Immunity
Article

Interleukin-15-Dependent NKp46$^+$ Innate Lymphoid Cells Control Intestinal Inflammation by Recruiting Inflammatory Monocytes

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DOI 10.1016/j.immuni.2012.05.013

SUMMARY

With the goal in mind to define how interleukin-15 (IL-15) contributes to acute intestinal inflammation, we have used a mouse model of ileitis induced by oral infection with *Toxoplasma gondii*. We observed that a crosstalk between IL-15 and interleukin-18 (IL-18) promoted intestinal recruitment of inflammatory monocytes, where these cells participated in parasite control but also in tissue damage. A stromal source of IL-15 controlled the development of lamina propria NKp46$^+$NK1.1$^+$ cells, whereas IL-18 produced during *T. gondii* infection stimulated their production of the chemokine CCL3. In turn, CCL3 attracted inflammatory monocytes via their chemokine receptor CCR1, which was indispensable for their recruitment into the inflamed gut. Collectively, these results identify the IL-15-dependent subset of intestinal NKp46$^+$ cells as an important source of CCL3, which can amplify intestinal inflammation via the recruitment of CCR1$^+$ inflammatory monocytes. Preliminary evidence suggests that this pathway might operate in Crohn's disease.

INTRODUCTION

Interleukin 15 (IL-15) is a pleiotropic cytokine with a large range of functions at the interface between innate and adaptive immunity. An essential role in the differentiation, survival, and/or activation of natural killer (NK), NKT cells, $\gamma\delta$ T cell receptor$^+$ (TCR) intraepithelial lymphocytes (IELs), and CD8$^+$ memory T cells is firmly established in mice lacking IL-15 or IL-15 receptor α chain (IL-15Rα) (Kennedy et al., 2000; Lodolce et al., 1998) or, conversely, overexpressing IL-15 (Fehniger et al., 2001; Ohta et al., 2002). In humans, IL-15 is thought to participate in the pathogenesis of a spectrum of inflammatory or autoimmune diseases. In rheumatoid arthritis, increased IL-15 concentrations are found in the synovium, and IL-15 is suggested to exert direct chemoattractant activity toward synovial T cells to stimulate their proliferation and their production of tumor necrosis factor-α (TNF-α). IL-15-activated T cells can also stimulate TNF-α production by peripheral blood monocytes and tissue macrophages via a contact-dependent mechanism (McInnes et al., 1996, 1997). A similar mechanism has been suggested in Crohn's disease (CD) (Liu et al., 2000). In CD, increased numbers of IL-15-producing mononuclear cells are observed in the intestinal lamina propria (LP), and augmented serum concentrations of IL-15 are found to predict patients' response to anti-TNF-α treatment (Bouchaud et al., 2010). Alternatively, we and others have suggested that in celiac disease, chronic overexpression of IL-15 by intestinal epithelial cells drives the expansion and activation of cytotoxic CD8$^+\alpha\beta$TCR$^+$ IELs that may destroy epithelial cells via an NK-like mechanism involving the NKG2D receptor (Hüe et al., 2004; Meresse et al., 2004). This mechanism has also been invoked to explain epithelial destruction in a mouse model of acute intestinal inflammation induced by intraperitoneal injection of poly(I:C) (Zhou et al., 2007). Finally, other studies suggest that IL-15 might impair immunoregulatory mechanisms and thereby promote CD8$^+$ and CD4$^+$ T cell effector responses (Ben Ahmed et al., 2009; Benahmed et al., 2007; DePaolo et al., 2011; Hmida et al., 2012).

To delineate the role of IL-15 in intestinal inflammation, we have used a mouse model of acute ileitis induced by oral infection of C57BL/6 mice with *Toxoplasma gondii*. In this strain of mice, the intestinal immune reaction is necessary to eliminate the parasite but is excessive and results in lethal jejuno-ileitis. Intestinal inflammation depends on the activation of CD4$^+$ T helper 1 (Th1) intestinal LP cells specific for the triggering antigen

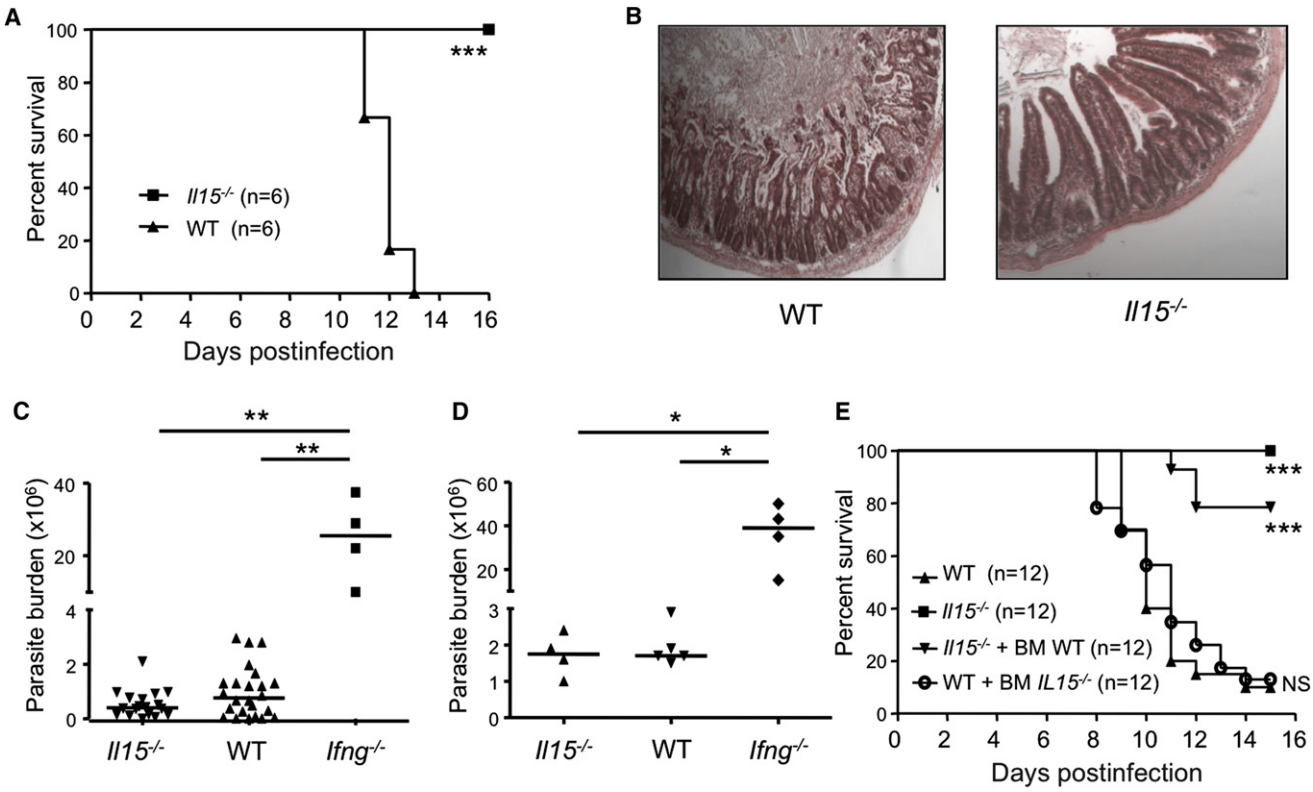

Figure 1. IL-15 Controls *T. gondii*-Induced Intestinal Inflammation

(A and E) Comparison of survival curves after oral infection by *T. gondii* in $Il15^{-/-}$ versus WT mice (A) and in irradiated WT or $Il15^{-/-}$ mice reconstituted with $Il15^{-/-}$ or WT bone marrow (BM) (E).

(B) Representative sections of paraffin-embedded jejunum stained with hematoxylin-eosin in WT and $Il15^{-/-}$ at day 7 p.i.

(C and D) Parasite load per 30 mg tissue as quantified by qRT-PCR on day 7 p.i. in gut (C) and MLN (D) from $Il15^{-/-}$, WT, and $Ifng^{-/-}$ mice.

*p < 0.05; **p < 0.005; ***p < 0.0005. See also Figure S1.

(Liesenfeld et al., 1996; Rachinel et al., 2004), but several complementary mechanisms are believed to participate in both pathogen exclusion and inflammation, notably the activation of $CD8^+\alpha\beta TCR^+$ IELs (Chardès et al., 1994; Egan et al., 2009) and that of inflammatory monocytes (Dunay et al., 2008). Furthermore, a prior report with $Il15^{-/-}$ mice has suggested that IL-15 may be necessary for both protection and inflammation during *T. gondii* infection (Combe et al., 2006).

We have shown herein that IL-15, although not required to control parasite replication, is necessary for the development of inflammation and tissue destruction. The proinflammatory effect of IL-15 did not depend on IEL activation and IL-15 was dispensable for the development of the $CD4^+$ Th1 cell response. In contrast, IL-15 amplified the recruitment of $CCR1^+$ inflammatory monocytes. We have shown that a nonhematopoietic source of IL-15 controlled the development and function of a subset of intestinal $NKp46^+$ innate lymphoid cells that produced CCL3 in response to inflammatory cytokines, and notably to IL-18 during *T. gondii* infection. In turn, CCL3 attracted $CCR1^+$ inflammatory monocytes via the CCR1 receptor, which was indispensable for the recruitment of the latter cells toward the inflamed gut. Preliminary evidence suggests that this pathway may operate in CD.

