C. rodentium*-induced inflammation

To further elucidate the physiologic effect of CD4⁺ T cell-derived IL10 on the intestinal inflammation associated with *C. rodentium* infection and on bacterial clearance, we used mice with a specific depletion of IL10 in all CD4⁺ T cells (IL10^{fllox/fllox} × CD4-Cre, referred to as CD4-IL10ko mice). We infected these mice and WT littermate control mice with *C. rodentium* and analyzed at day 10 after infection, a time point at which bacterial burden, intestinal inflammation, and IL10 expression by CD4⁺ T cells are highest (Fig. 1). Determination of the bacterial burden in the feces during the course of infection showed that bacterial loads were similar in both types of mice on day 3 after infection. However, mice deficient in IL10-producing CD4⁺ T cells exhibited enhanced control of the infection at day 7 and significantly lower bacterial numbers at day 10 than did infected WT mice (Fig. 4A). Interestingly,

Fig. 4. Influence of CD4⁺ T cell-derived IL10 on *C. rodentium*-induced colitis. CD4-IL10ko mice or control littermates (WT) were infected with CR or given PBS by orogastric gavage. CR burdens in fecal pellets harvested from infected WT or CD4-IL10ko mice were analyzed throughout the course of infection (A). Spleen weight (B) and colon weight-to-length ratio (C) were determined on day 10 post-infection. (A-C: WT n=12, CD4-IL10ko n=12). Histologic examination of infection-induced inflammation in H&E-stained sections of oral (O), middle (M), and rectal (R) colon regions was performed on day 10 after infection (D-G). Representative H&E-stained sections from M and R colon regions of CR-infected or PBS-treated WT or CD4-IL10ko mice are shown in D. Scale bars represent 100-μm increments, and black bars indicate crypt length. Sections were semiquantitatively scored (0-3) and measured for rectal crypt length (in μm) (E) and crypt hyperplasia (F) and overall inflammatory parameters (G) were quantified in O, M, and R colon regions. (E-G: WT n=4, CD4-IL10ko n=5) Data shown in the graphs are given as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

accelerated bacterial eradication in CD4-IL10ko mice was associated with exaggerated inflammation, characterized phenotypically by significantly higher spleen weight (Fig. 4B) and significantly larger colon weight-to-length ratio (Fig. 4C) than in infected WT mice. In addition, histologic analysis of the colon showed that *C. rodentium*-infected CD4-IL10ko mice exhibited severer crypt elongation and crypt hyperplasia (Fig. 4D-F) and a higher inflammation score than did control mice (Fig. 4G). These findings suggest that the presence

Fig. 5. CD4⁺ T cell-derived IL10 controls *C. rodentium*-induced IL17 responses. CD4-IL10ko mice (n=10) or control littermates (WT, n=10) were orally infected with CR or given PBS by orogastric gavage and were analyzed on day 10 post-infection. Splenocytes and cells from MLNs were analyzed by FACS for CD69 (A) expression (gated on CD4⁺ T-cells). After *ex vivo* stimulation of isolated splenocytes and MLN cells with anti-CD3 mAb for 72 h, TH17 (B), and TH1 (C) cell responses were measured by FACS (gated on CD4⁺ T cells). Cell culture supernatants from anti-CD3 mAb-stimulated splenocytes and MLNs were recovered for measurement of IL17 (B) and IFNγ (C) levels by Luminex. Data shown in the graphs are given as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

of CD4⁺ T cell-derived IL10 is important not for the eradication of *C. rodentium* but rather for the control of infection-associated inflammation.

IL10 deficiency in CD4⁺ T cells enhances the C. rodentium-induced IL17 response and facilitates an IFNγ response in the colon

To assess the effects of IL10-producing CD4⁺ T cells on effector T cells during *C. rodentium* infection, we isolated lymphocytes from the spleen and the MLNs of *C. rodentium*-infected CD4-IL10ko mice and WT mice on day 10 after infection and used flow cytometry

