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Differential Activity of IL-12 and IL-23 in Mucosal and Systemic Innate Immune Pathology

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Summary

The CD40-CD154 pathway is important in the pathogenesis of inflammatory bowel disease. Here we show that injection of an agonistic CD40 mAb to T and B cell-deficient mice was sufficient to induce a pathogenic systemic and intestinal innate inflammatory response that was functionally dependent on tumor necrosis factor-α and interferon-γ as well as interleukin-12 p40 and interleukin-23 p40 secretion. CD40-induced colitis, but not wasting disease or serum proinflammatory cytokine production, depended on interleukin-23 p19 secretion, whereas interleukin-12 p35 secretion controlled wasting disease and serum cytokine production but not mucosal immunopathology. Intestinal inflammation was associated with IL-23 (p19) mRNA-producing intestinal dendritic cells and IL-17A mRNA within the intestine. Our experiments identified IL-23 as an effector cytokine within the innate intestinal immune system. The differential role of IL-23 in local but not systemic inflammation suggests that it may make a more specific target for the treatment of IBD.

Introduction

The inflammatory bowel diseases (IBD) encompassing Crohn's disease (CD) and ulcerative colitis are chronic inflammatory disorders of the gastrointestinal tract that affect approximately 0.1% of Western populations (Bouma and Strober, 2003; Shanahan, 2002). Available evidence suggests that IBD involves an aberrant inflammatory response to intestinal bacteria in genetically susceptible individuals. The immune pathogenesis of CD is associated with increased inflammatory cytokines including tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), and interleukin-12 and interleukin-23 p40 (IL-12p40, IL-23p40). Accordingly, anti-TNF-α therapy has been shown to be beneficial in CD patients (Sandborn and Faubion, 2004). However, a limitation of this approach is that TNF-α also plays a pivotal role in protection from infection. Indeed, sustained blockade of TNF-α has been reported to increase susceptibility to infection such as reactivation of *Mycobacterium tuberculosis* (Sandborn and Faubion, 2004). These findings highlight the need to develop more specific strategies that discriminate the local pathogenic inflammatory response from systemic host protective immunity.

Interactions between CD40 and CD154 (CD40L) are important in the initiation and maintenance of T cell-mediated intestinal inflammation. In the inflamed intestinal tissue of human IBD patients, as well as in mouse models of IBD, $CD40^+$ antigen-presenting cells (APC) are found in association with $CD40L^+$ T cells (Liu et al., 1999, 2000; Polese et al., 2002). Importantly, blockade of CD40-CD40L interactions inhibits the development of colitis and can ameliorate established disease in mouse models (Cong et al., 2000; De Jong et al., 2000; Kelsall et al., 1996; Liu et al., 2000). These results suggest that after antigen encounter, activated $CD4^+CD40L^+$ T cells stimulate $CD40^+$ APC via the CD40L-CD40 pathway. This in turn leads to further activation of APC and T cells, establishing a positive feedback loop of immune activation (Diehl et al., 2000; van Kooten and Banchereau, 2000). In this type of inflammatory cascade, both $CD4^+$ T cells and APC can contribute to immunopathology with effector mechanisms including cytokine production (Strober et al., 2002).

CD40 stimulation of myeloid cells can lead to IL-12 production (Stuber et al., 1996). This cytokine plays a key role in the inflammatory response primarily due to its ability to induce IFN-γ production by T cells and NK cells (Trinchieri et al., 2003). In intestinal inflammation, administration of neutralizing IL-12p40 mAbs ameliorates colitis in several different models (Neurath et al., 1996; Simpson et al., 1998). This therapeutic effect is linked to reductions in IL-12-driven IFN-γ secretion as well as the induction of Fas-mediated apoptosis of Th1 cells (Fuss et al., 1999). In a recent study, anti-IL-12p40 treatment in CD led to an amelioration of inflammation in some patients (Mannon et al., 2004). Clinical improvement was associated with a reduction in the production of IL-12 and IFN-γ by mononuclear cells from the intestine. However, it is now known that IL-12p40 forms heterodimers not only with IL-12p35 (IL-12p35p40; IL-12p70) but also with IL-23p19 (IL-23p19p40). These results raise the possibility that activities previously ascribed to IL-12 may in fact be attributable to IL-23. Indeed, the immune pathological response in experimental autoimmune encephalomyelitis (Cua et al., 2003) and collagen-induced arthritis (Murphy et al., 2003) has been shown to be functionally dependent on IL-23 and not IL-12.