RESULTS

IL-15 Promotes the Severity of Intestinal Inflammation Induced by *T. gondii* in C57BL/6 Mice

In wild-type male C57BL/6 mice, oral gavage with 35 cysts of *T. gondii* strain 76K induced a severe jejuno-ileitis leading rapidly to death (Figure 1A). In $Il15^{-/-}$ mice, oral infection induced weight loss (Figure S1A available online), but all animals survived the early phase of infection and the severity of intestinal lesions was markedly attenuated (Figure 1B). In contrast, no increase in parasite load was observed in the intestine, mesenteric lymph nodes (MLNs) (Figures 1C and 1D), liver, or spleen of $Il15^{-/-}$ mice (Figures S1C and S1D) and the majority of $Il15^{-/-}$ mice were still alive at 40 days postinfection (p.i.) (Figure S1B), indicating that IL-15 is redundant to control parasite replication but contributes to intestinal inflammation in this model. These results differentiate the role of IL-15 from that of interferon-γ (IFN-γ), which is necessary not only to induce intestinal inflammation (Liesenfeld et al., 1996) but also to control local and systemic replication of the parasite (Figures 1C and 1D). IL-15 can be produced by many cell types, including enterocytes, dendritic cells (DCs), and macrophages. Accordingly, upregulation of IL-15 mRNA after *T. gondii* infection was observed in enterocytes as well as

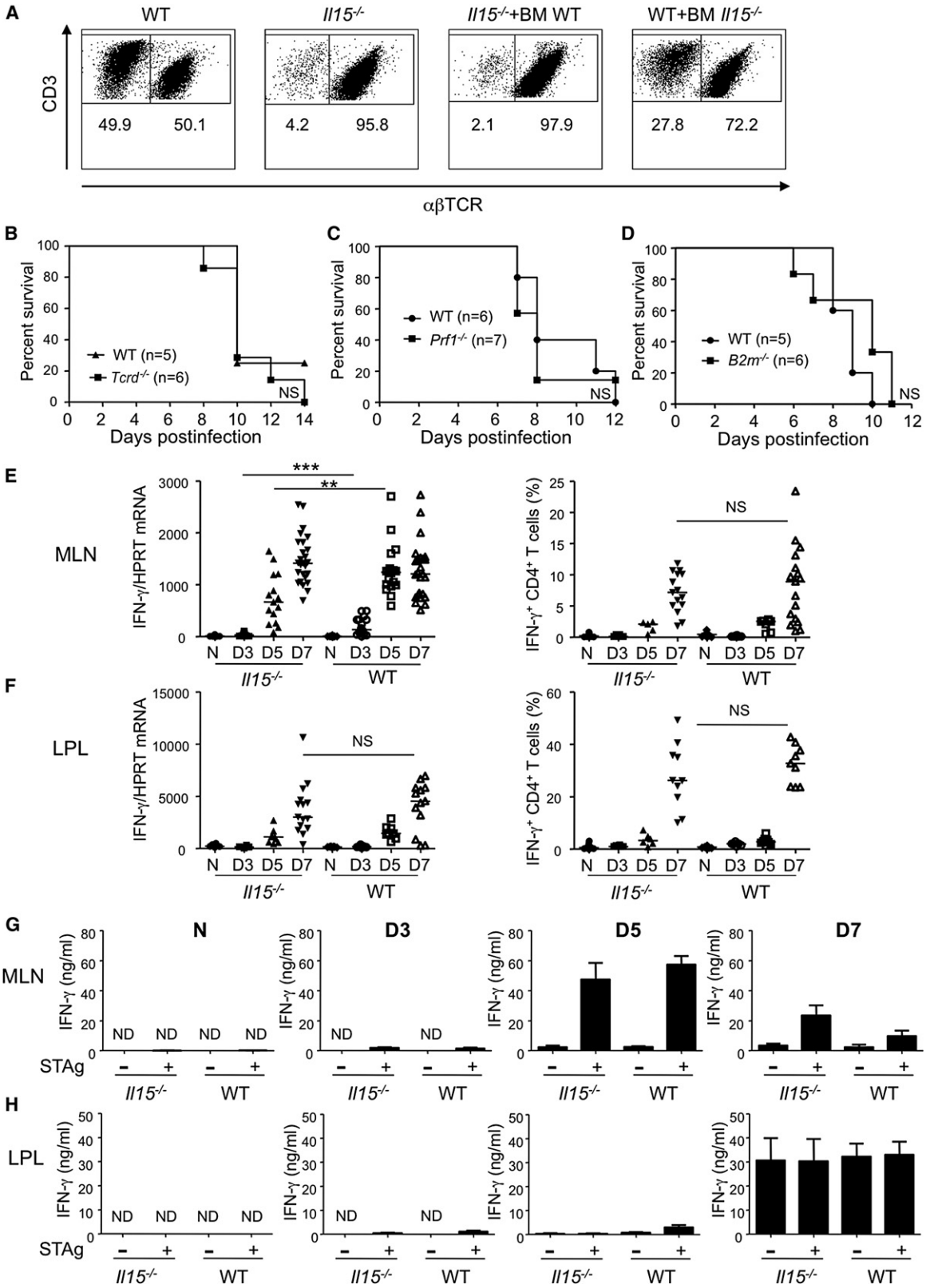

Figure 2. The Enhancing Role of IL-15 in *T. gondii*-Induced Intestinal Inflammation Depends neither on IELs nor on CD4$^+$ Th1 Cells
(A) Comparison of IEL subsets in WT and *Il15*$^{-/-}$ mice and in *Il15*$^{-/-}$ and WT chimeras reconstituted with BM from WT and *Il15*$^{-/-}$ mice, respectively. (B–D) Survival curves of *T. gondii*-infected *Tcrd*$^{-/-}$ (B), *Prf1*$^{-/-}$ (C), and *B2m*$^{-/-}$ (D) mice compared to WT mice.

in CD11c$^+$CD11b$^{+/-}$ DCs isolated from LP and MLN (Figures S1E–S1G). To determine which source of IL-15 controls the severity of the ileitis, the outcome of *T. gondii* infection was compared in hematopoietic chimeras. As shown in Figure 1E, all irradiated WT mice reconstituted with *Il15*$^{-/-}$ bone marrow (BM) developed a lethal ileitis whereas 80% of *Il15*$^{-/-}$ mice reconstituted with WT BM survived ($p < 0.005$), indicating that a nonhematopoietic source of IL-15, presumably epithelial cells, controls the intensity of intestinal inflammation.

IELs Are Not Central to the Enhancing Effect of IL-15 on Intestinal Inflammation during *T. gondii*-Induced Ileitis

A stromal source of IL-15 is necessary for the expansion of CD8$\alpha\alpha^+$ IELs and more particularly of $\gamma\delta$TCR$^+$ IELs (Schluns et al., 2004). Accordingly, $\gamma\delta$TCR$^+$ IELs were almost absent in *Il15*$^{-/-}$ mice reconstituted or not with WT BM, which developed an attenuated form of ileitis (Figure 2A). Adoptive transfer of 5×10^6 $\gamma\delta$TCR$^+$ IELs did not aggravate the course of the ileitis in *Il15*$^{-/-}$ recipients (Figure S2A) and, in keeping with previous observations (Liesenfeld et al., 1996), *Tcrd*$^{-/-}$ mice developed a lethal ileitis (Figure 2B). Therefore, it is unlikely that $\gamma\delta$TCR$^+$ IELs contributed to IL-15-driven inflammation.

Nonhematopoietic cell-derived IL-15 might also activate cytotoxic CD8$^+\alpha\beta$TCR$^+$ IELs. After oral infection with *T. gondii*, this subset of IELs can kill *T. gondii*-infected targets in vitro (Chardès et al., 1994). In addition, IL-15 can stimulate IEL cytotoxicity by inducing perforin and granzymes (Mention et al., 2003) or by enhancing expression of and/or signaling via the activating NKG2D receptor (Hüe et al., 2003; Meresse et al., 2004). We found that NKG2D expression increased significantly in LP CD8$^+\alpha\beta$TCR$^+$ lymphocytes from WT but not *Il15*$^{-/-}$ mice (Figure S2B) but IELs remained largely NKG2D negative during *T. gondii* ileitis (data not shown) and administration of a blocking NKG2D antibody (Ogasawara et al., 2004) did not modify the course of the disease in WT mice (Figure S2C). Finally, neither the course of the ileitis (Figures 2C and 2D) nor the parasitic load (data not shown) were modified in either *Prf1*$^{-/-}$ or *B2m*$^{-/-}$ mice, arguing against a major contribution of cytotoxic CD8$^+$ T cells to early protection against the parasite. These results also point to a need for IL-15-mediated amplification of intestinal inflammation in this acute model.

IL-15 Is Dispensable for the Induction of *T. gondii*-Specific Th1 Cell Responses

A previous study suggested that IL-15 promotes a specific Th1 cell response during *T. gondii* infection (Combe et al., 2006). This response is thought to be initiated by infected mucosal CD11c$^+$ DCs, which migrate to MLNs between days 3 and 7 p.i. (Courret et al., 2006) and elicit specific CD4$^+$ Th1 cells that home to the LP. IFN-γ mRNA and protein expression were therefore compared on days 0, 3, 5, and 7 in jejunal biopsies (Figures S2D and S2E) and in cells isolated from MLN and LP of *Il15*$^{-/-}$ and WT mice (Figures 2E and 2F, left). Upregulation of IFN-γ mRNA was slightly delayed in MLN lymphocytes of *Il15*$^{-/-}$ mice and a small amount of IFN-γ secretion was induced by soluble *T. gondii* antigen (STAg) from LPL in WT but not *Il15*$^{-/-}$ mice on day 5 (Figures 2E and 2G). Yet, on day 7, LPL from both groups of animals spontaneously produced massive and comparable amounts of IFN-γ, which did not further increase in the presence of STAg (Figure 2H). Furthermore, no difference could be demonstrated between WT and *Il15*$^{-/-}$ mice in the percentages of IFN-γ-producing CD4$^+$ T cells in either the MLN or LPL at all time points (Figures 2E and 2F, right). Finally, no difference could be demonstrated in the percentage of CD4$^+$CD25$^+$Foxp3$^+$ T cells in the LP of WT and *Il15*$^{-/-}$ mice on days 5 and 7 p.i. (Figure S2F). Altogether, these results indicate that WT and *Il15*$^{-/-}$ mice develop comparable CD4$^+$ Th1 cell responses, a finding in keeping with comparable parasitic burden in both animal groups.