Fig. 6. CD4⁺ T cell-derived IL10 controls IL17 and IFN γ production in colonic tissues *after* *C. rodentium* infection. CD4-IL10ko mice (n=6) or control littermates (WT, n=6) were orally infected with CR and analyzed on day 10 post-infection (p.i.). Biopsy samples from rectal colon tissue were analyzed by real-time PCR for *IL17* (A) *IFN γ* (B), *IL22* (C) and *IL-1 β* (D) mRNA expression. Gene expression was normalized to the *RPS9* and is shown as relative expression. Data shown in the graphs are given as mean $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

to analyze them phenotypically. The expression of the early activation marker CD69 by CD4⁺ T cells in the spleen and in MLNs was significantly upregulated in CD4-IL10ko mice but not in infected WT mice (Fig. 5A), a finding demonstrating the exaggerated activation status of these cells. Because *C. rodentium* infection of BALB/c mice is characterized by elevated TH17 responses, we determined the percentage of IL17-producing CD4⁺ T cells in spleens and MLNs from infected CD4-IL10ko mice and WT mice. We found that IL10 depletion in CD4⁺ T cells significantly increased the percentage of IL17-producing T cells and IL17 secretion after *in vitro* stimulation of lymphocytes isolated from infected CD4-IL10ko mice (Fig. 5B). Consequently, quantitative PCR analysis of colonic biopsy samples showed increased expression of IL17, IL22, and IL1 β in CD4-IL10ko mice (Fig. 6A,C and D). The percentage of IFN γ -producing CD4⁺ T cells and IFN γ -secretion was not altered in the spleens and MLN of infected mice (Fig. 5C) but significantly increased in the colon of *C. rodentium* infected CD4-IL10ko mice (Fig. 6B). These results suggest that IL10 significantly contributes to the control of *C. rodentium*-induced colitis.

IL10 does not directly alter IL17 expression in vitro

In order to understand the immunoregulatory mechanism of IL10 on TH1 and TH17 cells, we first analyzed whether TH1 and TH17 cells express the IL10 receptor (IL10R), and could therefore respond to IL10 directly. We included *in vitro* polarized TH1 and TH17 cells, naïve CD4⁺ T cells and Tregs, known to express high levels of IL10R [30]. Interestingly, the expression of IL10R was strongly downregulated on *in vitro* polarized TH1 and TH17 cells compared to naïve CD4⁺ T cells and Tregs (Fig. 7A), a finding suggesting that IL10 may not directly act on activated polarized TH1 and TH17 cells. To examine whether IL10 may influence the differentiation of TH cells we performed polarization experiments in the

Fig. 7. IL10 does not influence *in vitro* polarization of TH17 cells. CD4⁺ CD25⁻ T cells or CD4⁺ CD25^{hi} Tregs were FACS sorted from spleens of BALB/c mice. CD4⁺ CD25⁻ T cells were either left untreated (naïve) or were polarized under TH1 and TH17 cell conditions for 3 days. Relative expression of *IL10 receptor* (IL10R) was analyzed by real-time PCR. Gene expression was normalized and is shown as fold change over naïve. Data shown in the graphs are given as mean ± SEM. The results are summarized from two independent experiments. (A). Sorted CD4⁺ CD25⁻ T cells were either left untreated (naïve) or were polarized under TH17 cell conditions in the presence of recombinant IL10 (rmIL10, 250ng/ml) or a neutralizing IL10 antibody (αIL10, 10μg/ml). Relative expression of *IL17* was analyzed by real-time PCR. Gene expression was normalized and is shown as fold change over naïve. Data shown in the graphs are given as mean ± SEM. The results are summarized from two independent experiments (B). Sorted CD4⁺ CD25⁻ T cells were either left untreated (naïve)

or were polarized under TH17 cell conditions in the presence of recombinant IL10 (rmIL10, 250ng/ml) or a neutralizing IL10 antibody (αIL10, 10μg/ml). IL-17 expression was determined by intracellular FACS staining (C). One representative dot plot out of three is shown.

presence and absence of recombinant IL10 or anti-IL10 antibodies. Of note, the presence or absence of IL10 during polarization does not alter the IL17 response on mRNA level and on protein level indicating that IL10 may act indirectly on TH17 (Fig. 7B and C).