To assess the role of CD40-mediated effector function in the innate response and to further probe the

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role of IL-12 and IL-23 in this pathway, we injected T and B cell-deficient mice with an agonistic CD40 mAb. Anti-CD40 stimulation led to a systemic and local inflammatory disease characterised by wasting disease, splenomegaly, increases in serum inflammatory cytokines, and colitis. Functional analysis showed that the systemic inflammatory response was driven by IL-12 and not IL-23, whereas local intestinal inflammation required the presence of IL-23 and was independent of IL-12. These results newly identify IL-23 as a key effector cytokine within the innate intestinal immune system and point to divergent roles for IL-12 and IL-23 in local and systemic inflammation.

Results

Anti-CD40 Induces Wasting Disease and Colitis in T Cell-Deficient Mice

To assess the effects of CD40 stimulation on the activation of the innate immune system, we injected immunodeficient recombinase-activating gene 1-deficient (Rag1 KO) mice with 200 μg CD40 mAb. Clinically, the mice developed wasting disease as well as gastrointestinal symptoms including diarrhea and anal inflammation. CD40 mAb-treated Rag1 KO mice developed rapid weight loss, up to 20% within the first 4 days (Figure 1A). 7 days after antibody challenge, the weight was still substantially decreased compared to isotype- or PBS-treated control mice. At this time, all CD40 mAb-treated mice developed splenomegaly. Other pathological changes in these mice included hepatopathy, lymphadenomegaly of the mesenteric lymph node (MLN), and colon pathology, as indicated by swelling of the colon wall and presence of edema. 10 days after CD40 stimulation, the mice recovered clinically but still showed macroscopical signs of colon pathology, whereas 3 weeks after the initiation of the immune response, no pathology was observed (data not shown). None of these changes were seen when two different isotype control antibodies or PBS injections were used. C.B-17 SCID mice developed similar pathology after anti-CD40 stimulation (data not shown).

Consistent with macroscopic observations, 7 days after CD40 mAb challenge, mice had histological signs of colitis with pronounced epithelial hyperplasia and marked leukocyte infiltration within the lamina propria, goblet cell depletion, and epithelial cell destruction (Figures 1B and 1C and Figures S1A–S1C in the Supplemental Data available with this article online). 10 days after CD40 activation, there was still marked leucocytic infiltration, but by 3 weeks after CD40 stimulation, all histological changes had resolved (data not shown).

In summary, our results indicate that CD40 mAb treatment in immunodeficient mice induces weight loss and intestinal pathology.

Innate Immune Activation after Anti-CD40 Stimulation In Vivo

In line with the pathological and histopathological observations, marked increases in total leukocytes were found in the spleen and locally in the colon in anti-CD40-treated mice (Figure S1C). CD40 is abundantly expressed on CD11c^{hi} dendritic cells (DCs) of SCID or Rag1 KO mice (Figure 2A), and these cells became activated with increases in MHC-II, CD80, and CD86 expression at early time points after anti-CD40 stimulation (Figure 2A). There was also a marked increase in the density of CD11c^{hi} DC in leucocytic clusters in the colon (Figure 2B).

Changes in serum cytokines and chemokines were analyzed to determine whether these correlate with the development of anti-CD40-induced disease. Serum was assayed at day 3, when the anti-CD40-stimulated mice had their maximum weight loss and the first signs of colon pathology, as well as at day 7, when intestinal pathology peaked. Increased amounts of TNF-α, IL-6, IFN-γ, MCP-1, and IL-12p70 were found in CD40 mAb-treated mice. There was no significant change in IL-10 (Figure 2C).

Anti-CD40 treatment induced comparable local and systemic immune pathology in germ-free and specific pathogen-free (SPF) housed animals, suggesting that the CD40-mediated activation pathway is not dependent on the preactivation of the innate immune system by replicating resident bacteria (Supplemental Data and Figure S2).

Anti-CD40-Induced Immune Pathology Depends on Cytokine Secretion

To test whether proinflammatory cytokines played a functional role in the development of the anti-CD40-mediated immunopathology, Rag1 KO mice were coinjected with CD40 mAb together with anti-TNF-α, anti-IL-12 and IL-23 p40 (anti-p40), or anti-IFN-γ. Anti-p40 had the most marked effect, leading to inhibition of wasting disease, increases in serum cytokines, and colon immune pathology (Figures 3 and 4). Anti-TNF-α and anti-IFN-γ also significantly inhibited wasting, although neither afforded complete protection from increases in serum cytokine levels or colitis (Figure 3). Together the data indicate that proinflammatory cytokines play a key role in anti-CD40-induced systemic and intestinal immune pathology.