Recruitment of Inflammatory Monocytes Is Impaired in *Il15*$^{-/-}$ Mice during *T. gondii*-Induced Ileitis

Inflammation in WT and *Il15*$^{-/-}$ mice was next assessed by comparing intestinal IL-1β, TNF-α, and IL-6 production during infection. Upregulation of IL-1β, TNF-α, and IL-6 mRNA was first observed in intestinal biopsies and in isolated LP cells on day 5 p.i. and increased markedly on day 7 when copious amounts of IL-1β, TNF-α, and IL-6 were spontaneously released in the supernatants of biopsies (Figures S3A and S3B) or isolated LP (Figures 3A and S3C). Upregulation of inflammatory cytokines was observed in all mice but was significantly higher in WT than in *Il15*$^{-/-}$ mice.

To identify the cellular source of inflammatory cytokines, LP cells were isolated from infected WT mice on day 7 and sorted into CD3$^+$, CD19$^+$, CD3$^-$NK1.1$^+$, or CD45$^+$CD3$^-$CD19$^-$NK1.1$^-$ cells. Reverse transcriptase-polymerase chain reaction (RT-PCR) and ELISA analysis showed that IL-1β, TNF-α, and IL-6 mRNA and IL-1β and TNF-α secretion were upregulated only in CD45$^+$CD3$^-$CD19$^-$NK1.1$^-$ LP cells (Figures 3B and S3D). The latter subset was further separated into neutrophils (Ly6G$^+$Ly6C$^+$CD11b$^+$), inflammatory monocytes (Ly6C^{hi}F4/80$^+$Ly6G$^-$CD11b$^+$CD11c$^-$), and resident macrophages (F4/80$^+$CD11b$^+$Ly6C$^-$Ly6G$^-$) and their secretion tested by ELISA. Inflammatory monocytes released conspicuous amounts of IL-1β, TNF-α, and IL-6 whereas much smaller amounts of TNF-α and IL-6 were produced by neutrophils and macrophages, respectively (Figure S3E). Flow cytometry analysis confirmed that inflammatory monocytes were the main producers of TNF-α (Figure 3C). These data suggested that IL-15 was necessary to promote the activation and/or the recruitment of inflammatory monocytes. IL-15 did not induce the production of TNF-α and/or IL-1β in peritoneal macrophages from WT mice (naive or infected with *T. gondii*) (not shown). IL-15 also failed to stimulate the production of inflammatory cytokines by LP cells from naive WT mice even after stimulation by LPS or CD3 and CD28 antibodies (Figures S3F–3H). In contrast, the percentages as well as the absolute numbers of inflammatory

(E and F) Synthesis of IFN-γ by MLN (E) and LPL (F) from naive and infected *Il15*$^{-/-}$ and WT mice. Left: qRT-PCR quantification of IFN-γ transcripts. Right: percentages of isolated CD4$^+$ T cells spontaneously expressing intracellular IFN-γ.
(G and H) IFN-γ secretion evaluated by ELISA after a 72 hr culture with (+) or without (–) 15 μg/ml of soluble *T. gondii* antigen (STAg) in supernatants of lymphocytes isolated from MLN (G) or LP (H) in naive (N) and infected *Il15*$^{-/-}$ and WT mice.
NS, not significant; *$p < 0.05$; **$p < 0.005$; ***$p < 0.0005$. See also Figure S2.

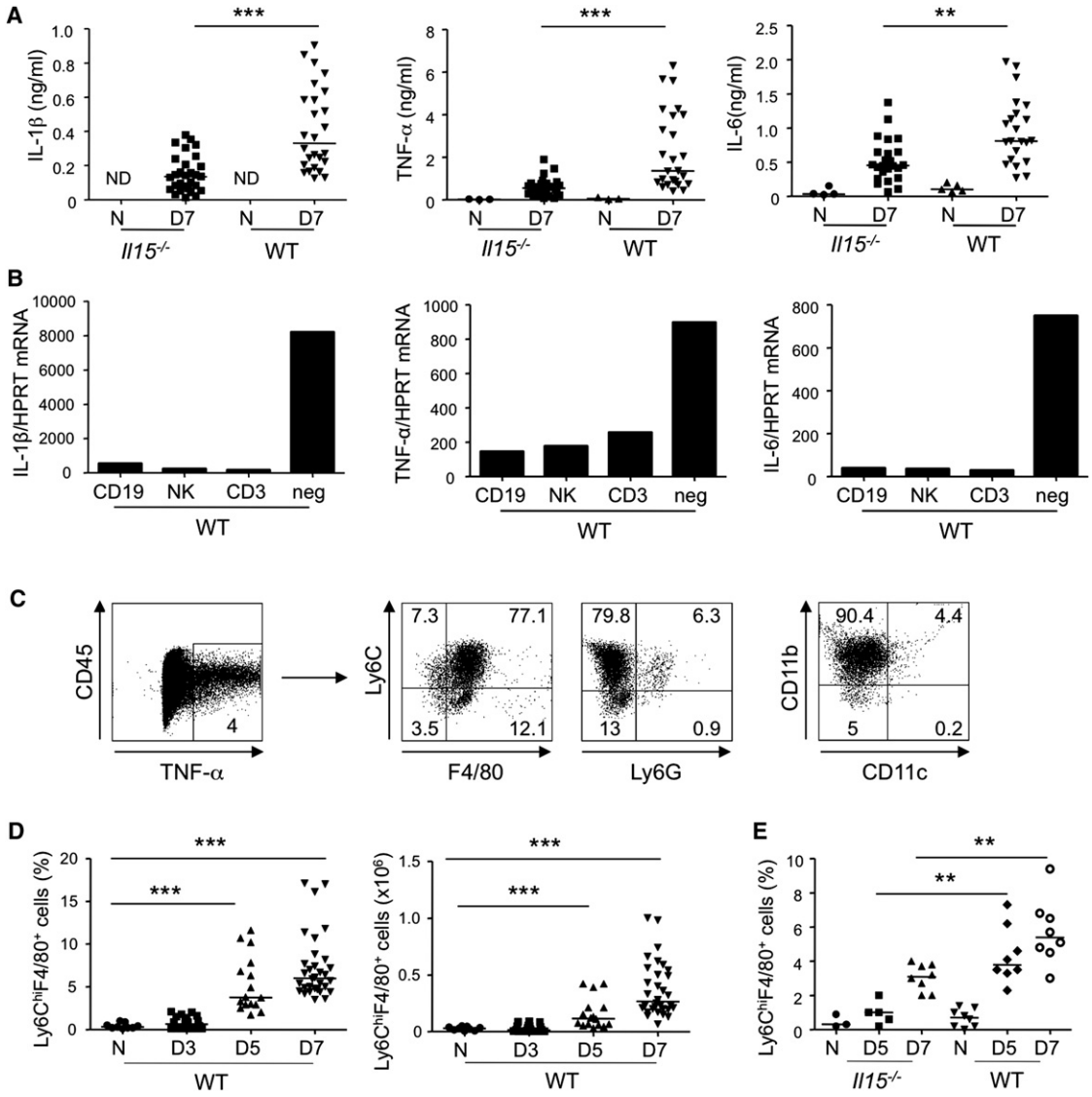

Figure 3. The Recruitment of Inflammatory Monocytes Is Impaired in *Il15*$^{-/-}$ Mice during *T. gondii*-Induced Ileitis

(A) Spontaneous secretion of IL-1β, TNF-α, and IL-6 evaluated by ELISA in 72 hr culture supernatants of LP cells from *Il15*$^{-/-}$ and WT mice either naive or on day 7 p.i.

(B) qRT-PCR quantification of IL-1β, TNF-α, and IL-6 transcripts in flow cytometry-sorted CD45$^+$ LP B (CD19$^+$), NK (CD3$^-$NK1.1$^+$), and T (CD3$^+$) cells and in the negative CD45$^+$CD3$^-$CD19$^-$NK1.1$^-$ LP cells (neg) on day 7 p.i. in *Il15*$^{-/-}$ and WT mice. Results from one experiment with a pool of ten mice.

(C) Surface phenotype of LP cells containing intracellular TNF-α on day 7 p.i. in WT mice: one representative out of five experiments.

(D) Percentages and absolute numbers of LP Ly6C^{hi}F4/80$^+$ inflammatory monocytes in WT mice on days 3, 5, and 7 p.i.

(E) Percentages of LP Ly6C^{hi}F4/80$^+$CCR1$^+$ inflammatory monocytes in *Il15*$^{-/-}$ and WT mice on days 5 and 7 p.i.

NS, not significant; *p < 0.05; **p < 0.005; ***p < 0.0005. See also Figure S3.

monocytes, which increased on day 5 and even more on day 7 p.i. in infected WT mice (Figure 3D), were significantly reduced in *Il15*$^{-/-}$ mice, suggesting that IL-15 stimulates the recruitment of inflammatory monocytes (Figure 3E and data not shown).