Discussion

The results of this study show that CD4⁺ T cell-derived IL10 is involved in the control of *C. rodentium*-induced colitis in BALB/c mice. IL10 expression was upregulated in CD4⁺ T cells from the colonic tissue of mice infected with *C. rodentium*. Infected mice, deficient in CD4⁺ T cell-derived IL10 exhibited faster clearance of the bacterial burden but experienced worse colitis, crypt hyperplasia, and pathology than did WT mice. Importantly, the depletion of CD4⁺ T cell-derived IL10 was accompanied by an accelerated colonic IFNγ and IL17 response in infected animals.

IL10 is produced by many cell types, including T cells, B cells, and most myeloid-lineage cells [31]. It also acts on a variety of immune cells, controlling the activity of effector T cells [32], NK cells [33], and APCs [34, 35]. IL10 is an important mediator of intestinal homeostasis [36] and has emerged as a key immunoregulator during infection with various pathogens, ameliorating the excessive T-cell responses that are responsible for much of the immunopathology associated with these infections [37]. After *C. rodentium* infection, CD4⁺ T cells, including regulatory and effector T cells, exhibit increased production of IL10, although

the relative importance of each of these T-cell types is debated and may change during the infection process. However, we found that the depletion of IL10 from FoxP3⁺ regulatory T cells did not alter bacterial eradication or affect the development of *C. rodentium*-associated colitis. In contrast, *C. rodentium* infection of mice with IL10 deficiency in all CD4⁺ T cells resulted in faster bacterial clearance but severer inflammation of the colon.

Several mechanisms that control the immune responses have been described. In *C. rodentium* infection of C57BL/6 mice, overproduction of both TH1-derived IFN γ and TH17-derived IL17 mediates the pathology [8, 38]. Importantly, we show that the infection of WT BALB/c mice with *C. rodentium* leads to an enhanced TH17 response in the colon but no alteration in the TH1 response was detectable, showing that the genetic background significantly defines the susceptibility and the immune response to infection. However, after depletion of IL10 in CD4⁺ T cells we detected a significant increase in IFN γ -, IL17-, and IL22-expression in the colon of infected CD4-IL10ko mice compared to infected WT mice. Interestingly, *in vitro* and *in vivo* studies have demonstrated that IL10 can control both TH1 and TH17 immune responses [39, 40]. In particular, IL10 inhibits the differentiation and proliferation of TH1 cells by selectively inhibiting the function of APCs [34, 35, 41]. This is well in line with our data, as we showed that polarized TH1 cells express low levels of IL10R. Huber and colleagues demonstrated that *ex vivo* freshly isolated TH17 CD4⁺ T cells express IL10R [42]; this expression makes them sensitive to IL10, and IL10 sensitivity in turn leads to the inhibition of TH17 effector functions [30]. However, in the present study we detected that *in vitro* polarized, recently activated TH17 down-regulate IL10R expression. In addition, the *in vitro* differentiation of TH17 cells seems not be influenced by IL10 as polarization of TH17 T cells in the presence of IL10 *in vitro* was not altered. These results imply that IL10 may also act indirectly on the differentiation by selectively inhibiting the function of APCs which is in accordance with a study by Gu et al. [43], showing that APCs contribute to the negative regulation of TH17 cell differentiation by IL10. However, our data do not exclude a possible further direct contribution of IL10 on T cells *in vivo* as it was described by Huber et al. [42].

The differentiation and maintenance of TH17 cells depend on a specific combination of cytokines, including TGF- β , IL-6, IL-23, and IL1 β [44-46]. In the present study we found an increase in IL1 β expression. IL1 β is a proinflammatory cytokine that is produced after pattern recognition receptors are activated by microbial products; IL1 β induces the recruitment of neutrophils and macrophages and activates the release of other cytokines important to host defense. Chung and colleagues demonstrated that IL1 β signaling drives an early TH17 differentiation [47]. In addition it was shown that balanced IL1 β activity is required for the host response to *C. rodentium* infection as IL1 β improved the bacterial clearance but also increased tissue damage [48]. These results are in accordance with those of our study as we found that increased expression of IL17 and IL22 in the colon of *C. rodentium*-infected CD4-IL10ko mice was accompanied by elevated IL1 β expression.

Taken together, this study indicates that bacterial eradication requires a coordinated response in which initial proinflammatory mechanisms clear the pathogen and are then down-regulated by IL10 before pathology occurs. Thus, both the timing and the relative amounts of proinflammatory and antiinflammatory cytokine production are crucial for safe clearance of the bacterial burden. Therapeutic strategies augmenting IL10 to reduce host injury during infection must therefore be combined with effective antimicrobial agents to ensure the safe elimination of the pathogen.