Differential Roles for IL-12 and IL-23 in Systemic and Gut Immunopathology

Anti-IL-12p40 neutralizes the activity not only of IL-12 (p35p40) but also IL-23, which is composed of a heterodimer of p40 with p19 (Trinchieri et al., 2003). To unravel the role of IL-23 in this system, immunodeficient mice were coinjected with anti-CD40 together with neutralizing anti-IL-12 and -23p40 (anti-p40), anti-IL-23p19 (anti-p19), or an isotype control antibody. Anti-p40 inhibited systemic immune activation as well as local colonic pathology (Figure 4). In contrast, anti-CD40 and anti-p19 coinjected mice developed wasting disease (Figure 4A) and showed serum amounts of the inflammatory mediators MCP-1, IL-6, and TNF-α that were similar to anti-CD40-treated controls (Figure 4B). Strikingly, anti-IL-23p19-treated mice did not develop signs of intestinal inflammation (Figure 4C). These experiments suggest differential roles for IL-12 and IL-23 in systemic and mucosal innate immunopathology.

To further investigate this, we crossed mice that are deficient for IL-12p35 or IL-23p19 as well as IL-12 and IL-23p40 onto a Rag1-deficient background (Rag1 p35 DKO, Rag1 p19 DKO, Rag1 p40 DKO). These double knockout mice were injected with anti-CD40. Rag1 p40

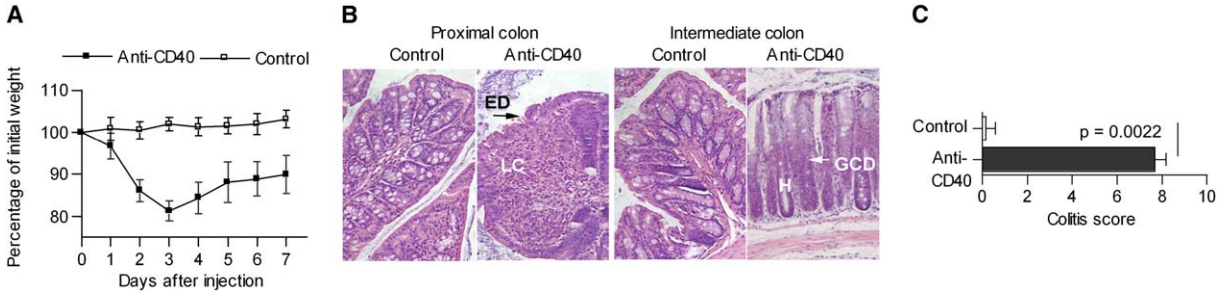

Figure 1. Anti-CD40 Stimulation Induces Wasting Disease and Intestinal Inflammation

Rag1 KO mice received 200 μg CD40 mAb i.p. Control mice received isotype control antibodies or PBS.

(A) Weight as a percentage of the initial weight at day 0. Data represent the mean weight ± 1 SD and is pooled from ≥5 independent experiments (n = 19–35 mice per group).

(B) H&E staining of proximal and intermediate colon. Epithelial hyperplasia (H), epithelial cell damage (ED), leucocytic cell clusters (LC) in the lamina propria, and a depletion of goblet cells (GCD) were found after CD40 stimulation. Original magnification 200×. Histology is representative for >20 mice per group.

(C) Colitis score. Data represent the mean ± 1 SD of six mice per group.

DKO mice were protected from anti-CD40-induced wasting disease and splenomegaly, had reduced serum cytokine concentrations, and were protected from intestinal pathology (Figures 5A–5D). Rag1 p35 DKO did not develop wasting disease after anti-CD40 stimulation, but intestinal inflammation was present (Figures 5A and 5D). In these mice, reduced splenomegaly was detected, and significantly reduced serum TNF-α was found compared to anti-CD40-treated Rag1 KO mice (Figures 5B and 5C). In contrast, anti-CD40-treated Rag1 p19 DKO mice developed wasting disease, splenomegaly, and elevated serum cytokine amounts (Figures 5A–5C). Despite this systemic immune activation, these mice were protected from intestinal pathology (Figure 5D). To test whether the activity of IL-12 and IL-23 involves the modulation of the intestinal cytokine response, we determined proinflammatory cytokine concentrations within the intestine in the anti-CD40-treated mice. Compared to control mice, anti-CD40-treated mice had increased amounts of colonic TNF-α, MCP-1, and IL-6 (Figure 5E). Similar or greater increases were also present in Rag1 p35 DKO mice, which is in line with the presence of colitis in these mice. In contrast and again consistent with disease score, elevations in inflammatory cytokines were abrogated in anti-CD40-treated Rag1 p40 DKO and Rag1 p19 DKO mice (Figure 5E).