IL-15 Promotes CCR1- and CCL3-Dependent Recruitment of Inflammatory Monocytes during *T. gondii*-Induced Ileitis

One attractive hypothesis to explain the role of IL-15 was the induction of chemokines able to stimulate intestinal migration of inflammatory monocytes. mRNA for such chemokines including CCL2, CCL3, CCL4, CCL5, and CXCL10 were markedly increased in intestinal biopsies and in isolated LP cells on day 7 p.i. compared to naive animals. The increase was comparable in WT and *Il15*$^{-/-}$ mice except for CCL3 mRNA, which were less induced in *Il15*$^{-/-}$ mice (Figures 4A, 4B, and S4A–S4C). Accordingly, CCL3 concentrations were significantly reduced in supernatants of LP cells from infected *Il15*$^{-/-}$mice (Figure 4C). Confirming a role of CCL3 in inflammatory monocytes recruitment, treatment with a blocking CCL3 antibody

significantly reduced the percentage of Ly6C^{hi}F4/80^{+} LP cells (Figure 4D) and the production of TNF-α (Figure 4E). Anti-CCL3 treatment also reduced the severity of histological lesions (Figure 4F) but did not affect overall survival (Figure 4G).

CCL3 binds two distinct receptors, CCR1 and CCR5, both of which can be expressed by inflammatory monocytes. On day 7 p.i., CCR5 mRNA were similarly increased in jejunal biopsies and in LP cells from WT and *Il15*$^{-/-}$ mice. In contrast, CCR1 mRNA was significantly lower in *Il15*$^{-/-}$ than in WT mice (Figure 4H). The role of CCR1 in the recruitment of inflammatory monocytes was therefore assessed with *Ccr1*$^{-/-}$ mice. Contrasting with their recruitment in infected WT mice, no increase in Ly6C^{hi}F4/80^{+} LP cells nor in TNF-α^{+}CD45^{+} LP cells was observed in *Ccr1*$^{-/-}$ mice during *T. gondii* infection and the percentage of such cells on day 7 were even lower than in *Il15*$^{-/-}$ mice (Figures 4I and 4J). Accordingly, IL-1β production by LP cells was abolished (Figure 4K). The small residual amounts of TNF-α may derive from neutrophils as indicated by the fact that these cells were normally recruited in the intestine of *Ccr1*$^{-/-}$ mice (Figure S4D) and produced small amounts of TNF-α when purified on day 7 (Figure S3E). As already described in *Ccr1*$^{-/-}$ mice (Khan et al., 2001), intestinal lesions were less severe on day 7 p.i. than in WT mice (Figure 4L) but the mice died, probably because of 20- to 100-fold higher parasitic loads in MLN, spleen, and liver than WT mice (Figures S4E–S4G). Altogether these results indicated that the intestinal recruitment of Ly6C^{hi}F4/80^{+} inflammatory monocytes during *T. gondii*-induced ileitis requires CCR1 and can be stimulated via CCL3.

IL-15-Dependent NKp46^{+}NK1.1^{+}CD127^{-} Cells Are an Early and Major Source of CCL3 during *T. gondii*-Induced Ileitis

CCL3 can be produced by cells of hematopoietic or epithelial origin. CCL3 transcripts increased 100- to 200-fold more in LP cells than in enterocytes of infected mice (Figure 5A). Analysis of LP subsets sorted on day 7 p.i. indicated that CCL3 mRNA was strongly upregulated in a subset of CD3^{-}NKp46^{+}NK1.1^{+} cells (Figure 5B). In these cells, upregulation of CCL3 RNA could already be detected at day 3 p.i., which is prior to CCR1^{+} inflammatory monocyte recruitment (Figure 5C).

Murine LP contains two distinct lineages of NKp46^{+} innate lymphoid cells: IL-7-dependent Rorc^{+}NKp46^{+}NK1.1^{-}CD127^{+} cells that produce IL-22 and NKp46^{+}NK1.1^{+}CD127^{-} cells that depend on IL-15 and thus resembles classical splenic NK cells (reviewed in Colonna, 2009; Spits and Di Santo, 2011). Consistent with the hypothesis that LP NKp46^{+}NK1.1^{+}CD127^{-} cells were the IL-15-dependent source of CCL3, their absolute number was significantly decreased in naive and infected *Il15*$^{-/-}$ mice compared to WT mice (Figure 5D) whereas the numbers of NKp46^{+}NK1.1^{-}CD127^{+} and of IL-22-containing NKp46^{+} cells were unchanged (Figures S5A and S5B). This hypothesis was confirmed by two complementary sets of experiments. First, by using hematopoietic chimeras, we observed that differentiation and/or homeostasis of LP CD3^{-}NKp46^{+} NK1.1^{+} cells required a stromal but not a hematopoietic source of IL-15 (Figures 5E and 5F). CCL3 mRNA, recruitment of LP Ly6C^{hi}F4/80^{+} inflammatory monocytes, and ileitis severity were significantly reduced in irradiated *Il15*$^{-/-}$ mice reconstituted with WT bone marrow cells that lacked LP CD3^{-}NKp46^{+}NK1.1^{+} cells but not in irradiated WT reconstituted with *Il15*$^{-/-}$ bone marrow, which had normal percentages of LP CD3^{-}NKp46^{+} NK1.1^{+} cells (Figures 5G, 5H, and 1E). Second, treatment of mice with a depleting NK.1.1 antibody efficiently depleted LP NKp46^{+}NK1.1^{+}CD127^{-} cells (Figures S5C and S5D) and simultaneously reduced CCL3 production by LP cells (Figure 5I). The percentage of LP Ly6C^{hi}F4/80^{+} inflammatory monocytes and IL-1β and TNF-α mRNA were significantly decreased at day 7 p.i. and epithelial damage was attenuated (Figures 5J–5L). Altogether these data indicate that LP NKp46^{+}NK1.1^{+} CD127^{-} cells depend on a stromal source of IL-15 for their differentiation and/or homeostasis and are an important source of CCL3 during *T. gondii* infection that contributes to CCR1^{+} inflammatory monocyte recruitment. Some CCL3 transcripts were also observed in inflammatory monocytes and neutrophils (Figure S5E). A major contribution of neutrophil-derived CCL3 for CCR1^{+} inflammatory monocytes recruitment seemed unlikely because these cells were observed only late in infection and were unaltered in the absence of IL-15 (Figure S5F). Moreover, their efficient depletion by Ly6G antibody did not reduce CCL3 production or CCR1^{+} inflammatory monocyte recruitment (Figures S5G–S5I). However, it remains possible that CCR1^{+} inflammatory monocyte-derived CCL3 observed late in infection might amplify their recruitment.

CCL3 Production by CD3^{-}NKp46^{+}NK1.1^{+} Cells Is Stimulated by IL-18

In keeping with previous observations (Dorner et al., 2004; Fehniger et al., 1999), we found that CCL3 was induced in purified murine spleen NKp46^{+} cells by IL-15 and IL-18 and to a lesser extent by α-IFN but not by IFN-γ, IL-12, IL-21, or IL-23, nor by STAg that may stimulate NK cells (Figure 6A; Sher et al., 1993). A transient increase IL-15 mRNA was detected during the first 3 days p.i. in enterocytes and on day 2 in LP CD11c^{+} DCs (Figures S1E and S1F). IL-15 protein was not detected, perhaps because of its known binding to IL-15Rα that confines its expression to the surface of producing cells and/or hampers detection (Stonier and Schluns, 2010). No increase in IL-18 mRNA was observed but conspicuous amounts of mature IL-18 protein were detected on days 3 and thereafter in both WT and *Il15*$^{-/-}$ mice, which probably derived from enterocytes as indicated by the fact that it was detected in the supernatants of intestinal biopsies but not of LP cells (Figure 6B and not shown). Consequently, we assessed the roles for IL-15 and IL-18 in activating CCL3 production by NK cells by using a short treatment with neutralizing antibody. CCL3 production and recruitment of inflammatory monocytes were modestly but not significantly reduced by IL-15 antibody (Figures 6C–6E) whereas IL-18 antibody treatment significantly reduced both parameters (Figures 6F and 6G). These results indicate that IL-18 promotes induction of CCL3 in vivo during *T. gondii* infection. In contrast, IL-15 plays a critical role in the homeostasis of intestinal NK cells, which are the main CCL3 producers, whereas its role in CCL3 upregulation is less clear. Contrasting with lesser inflammatory monocytes recruitment, histology was not improved but rather impaired in anti-IL-18-treated mice (data not shown), a paradoxical result probably explained by the protective role of IL-18 against epithelial damage (Elinav et al., 2011). IL-18 was significantly less