Abbreviations

CR (*Citrobacter rodentium*); dpi (days post-infection); FIC (Fox-IRES-Cre); IBD (inflammatory bowel disease); LP (lamina propria); LPL (lamina propria lymphocytes); p.i. (post-infection); RPS9 (ribosomal protein S9).

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Disclosure Statement

The authors declare no commercial or financial conflict of interest.

References

- 1 Kaser A, Zeissig S, Blumberg RS: Inflammatory bowel disease. *Annu Rev Immunol* 2010;28:573-621.
- 2 Neish AS: Microbes in gastrointestinal health and disease. *Gastroenterology* 2009;136:65-80.
- 3 Nell S, Suerbaum S, Josenhans C: The impact of the microbiota on the pathogenesis of IBD: lessons from mouse infection models. *Nat Rev Microbiol* 2010;8:564-577.
- 4 Eckmann L: Animal models of inflammatory bowel disease: lessons from enteric infections. *Ann N Y Acad Sci* 2006;1072:28-38.
- 5 Mundy R, MacDonald TT, Dougan G, Frankel G, Wiles S: *Citrobacter rodentium* of mice and man. *Cell Microbiol* 2005;7:1697-1706.
- 6 Deng W, Vallance BA, Li Y, Puente JL, Finlay BB: *Citrobacter rodentium* translocated intimin receptor (Tir) is an essential virulence factor needed for actin condensation, intestinal colonization and colonic hyperplasia in mice. *Mol Microbiol* 2003;48:95-115.
- 7 Higgins LM, Frankel G, Douce G, Dougan G, MacDonald TT: *Citrobacter rodentium* infection in mice elicits a mucosal Th1 cytokine response and lesions similar to those in murine inflammatory bowel disease. *Infect Immun* 1999;67:3031-3039.
- 8 Symonds EL, Riedel CU, O'Mahony D, Laphorne S, O'Mahony L, Shanahan F: Involvement of T helper type 17 and regulatory T cell activity in *Citrobacter rodentium* invasion and inflammatory damage. *Clin Exp Immunol* 2009;157:148-154.
- 9 Bry L, Brigl M, Brenner MB: CD4⁺-T-cell effector functions and costimulatory requirements essential for surviving mucosal infection with *Citrobacter rodentium*. *Infect Immun* 2006;74:673-681.
- 10 Maaser C, Housley MP, Iimura M, Smith JR, Vallance BA, Finlay BB, Schreiber JR, Varki NM, Kagnoff MF, Eckmann L: Clearance of *Citrobacter rodentium* requires B cells but not secretory immunoglobulin A (IgA) or IgM antibodies. *Infect Immun* 2004;72:3315-3324.
- 11 Simmons CP, Clare S, Ghaem-Maghami M, Uren TK, Rankin J, Huett A, Goldin R, Lewis DJ, MacDonald TT, Strugnell RA, Frankel G, Dougan G: Central role for B lymphocytes and CD4⁺ T cells in immunity to infection by the attaching and effacing pathogen *Citrobacter rodentium*. *Infect Immun* 2003;71:5077-5086.
- 12 Spahn TW, Ross M, von EC, Maaser C, Spieker T, Kannengiesser K, Domschke W, Kucharzik T: CD4⁺ T cells transfer resistance against *Citrobacter rodentium*-induced infectious colitis by induction of Th 1 immunity. *Scand J Immunol* 2008;67:238-244.
- 13 Moore KW, de Waal MR, Coffman RL, O'Garra A: Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 2001;19:683-765.
- 14 Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W: Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* 1993;75:263-274.
- 15 Begue B, Verdier J, Rieux-Laucat F, Goulet O, Morali A, Canioni D, Hugot JP, Daussy C, Verkarre V, Pigneur B, Fischer A, Klein C, Cerf-Bensussan N, Ruemmele FM: Defective IL10 signaling defining a subgroup of patients with inflammatory bowel disease. *Am J Gastroenterol* 2011;106:1544-1555.