In summary, our experiments demonstrate that CD40 stimulation initiates a complex innate immune cascade that drives the development of systemic and local gut immune pathology. In this model, we find that the inflammatory cytokines TNF-α and IL-12 are preferentially linked to the systemic innate immune response, whereas the activity of IL-23 is linked primarily to the intestinal inflammatory response with little functional role in systemic immune activation.

Differential Expression of IL-12 versus IL-23 in Spleen and Colon

To determine whether IL-23 and IL-12 are produced in the intestine, we analyzed the expression of IL-23 p19, IL-12 p35, and IL-12 p40 mRNA in colon and spleen at D7 after CD40 mAb injection via real-time reverse-transcription polymerase chain reaction (RT-PCR). Relative to untreated control spleen or colon, there was an increase in both IL-23 p19 and IL-12 p35 subunits in both compartments after anti-CD40 stimulation (Figure 6A). We did not observe an increase in IL-12 p40 subunit, because p40 mRNA was expressed at a high amount prior to injection (data not shown). Consistent with the mRNA expression data, increased concentrations of IL-23 protein were detected in the culture supernatants of colon from anti-CD40-treated mice compared to controls (Figure 6B).

Anti-CD40 stimulation led to myeloid cell activation and accumulation, so we next investigated whether these cells are a potential source of IL-23. $CD11c^{hi}$ (DC) and $CD11b^{+}CD11c^{-}$ (monocytic) cells were isolated by FACS sorting from spleen and colon 7 days after anti-CD40 stimulation, and IL-23 p19, IL-12 and IL-23 p40, and IL-12 p35 gene expression was determined by real-time RT-PCR. Relative to HPRT, the highest amount of anti-CD40-induced IL-23 p19 mRNA was found among colonic DC (Figure S3). These cells expressed a striking 568-fold increase compared to resting splenic DC and 69-fold higher amount than activated splenic DC (Figure S3). This is likely to reflect functional IL-23, because both splenic and colonic DC populations expressed high amounts of IL-12 and IL-23 p40 (Figure S3). Colonic monocytes may also contribute to local IL-23 production, as indicated by the fact that they too expressed high amounts of IL-23 p19 mRNA (Figure S3). As expected, there was also an increase in IL-12 p35, relative to amounts in resting splenic populations, among DC and monocyte or macrophages isolated from the spleen and colon of anti-CD40-treated mice. The IL-12 p35 increase was largest among colonic monocyte or macrophages (106-fold higher). It is worth noting, however, that for activated colonic DC, the increase in IL-12 p35 (7-fold) was substantially lower than the increase in IL-23 p19 (568-fold). Together, the data suggest that anti-CD40 stimulation induces IL-23 expression by both DC and monocyte or macrophage populations and that for the former this is most pronounced in the colon.

Immune pathological responses associated with IL-23 are thought to involve promotion of IL-17-producing T cells (Hunter, 2005; McKenzie et al., 2005). To determine whether IL-23-mediated innate inflammation