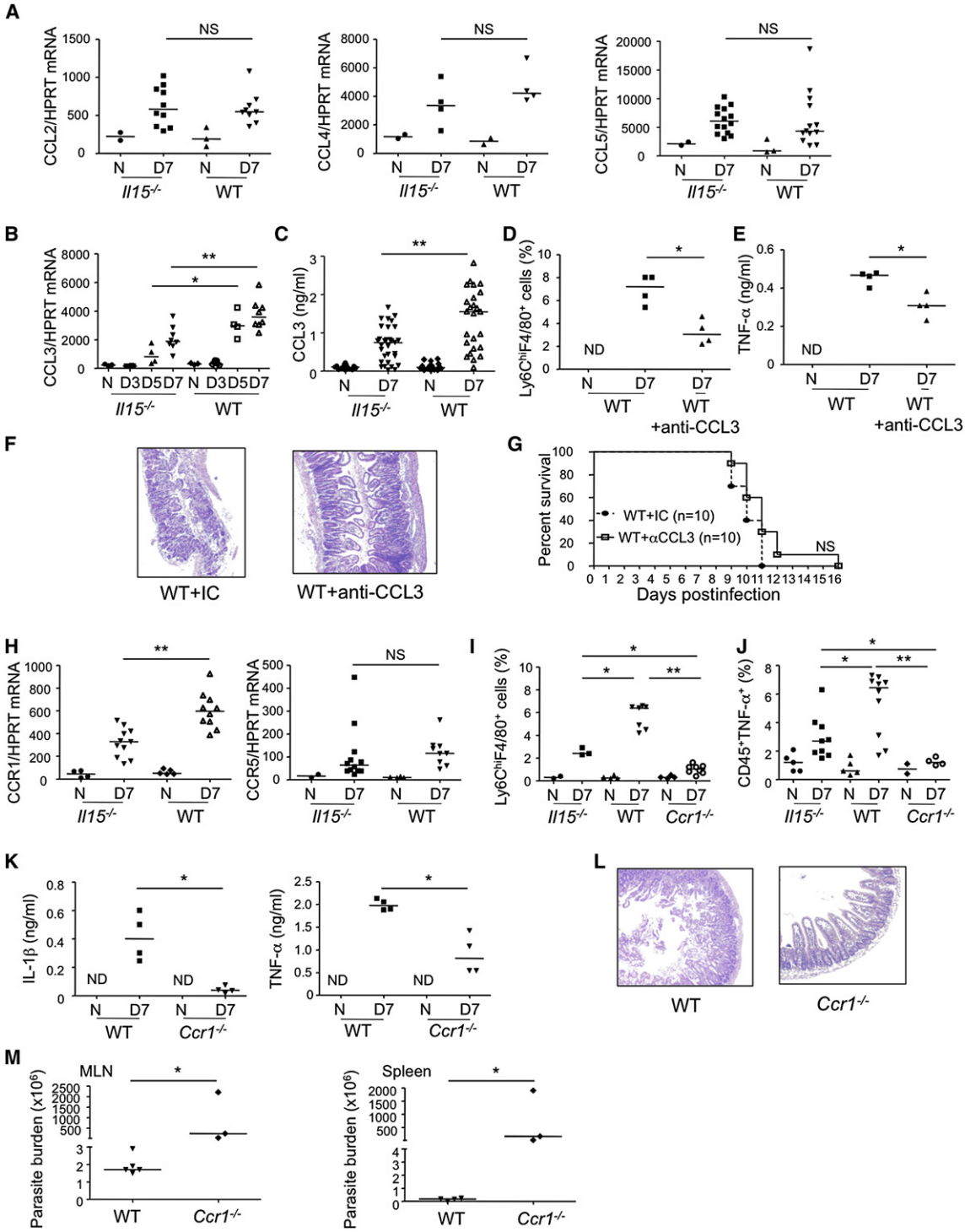

Figure 4. IL-15 Promotes CCR1-CCL3-Dependent Recruitment of Inflammatory Monocytes during *T. gondii*-Induced Ileitis

(A, B, and H) qRT-PCR quantification of CCL2-5 transcripts (A, B) or CCR1 and CCR5 transcripts (H) in LPL from *Il15*$^{-/-}$ and WT mice either naive or on indicated p.i. days.

(C) Spontaneous secretion of CCL3 in 72 hr supernatants of LP from *Il15*$^{-/-}$ and WT mice either naive or on day 7 p.i.

(D–F) Percentage of LP Ly6C^{hi}F4/80^{+}CCR1^{+} inflammatory monocytes (D), spontaneous secretion of TNF-α (E), and representative jejunal sections (F) on day 7 p.i. in *Il15*$^{-/-}$ and WT mice treated or not with CCL3 mAb.

(G) Survival curves of WT mice treated with CCL3 mAb or control isotype.

(I and J) Percentage of LP Ly6C^{hi}F4/80^{+}CCR1^{+} inflammatory monocytes (I) and of CD45^{+} LP cells stained with TNF-α mAb (J) on day 7 p.i. in *Il15*$^{-/-}$, WT, and *Ccr1*$^{-/-}$ mice.

produced at late time points during infection in *Il15*$^{-/-}$ compared to WT mice (Figure 6B), probably as a consequence of lesser epithelial damage. However, a direct role for IL-15 in regulating epithelial IL-18 production cannot be excluded.

CCL3 and CCR1 Expression Are Upregulated in Crohn's Disease

In Crohn's disease, TNF-α-producing inflammatory monocytes play a central role in gut inflammation as attested by the efficiency of therapeutic administration of TNF-α antibodies. Recent work has identified their CD33^{+}CD14^{+} phenotype and shown their expression of several chemokine receptors, including CCR1, which is absent on gut-resident CD33^{+}CD14^{-} myeloid cells (Kamada et al., 2008). In keeping with these data, IL-1β, TNF-α, and CCR1 mRNA were significantly upregulated in inflamed biopsies from Crohn's disease patients (Figures 7A and 7B). CCL3 (but not CCL5) mRNA was also significantly increased in the same biopsies (Figure 7C) and there was a positive correlation between CCL3 and CCR1 (spearman coefficient r: 0.64) but not of CCL3 and CCR5 mRNA (Figure 7D). Confirming recent work (Takayama et al., 2010), NKp46^{+}CD56^{+}CD127^{-}CD122^{+} NK cells were present in the intestine of Crohn's disease patients and their proportion was increased in inflamed compared to unaffected mucosa (Figures 7E and S6). IL-15 and IL-18, known to be upregulated in the intestine of CD patients (Liu et al., 2000; Monteleone et al., 1999b; Pizarro et al., 1999), could synergize and stimulate CCL3 production in CD45^{+}CD3^{-}NKp46^{+}CD56^{+} cells derived either from control blood or from the mucosa of Crohn's disease patients (Figures 7F and 7G). Interestingly, CCL3 production was more readily induced by IL-15 and/or IL-15 and IL-18 in NK cells from inflamed than from unaffected CD mucosa (Figure 7G). Therefore, in Crohn's disease, IL-15 and IL-18 might cooperate to promote the production of CCL3 by gut NK cells and enhance the recruitment of CCR1^{+} inflammatory monocytes. Overall, our data highlight how IL-15-dependent gut innate lymphoid cells might integrate local signals and tune the intensity of intestinal inflammation by influencing the recruitment of inflammatory monocytes.

DISCUSSION

In response to oral infection by the virulent 76K strain of *T. gondii*, C57BL/6 mice develop an immune response, which is a two-edged sword because it permits early control of parasite replication but results in uncontrolled intestinal inflammation lethal within 10 days (Liesenfeld et al., 1996; Mennechet et al., 2002). The role of IL-15 in the immune response against *T. gondii* infection is controversial. Combe et al. (2006) have suggested that intestinal inflammation is reduced and *T. gondii* infection less well controlled in orally infected *Il15*$^{-/-}$ mice. In contrast, Lieberman et al. (2004) did not detect any increase in cyst burden in *Il15*$^{-/-}$ mice after intraperitoneal infection. Our results confirm that intestinal inflammation is markedly attenuated in infected *Il15*$^{-/-}$ mice. Yet, no difference could be demonstrated in parasitic load in intestine, liver, and spleen between *Il15*$^{-/-}$ and WT mice, suggesting that in our conditions of infection, IL-15 did not impair early parasite control and therefore this model was valuable to analyze the mechanisms underlying the role of IL-15 in intestinal inflammation.

Previous work has suggested that IL-15 can promote a cytolytic attack of the gut epithelium by IELs. Our data indicate that γδTCR^{+} and CD8^{+}αβTCR^{+} IELs do not play a determinant role in ileitis severity. This conclusion is consistent with two previous studies (Denkers et al., 1997; Liesenfeld et al., 1996) but is at odds with a recent report suggesting the contribution of CCR2-dependent CD103^{+}CD11c^{+} IELs to *T. gondii*-induced gut inflammation (Egan et al., 2009). Yet, it is noticeable that during *T. gondii* infection, CCR2 may be involved not only in the recruitment of IELs but also in the migration of CCR1^{+} inflammatory monocytes which, as discussed below, play a central role in *T. gondii*-induced ileitis.

An alternative hypothesis is a role of IL-15 in the amplification of the *T. gondii*-specific Th1 cell response (Combe et al., 2006). Based on the analysis of MLN at day 10 postinfection, Combe et al. (2006) suggested that IL-15 is necessary for optimal priming of Th1 cell responses. Day 10, however, is late to evaluate priming because parasite-loaded dendritic cells are first detected in MLN on day 3 (Courret et al., 2006) and ileitis is full blown by day 7. Furthermore, another report observes normal serum response in *Il15*$^{-/-}$ mice after intraperitoneal infection (Lieberman et al., 2004). Herein, comparable amounts of IFN-γ were secreted by LP cells of WT and *Il15*$^{-/-}$ mice on day 7 p.i., pleading against a major enhancing effect of IL-15 on the Th1 cell response in our conditions of infection. Yet appearance of IFN-γ transcripts was slightly delayed in MLN of *Il15*$^{-/-}$ mice and, at day 5 p.i., STAg could stimulate a minor production of IFN-γ in LPL of WT but not of *Il15*$^{-/-}$ mice. Whether these subtle early differences participate in the enhancing effect of IL-15 on inflammation remains unclear.

Contrasting with the comparable production of IFN-γ in the intestinal mucosa at day 7 in both animal groups, secretion of IL-1β, TNF-α, and IL-6 was significantly diminished in *Il15*$^{-/-}$ mice. The cytokines were mainly produced by Ly6C^{hi}F4/80^{+}Ly6G^{-}CD11b^{+}CD11c^{-}CCR1^{+} inflammatory monocytes. In vitro studies have suggested that IL-15 enhances the secretion of TNF-α by human monocytes via a T cell contact-dependent mechanism (Liu et al., 2000; McInnes et al., 1996). Yet, adding IL-15 alone or in combination with other stimuli to in vitro culture of peritoneal or LP cells failed to enhance TNF-α and IL-1β secretion, arguing against a role of IL-15 in inflammatory monocyte differentiation. In contrast, IL-15 was necessary for maximal in vivo recruitment of CCR1^{+} inflammatory monocytes, as shown by the fact that their numbers increased significantly less in the LP of *Il15*$^{-/-}$ than of WT infected mice.