- 16 Duchmann R, Schmitt E, Knolle P, Meyer zum Buschenfelde KH, Neurath M: Tolerance towards resident intestinal flora in mice is abrogated in experimental colitis and restored by treatment with interleukin-10 or antibodies to interleukin-12. *Eur J Immunol* 1996;26:934-938.
- 17 Tilg H, van MC, van den Ende A, Kaser A, van Deventer SJ, Schreiber S, Gregor M, Ludwiczek O, Rutgeerts P, Gasche C, Koningsberger JC, Abreu L, Kuhn I, Cohard M, LeBeaut A, Grint P, Weiss G: Treatment of Crohn's disease with recombinant human interleukin 10 induces the proinflammatory cytokine interferon gamma. *Gut* 2002;50:191-195.
- 18 Buruiana FE, Sola I, Alonso-Coello P: Recombinant human interleukin 10 for induction of remission in Crohn's disease. *Cochrane Database Syst Rev* 2010;CD005109.
- 19 Belkaid Y, Hoffmann KF, Mendez S, Kamhawi S, Udey MC, Wynn TA, Sacks DL: The role of interleukin (IL)-10 in the persistence of *Leishmania major* in the skin after healing and the therapeutic potential of anti-IL-10 receptor antibody for sterile cure. *J Exp Med* 2001;194:1497-1506.
- 20 Murray HW, Lu CM, Mauze S, Freeman S, Moreira AL, Kaplan G, Coffman RL: Interleukin-10 (IL-10) in experimental visceral leishmaniasis and IL-10 receptor blockade as immunotherapy. *Infect Immun* 2002;70:6284-6293.
- 21 Brooks DG, Trifilo MJ, Edelmann KH, Teyton L, McGavern DB, Oldstone MB: Interleukin-10 determines viral clearance or persistence in vivo. *Nat Med* 2006;12:1301-1309.
- 22 Ejrnaes M, Filippi CM, Martinic MM, Ling EM, Togher LM, Crotty S, von Herrath MG: Resolution of a chronic viral infection after interleukin-10 receptor blockade. *J Exp Med* 2006;203:2461-2472.
- 23 Abel S, Luckheide N, Westendorf AM, Geffers R, Roers A, Muller W, Sparwasser T, Matuschewski K, Buer J, Hansen W: Strong impact of CD4+ Foxp3+ regulatory T cells and limited effect of T cell-derived IL-10 on pathogen clearance during *Plasmodium yoelii* infection. *J Immunol* 2012;188:5467-5477.
- 24 Roers A, Siewe L, Strittmatter E, Deckert M, Schluter D, Stenzel W, Gruber AD, Krieg T, Rajewsky K, Muller W: T cell-specific inactivation of the interleukin 10 gene in mice results in enhanced T cell responses but normal innate responses to lipopolysaccharide or skin irritation. *J Exp Med* 2004;200:1289-1297.
- 25 Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S: CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 2008;322:271-275.
- 26 Kamanaka M, Kim ST, Wan YY, Sutterwala FS, Lara-Tejero M, Galan JE, Harhaj E, Flavell RA: Expression of interleukin-10 in intestinal lymphocytes detected by an interleukin-10 reporter knockin tiger mouse. *Immunity* 2006;25:941-952.
- 27 Riedel CU, Casey PG, Mulcahy H, O'Gara F, Gahan CG, Hill C: Construction of p16Slux, a novel vector for improved bioluminescent labeling of gram-negative bacteria. *Appl Environ Microbiol* 2007;73:7092-7095.
- 28 Weigmann B, Tubbe I, Seidel D, Nicolaev A, Becker C, Neurath MF: Isolation and subsequent analysis of murine lamina propria mononuclear cells from colonic tissue. *Nat Protoc* 2007;2:2307-2311.
- 29 Galvez J, Garrido M, Merlos M, Torres MI, Zarzuelo A: Intestinal anti-inflammatory activity of UR-12746, a novel 5-ASA conjugate, on acute and chronic experimental colitis in the rat. *Br J Pharmacol* 2000;130:1949-1959.
- 30 Chaudhry A, Samstein RM, Treuting P, Liang Y, Pils MC, Heinrich JM, Jack RS, Wunderlich FT, Bruning JC, Muller W, Rudensky AY: Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. *Immunity* 2011;34:566-578.
- 31 Saraiva M, O'Garra A: The regulation of IL-10 production by immune cells. *Nat Rev Immunol* 2010;10:170-181.
- 32 O'Garra A, Vieira P: T(H)1 cells control themselves by producing interleukin-10. *Nat Rev Immunol* 2007;7:425-428.
- 33 D'Andrea A, Aste-Amezaga M, Valiante NM, Ma X, Kubin M, Trinchieri G: Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma-production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. *J Exp Med* 1993;178:1041-1048.
- 34 Fiorentino DF, Zlotnik A, Vieira P, Mosmann TR, Howard M, Moore KW, O'Garra A: IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol* 1991;146:3444-3451.
- 35 Fiorentino DF, Zlotnik A, Mosmann TR, Howard M, O'Garra A: IL-10 inhibits cytokine production by activated macrophages. *J Immunol* 1991;147:3815-3822.
- 36 Shouval DS, Ouahed J, Biswas A, Goettel JA, Horwitz BH, Klein C, Muise AM, Snapper SB: Interleukin 10 receptor signaling: master regulator of intestinal mucosal homeostasis in mice and humans. *Adv Immunol* 2014;122:177-210.