Mechanisms controlling inflammatory monocytes migration into the inflamed gut are not fully delineated. Previous work

(K) Spontaneous secretion of IL-1β and TNF-α by LP in WT and *Ccr1*$^{-/-}$ mice either naive or on day 7 p.i. (ND, not detectable).
(L) Representative jejunal sections on day 7 in WT and *Ccr1*$^{-/-}$ mice.
(M) Parasitic loads in MLN and spleen of WT and *Ccr1*$^{-/-}$ mice.
NS, not significant; *p < 0.05; **p < 0.005; ***p < 0.0005. See also Figure S4.

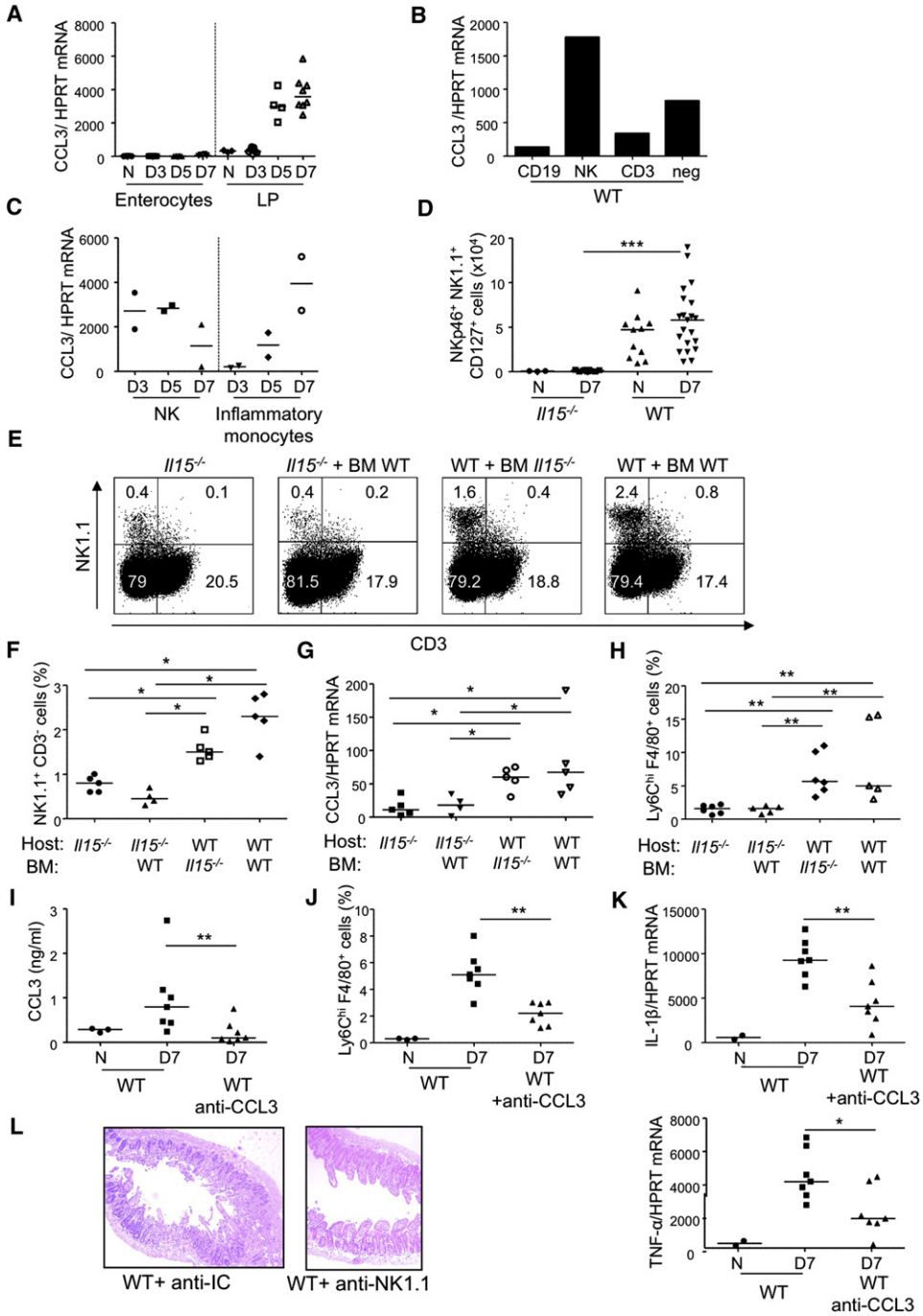

Figure 5. CCL3 Production during *T. gondii*-Induced Ileitis Depends on Intestinal IL-15-Dependent NKP46$^+$NK1.1$^+$CD127$^-$ Cells

(A–C) qRT-PCR quantification of CCL3 transcripts in enterocytes and LP cells (A), in flow cytometry-sorted CD45$^+$ LP B (CD19$^+$), NK (NK1.1$^+$), and T (CD3$^+$) cells (B), and in flow cytometry-sorted Ly6C^{hi}F4/80$^+$CCR1$^+$ inflammatory monocytes or CD3$^-$NKp46$^+$NK1.1$^+$ LP cells at indicated days in WT mice (C) (each point represents a pool of ten mice).

(D) Absolute numbers of NKP46$^+$NK1.1$^+$CD3$^-$ cells in naive and infected WT and *Il15*$^{-/-}$ mice.

(E) Comparison by flow cytometry of CD3$^-$NK1.1$^+$ and CD3$^+$NK1.1$^+$ cells in LP 2 months after reconstitution of irradiated *Il15*$^{-/-}$ mice with WT bone marrow (*Il15*$^{-/-}$ + BM WT) or irradiated mice WT with *Il15*$^{-/-}$ (WT + BM *Il15*$^{-/-}$) or WT bone marrow (WT + BM WT) compared to unmodified *Il15*$^{-/-}$ mice. One representative out of six experiments.

(F) Flow cytometry determination of LP CD3$^-$NKp46$^+$NK1.1$^+$ cells in hematopoietic chimera on day 7 p.i.

(G) qRT-PCR quantification of CCL3 transcripts in jejunum of hematopoietic chimeras on day 7 p.i.

(H and J) Flow cytometry determination of Ly6C^{hi}F4/80$^+$ LP CCR1$^+$ inflammatory monocytes on day 7 p.i. in hematopoietic chimeras (H) or in mice treated with NK1.1 antibody (J).

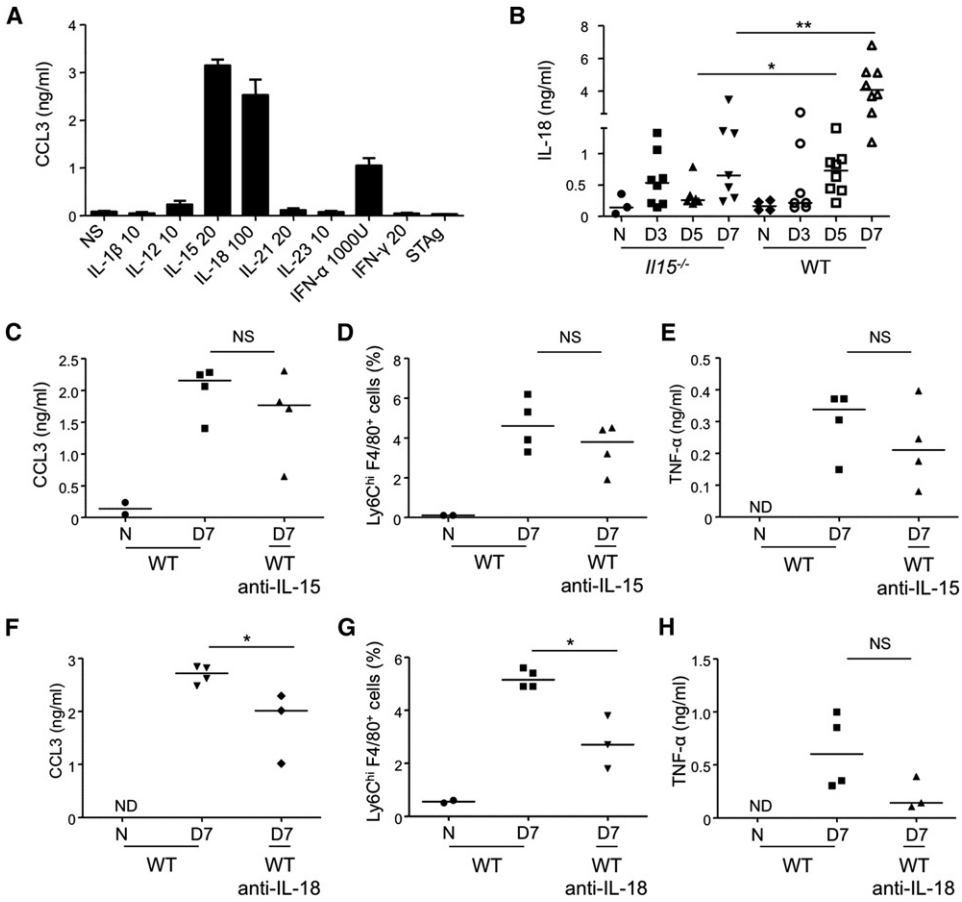

Figure 6. CCL3 Production by CD3$^-$NKp46$^+$NK1.1$^+$ Cells Is Stimulated by IL-15 and IL-18

(A) ELISA dosage of CCL3 in 72 hr supernatants of flow cytometry-sorted spleen CD3$^-$NKp46$^+$NK1.1$^+$ cells isolated from naive WT mice and stimulated with indicated cytokines.

(B) ELISA dosage of IL-18 in 24 hr supernatants of jejunal biopsies from *Il15*$^{-/-}$ and WT mice at indicated p.i. days.

(C–H) ELISA dosage of CCL3 (C, F) and TNF-α (E, H) in 72 hr supernatants of LP cells from WT mice treated or not with IL-15 (C–E) or IL-18 (F–H) antibodies.

(D and G) Flow cytometry determination of LP Ly6C^{hi}F4/80$^+$CCR1$^+$ inflammatory monocytes on day 7 p.i. in antibody-treated mice.

NS, not significant; *p < 0.05; **p < 0.005; ***p < 0.0005.

showed that inflammatory monocytes are not recruited in the intestine of *T. gondii*-infected *Ccr2*$^{-/-}$ mice (Dunay et al., 2008). Yet, CCR2 was dispensable for inflammatory monocyte migration into the inflamed gut and instead controlled monocyte emigration from bone marrow (Dunay et al., 2008). Herein, the lesser amounts of CCR1 and CCL3 mRNA in the intestine of infected *Il15*$^{-/-}$ than in that of WT mice pointed to their possible role in the local recruitment of inflammatory monocytes. Demonstrating the instrumental role of CCR1, LP Ly6C^{hi}F4/80$^+$ Ly6G$^-$CD11b$^+$CD11c$^-$ inflammatory monocytes were almost undetectable in infected *Ccr1*$^{-/-}$ mice, a result that contrasted with the normal recruitment of neutrophils. Consistent with previous observations in *Ccr1*$^{-/-}$ mice (Khan et al., 2001) and with the hypothesis that CCR1$^+$ inflammatory monocytes participate in tissue damage, histology was improved in *Ccr1*$^{-/-}$ mice. Yet, and as also observed by Khan et al. (2001), the parasitic load was markedly increased in spleen and liver, leading to the rapid death of infected animals. The latter finding confirms the important role of inflammatory monocytes in parasite control described in *Ccr2*$^{-/-}$ mice (Dunay et al., 2008). The extensive parasite replication observed in the intestine of *Ccr2*$^{-/-}$ mice was, however, not observed in *Ccr1*$^{-/-}$ mice, perhaps because of difference in strains and/or conditions of infection. Therefore, similar to CD4$^+$ Th1 cells, CCR1$^+$ inflammatory monocytes probably can serve a dual role in *T. gondii* infection and participate in both tissue damage and parasite control. CCR1 was

(I) ELISA dosage of CCL3 in 72 hr supernatants of LP cells isolated on day 7 p.i. from anti-NK1.1-treated mice.

(K) qRT-PCR quantification of IL-1β and TNF-α transcripts in LP cells from anti-NK1.1-treated mice.

(L) Representative jejunal sections of WT mice treated or not with anti-NK1.1 on day 7.

NS, not significant; *p < 0.05; **p < 0.005; ***p < 0.0005. See also Figure S5.

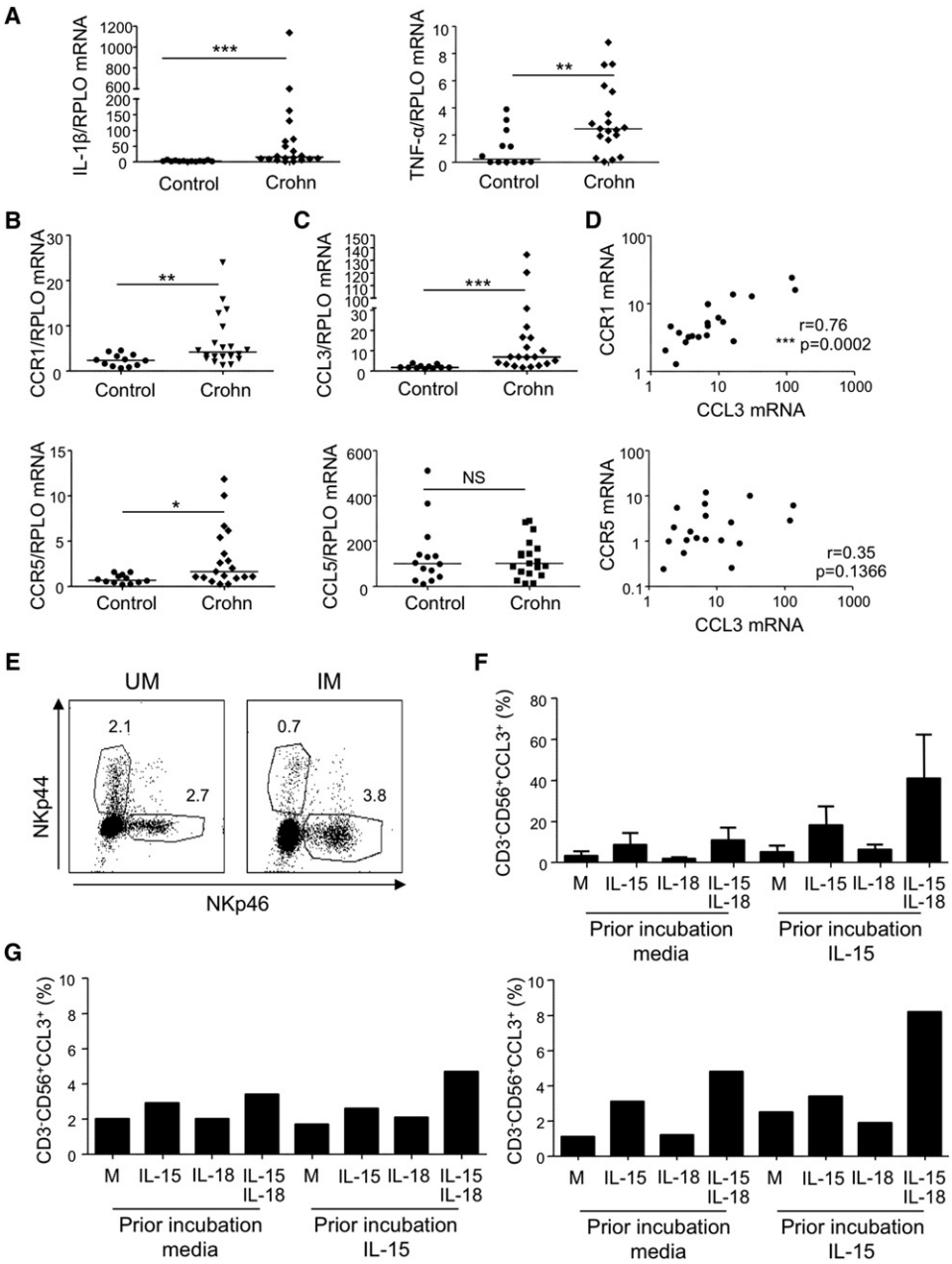

Figure 7. CCL3 and CCR1 Are Increased in Crohn's Disease

(A–C) qRT-PCR quantification of IL-1β, TNF-α (A), CCR1, CCR5 (B), CCL3, CCL5 (C), transcripts in intestinal biopsies from controls (n = 15) and from Crohn's disease patients (n = 19).

(D) Correlation between CCL3 and CCR1 transcripts (r = 0.76, p = 0.0002) and CCL3 and CCR5 transcripts (r = 0.35, p = 0.1366).

(E) Flow cytometry analysis showing an increase in $CD3^{-}NKp46^{+}CD56^{+}NKp44^{-}CD122^{+}CD127^{-}$ cells in inflamed ($CCR1^{+}$ inflamed mucosa) compared to unaffected (UM) mucosa from the same CD patient (one representative out of four experiments).

(F) Percentage of CCL3 producing peripheral $CD3^{-}NKP46^{+}CD56^{+}$ human NK cells after stimulation with 20 ng/ml IL-15 and/or IL-18 prior to or not 24 hr incubation with 20 ng/ml IL-15 (n = 3).

(G) Percentage of CCL3 producing $CD3^{-}NKP46^{+}CD56^{+}$ cells in unaffected (left) or inflamed (right) CD mucosa after the same stimulation as in (F) (one out of two experiments).

NS, not significant; *p < 0.05; **p < 0.005; ***p < 0.0005. See also Figure S6.

shown to play a prominent role for inflammatory monocyte migration into inflamed joints (Schall and Proudfoot, 2011). Our results provide evidence of a comparable role for CCR1 in the inflamed gut.

CCR1 has two main ligands, CCL3 and CCL5. Both chemokines were massively upregulated in the intestine of WT infected by *T. gondii* but only CCL3 was significantly less produced in $Il15^{-/-}$ mice, pointing to an IL-15-dependent source of CCL3.

Treatment of infected WT mice by a blocking CCL3 antibody decreased inflammatory monocytes recruitment, confirming a role for this chemokine. Reduction of inflammatory monocytes, however, was less drastic than in *Ccr1*$^{-/-}$ mice, either because of the complementary contribution of CCL5 and/or because of incomplete blockade by the antibody.

During *T. gondii* infection, CCL3 was first upregulated in LP NKp46^{+}NK1.1^{+}CD127^{-} NK cells and our results indicate that the latter cells were the source of CCL3 regulated by IL-15. Confirming and extending previous results (Satoh-Takayama et al., 2010), we observed that homeostasis of gut NK1.1^{+} NK cells depends on a stromal source of IL-15 that is presumably epithelial cells. Moreover, treatment with a depleting NK1.1 antibody reduced CCL3 production, inflammatory monocyte recruitment, and ileitis severity. The latter result is in keeping with a previous study showing that NK depletion via the asialoGM1 antibody markedly reduced intestinal inflammation during *T. gondii* infection although the mechanism was not elucidated (Khan et al., 2006). Our results indicate that NK-derived CCL3 controls inflammatory monocyte recruitment. NK cells produced CCL3 before and during inflammatory monocyte recruitment. At late time points, neutrophils and inflammatory monocytes became a complementary source of CCL3. CCL3 production by inflammatory monocytes may perhaps auto-amplify their recruitment but a major role of neutrophils was excluded because their depletion did not reduce the production of CCL3 or inflammatory monocyte recruitment.