- 37 Couper KN, Blount DG, Riley EM: IL-10: the master regulator of immunity to infection. *J Immunol* 2008;180:5771-5777.
- 38 Shiomi H, Masuda A, Nishiumi S, Nishida M, Takagawa T, Shiomi Y, Kutsumi H, Blumberg RS, Azuma T, Yoshida M: Gamma interferon produced by antigen-specific CD4+ T cells regulates the mucosal immune responses to *Citrobacter rodentium* infection. *Infect Immun* 2010;78:2653-2666.
- 39 Fitzgerald DC, Zhang GX, El-Behi M, Fonseca-Kelly Z, Li H, Yu S, Saris CJ, Gran B, Ciric B, Rostami A: Suppression of autoimmune inflammation of the central nervous system by interleukin 10 secreted by interleukin 27-stimulated T cells. *Nat Immunol* 2007;8:1372-1379.
- 40 McGeachy MJ, Bak-Jensen KS, Chen Y, Tato CM, Blumenschein W, McClanahan T, Cua DJ: TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. *Nat Immunol* 2007;8:1390-1397.
- 41 Ding L, Shevach EM: IL-10 inhibits mitogen-induced T cell proliferation by selectively inhibiting macrophage costimulatory function. *J Immunol* 1992;148:3133-3139.
- 42 Huber S, Gagliani N, Esplugues E, O'Connor W, Jr., Huber FJ, Chaudhry A, Kamanaka M, Kobayashi Y, Booth CJ, Rudensky AY, Roncarolo MG, Battaglia M, Flavell RA: Th17 cells express interleukin-10 receptor and are controlled by Foxp3(-) and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner. *Immunity* 2011;34:554-565.
- 43 Gu Y, Yang J, Ouyang X, Liu W, Li H, Yang J, Bromberg J, Chen SH, Mayer L, Unkeless JC, Xiong H: Interleukin 10 suppresses Th17 cytokines secreted by macrophages and T cells. *Eur J Immunol* 2008;38:1807-1813.
- 44 Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F: Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol* 2007;8:942-949.
- 45 Sutton C, Brereton C, Keogh B, Mills KH, Lavelle EC: A crucial role for interleukin (IL)-1 in the induction of IL-17-producing T cells that mediate autoimmune encephalomyelitis. *J Exp Med* 2006;203:1685-1691.
- 46 Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T, Levy DE, Leonard WJ, Littman DR: IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. *Nat Immunol* 2007;8:967-974.
- 47 Chung Y, Chang SH, Martinez GJ, Yang XO, Nurieva R, Kang HS, Ma L, Watowich SS, Jetten AM, Tian Q, Dong C: Critical regulation of early Th17 cell differentiation by interleukin-1 signaling. *Immunity* 2009;30:576-587.
- 48 Alipour M, Lou Y, Zimmerman D, Bording-Jorgensen MW, Sergi C, Liu JJ, Wine E: A balanced IL-1beta activity is required for host response to *Citrobacter rodentium* infection. *PLoS One* 2013;8:e80656.