In vitro analysis of the cytokines able to trigger CCL3 in murine NK cells suggested a role for IL-15 and/or IL-18. During *T. gondii* infection, an early and transient increase in IL-15 mRNA was documented whereas IL-18 secretion, probably derived from epithelial cells, was strongly induced later during infection. Neutralization of IL-15 or IL-18 in vivo by a transient antibody treatment demonstrated a role for IL-18 in CCL3 induction and inflammatory monocyte recruitment, although the role for IL-15 in this process was less clear. Surprisingly, blocking IL-18 did not improve but rather exacerbated intestinal lesions, consistent with recent evidence that epithelium-derived IL-18 serves a key protective role against intestinal epithelial damage (Elinav et al., 2011). In *T. gondii* ileitis, IL-18 may thus simultaneously promote the recruitment of inflammatory monocytes and protect against epithelial damage. Altogether, our results indicate that IL-15 controls the homeostasis of CCL3-producing intestinal NK cells whereas IL-18 induces CCL3 synthesis during *T. gondii* ileitis. Other complementary mechanisms may operate to stimulate CCL3 expression during *T. gondii* infection, including upregulation of IFN-α (Foureau et al., 2010) or induced ligands that may engage activating NK receptors (Ortaldo et al., 2001).

T. gondii-induced ileitis is one rare mouse model of acute inflammation affecting selectively the ileum and, as such, can be viewed as a possible model for Crohn's disease. Strikingly, TNF-α-producing inflammatory monocytes also play a central role and both IL-15 and IL-18 are upregulated in Crohn's disease-inflamed intestine (Liu et al., 2000; Monteleone et al., 1999a; Pizarro et al., 1999). Furthermore, it has recently been suggested that serum detection of IL-15 in Crohn's disease patients can predict their response to TNF-α antibodies (Bouchaud et al., 2010). Confirming previous work (Takayama et al., 2010), substantial numbers of LP NKp46^{+}CD56^{+}CD127^{-} NK cells were present in the intestine of Crohn's disease patients. Moreover, IL-15 and IL-18 exerted synergistic effects on the production of CCL3 by peripheral and intestinal NK cells, a result in keeping with the induction of IL-18R by IL-15 on human NK cells (Fehniger et al., 1999). Finally there was a positive correlation between the amounts of CCL3 and CCR1 mRNA in intestinal biopsies from Crohn's disease patients. It is therefore tempting to hypothesize that in Crohn's disease patients as well as in mice infected by *T. gondii*, intestinal NKp46^{+}CD56^{+} NK cells might produce CCL3 and thereby recruit TNF-α-producing CCR1^{+} inflammatory monocytes and enhance intestinal inflammation. Future work, however, is necessary to confirm this hypothesis and determine whether CCL3 or (more probably) CCR1 are meaningful therapeutic targets in Crohn's disease.

Recent work has enlightened the role of a spectrum of intestinal innate lymphoid cells in the early response to pathogens and/or intestinal inflammatory responses. A particular emphasis has been put on the subset that expresses NKp46^{+} in mice and NKp44^{+} in humans and produces IL-22, a cytokine important in the defense against bacteria (Colonna, 2009; Spits and Di Santo, 2011). Our results indicate that a distinct subset of IL-15-dependent gut NKp46^{+} cells may be activated during the response to intracellular pathogens and can, via CCL3, participate in the recruitment of CCR1^{+} inflammatory monocytes. IL-15-dependent NKp46^{+} cells may thereby participate in gut defense but also induce deleterious inflammation in and perhaps beyond the gut.

EXPERIMENTAL PROCEDURES

Mouse Models

8- to 12-week-old inbred male C57BL/6, *Il15*$^{-/-}$, *Prf1*$^{-/-}$, *B2m*$^{-/-}$, *Ifng*$^{-/-}$, *Tcrd*$^{-/-}$, and *Ccr1*$^{-/-}$ mice (provided by P. Murphy and J.L. Gao, NIAID, NIH, Bethesda, MD) were raised under specific-pathogen-free conditions in animal facilities of Institut Pasteur according to European guidelines. To produce hematopoietic chimeras, WT (CD45.1) or *Il15*$^{-/-}$ (CD45.2) recipient mice were lethally irradiated at 900 rads with a Ce^{137} source and intravenously injected with 5 × 10^{6} BM cells from either WT or *Il15*$^{-/-}$ donor mice. After 8 weeks, reconstitution was controlled with CD45.2 and CD45.1 markers on blood lymphocytes. For infection, mice were intragastrically gavaged with 35 cysts from *T. gondii* 76K strain and their weight and survival were monitored. Histology was evaluated on paraffin sections and parasitic load determined by quantitative PCR on day 7 p.i. (Reischl et al., 2003). For in vivo blocking or depletion experiments, WT mice received intraperitoneal injections of 100 μg of control isotypes or of CCL3 mAb (MAB450, IgG2a, R&D Systems) on days 1 and 2 p.i.; NK1.1 mAb (clone PK136, IgG2a, AbDserotec) on days −1 and +1; IL-18 rabbit-anti-mouse antibody (Acris) on days 1 and 2; and 50 μg of murine IL-15 mAb (clone M96, IgG1, gift from E. Butz, AMGEN) on days 3 and 4.

Controls and Patients

Peripheral blood was obtained from healthy adult volunteers. Endoscopic ileal biopsies from 19 patients with Crohn's disease and from 15 individuals undergoing a diagnostic procedure but with normal histology were obtained from patients (age 7–15 years) according to protocol AOM08087 approved by Ethical Committee Ile de France II. Ileal resection samples were from four additional Crohn's disease patients undergoing therapeutic surgery.

Lymphocyte Isolation and Cell Cultures

Mouse lymphocytes were isolated from blood, spleen, MLN, small intestinal epithelium, and LP as described (Mennechet et al., 2004). Peritoneal

mononuclear cells were harvested in cold PBS. Human PBL were isolated on Ficoll Hypaque gradient.

All cultures were performed in RPMI supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, 30 mM HEPES (GIBCO), and 0.05 mM β-mercaptoethanol (Biorad) at 37°C and in 5% CO_2. Intestinal biopsies (5 mm) were cultured alone for 24 hr in 48-well plates. Lymphocytes and peritoneal mononuclear cells (1 × 106/ml) were cultured in 96-well plates alone or in the presence of human IL-15 (R&D Systems) or of 10 μg/mL STAg (Grunvald et al., 1996). Production of IL-1β, IL-6, TNF-α, IFN-γ, or CCL3 was measured in culture supernatants by ELISA (R&D Systems). CCL3 secretion was induced with murine cytokines (R&D Systems) for mouse cells as indicated in Figure 6 or with human IL-15 and/or IL-18 (R&D Systems) for human cells.

Phenotyping and Lymphocyte Subset Isolation by Flow Cytometry

Isolated cells were incubated with Fc block (2.4G2, BD PharMingen) for 10 min and then stained with various mixes of directly coupled mAbs: Ly6C-FITC, Ly6G-PE, F4/80-PECy5, CD3-PE, CD3-APCCy7, NK1.1-APC, CD127-PECy7, NKP46-PECY5, CD11b-APCH7, CD11c-PECY7, αβTCR-APC (BD PharMingen), F4/80 PeCy5.5, CD45-eFluor450 (eBiosciences), Aqua view blue (Invitrogen), or control isotypes. For intracellular staining, LPL suspensions were incubated for 4 hr in culture medium added with 2 μl/ml Brefeldin A (Sigma-Aldrich) at 37°C. After surface staining, cells were fixed with 2% paraformaldhehyde (Euromedex), permeabilized with 0.5% saponin (Sigma), and stained intracellularly with IFN-γ-APC, TNF-α-APC (BD PharMingen), IL-22-PE (eBioscience) (murine cells), or CCL3-PE (human cells) mAbs (R&D systems). Cells were analyzed on a FACSCanto II or sorted with a FACSAria cell sorter II (BD Biosciences).

Quantitation of Gene Expression by Real-Time PCR

Tissues were harvested in RNA later and disrupted in RLT buffer (QIAGEN) with a FastPrep machine (MP Biomedicals). Cells were lysed in RLT buffer. RNA was extracted with RNAeasy kit (QIAGEN) and 1 μg was reversed transcribed to cDNA with Superscript II Reverse Transcriptase (Invitrogen). qRT-PCR was performed with TaqMan gene expression assays HPRT, IFN-γ, IL-1β, TNF-α, IL-6, CCL3-5, CCR1, CCR2, CCR5, and TaqMan Universal PCR master mix and GeneAmp 7000 machine (Applied Biosystems). cDNA samples were assayed in duplicates and expression was normalized relative to HPRT with Ct calculation.

Statistical Analysis

Groups were compared with GraphPad Prism software by unpaired Mann-Whitney test except for survivals, which were compared with Log-rank (Mantel-Cox) test.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at doi:10.1016/j.immuni.2012.05.013.

ACKNOWLEDGMENTS

The authors acknowledge funding from INSERM, Fondation Pour la Recherche Médicale, Agence Nationale pour la Recherche (ANR), Association François Aupetit, and Fondation Princesse Grace. J.S. was supported by a MNERT PhD fellowship. E.R.-P. and N.M. were supported by postdoctoral fellowships from Fundacion Espanola para la Ciencia y Tecnologia (FECYT), Fundacion Pedro I Pons, and from FRM and ANR, respectively. The authors are grateful to O. Cabaret and S. Bretagne (Hôpital Henri Mondor, Créteil) for help in quantifying parasitic loads and to C. Ducourneau (Faculté de Pharmacie, Tours) for help in preparing STAg.

Received: May 31, 2011
Revised: March 22, 2012
Accepted: May 3, 2012
Published online: June 14, 2012

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