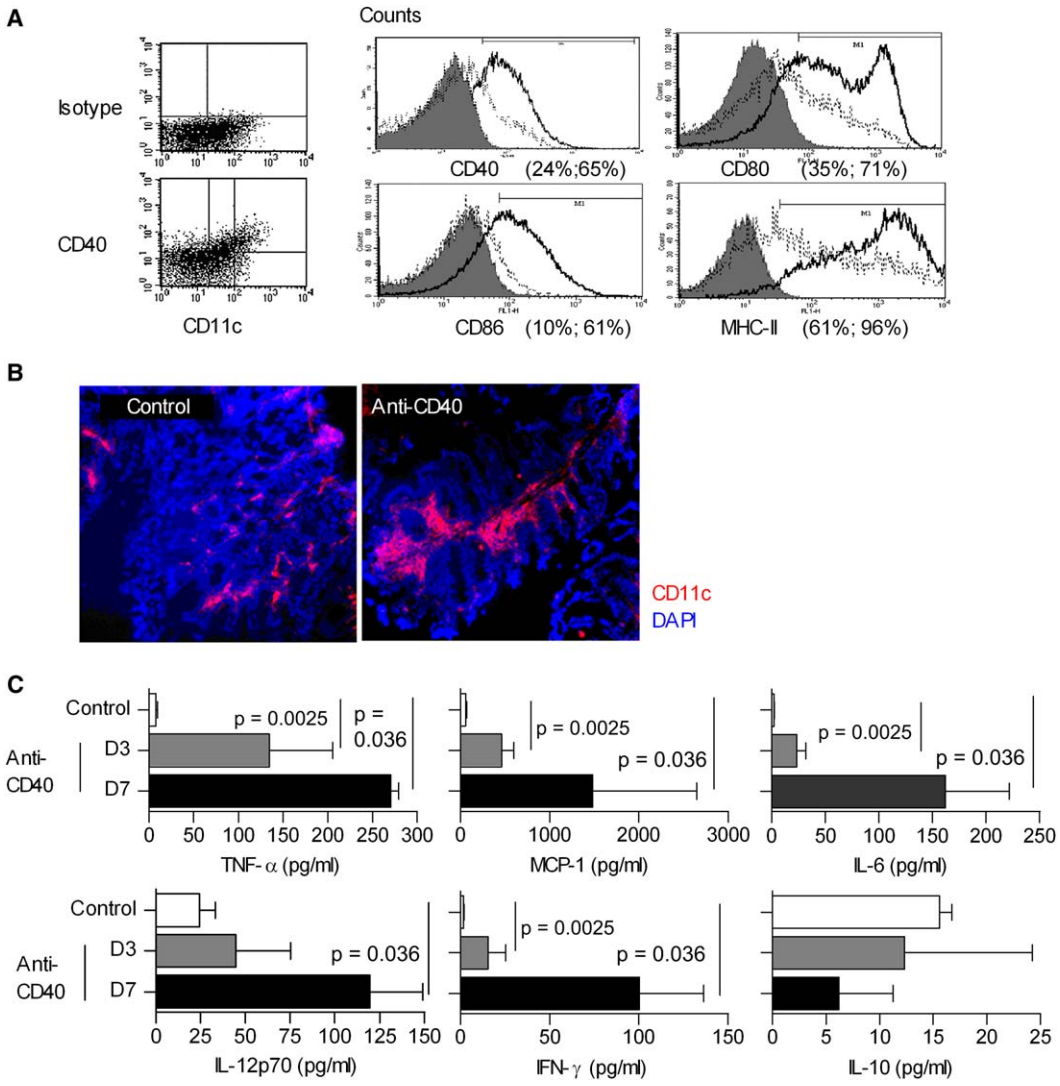

Figure 2. Immune Activation after Anti-CD40 Stimulation

Rag1 KO mice received 200 μg CD40 mAb i.p. or isotype antibody.

(A) Left: Dot plot of FACS staining for CD11c and isotype as well as CD11c and CD40 of mesenteric lymph node cells (MLN) of untreated SCID mice. Right: Rag1 KO MLN cells were isolated at day 3 after CD40 mAb and isotype treatment, pooled from six mice per group, and analyzed for CD40, CD80, CD86, and MHC-II on $CD11c^+$ gated cells. Histogram plots show activation markers of Rag1 KO mice after CD40 mAb (bold line) or isotype treatment (dotted line). Isotype control for the respective activation marker is shown for anti-CD40-treated mice (filled area). Isotype control-treated mice expressed a similar isotype staining as the CD40 mAb-treated mice (not shown). Numbers indicate the percentage of cells that are positive for the respective activation marker (Isotype; Anti-CD40). Similar results were obtained when spleen cells were analyzed.

(B) CD40 mAb treatment results in the accumulation of $CD11c^+$ cells in the colonic lamina propria of Rag1 KO mice. Large clusters of $CD11c^+$ cells are present in the proximal colon of anti-CD40-treated mice. The scattered distribution of $CD11c^+$ cells present in isotype control-treated mice is shown for comparison. Histological pictures are representative of $n \geq 4$ mice per group. Original magnification 200×. Isotype control gave no staining.

(C) CD40 mAb treatment leads to increased serum amounts of IL-6, MCP-1, IFN-γ, IL-12p70, and TNF-α. Serum cytokines concentrations were analyzed 3 and 7 days after injection. Data represent mean ± SD. Data points lower than standard curve data were set as 1 pg/ml. Significance was tested by the Mann and Whitney U test. Data from two independent experiments were pooled (n = 3–7 mice per group). A further independent experiment gave a similar result.

is also linked to increased IL-17 production, we examined the expression of IL-17 mRNA (now termed IL-17A) in colon and spleen of Rag1 KO mice after CD40 mAb injection. As shown in Figure 6C, compared to colons from unstimulated mice, there was a 65-fold increase in IL-17 transcripts after anti-CD40 stimulation. However, no such increase was observed in the spleen. As with other inflammatory cytokines, increases in colonic IL-17 mRNA were dependent on IL-23 and not IL-12 (Figure 6D). These results indicate that anti-CD40 stimulation induces IL-17 mRNA production by non-T cells and that this response is localized to the colon.

Discussion

The IBDs are of complex multifactorial pathogenesis and involve the activation of the innate and adaptive immune system. Animal models have suggested

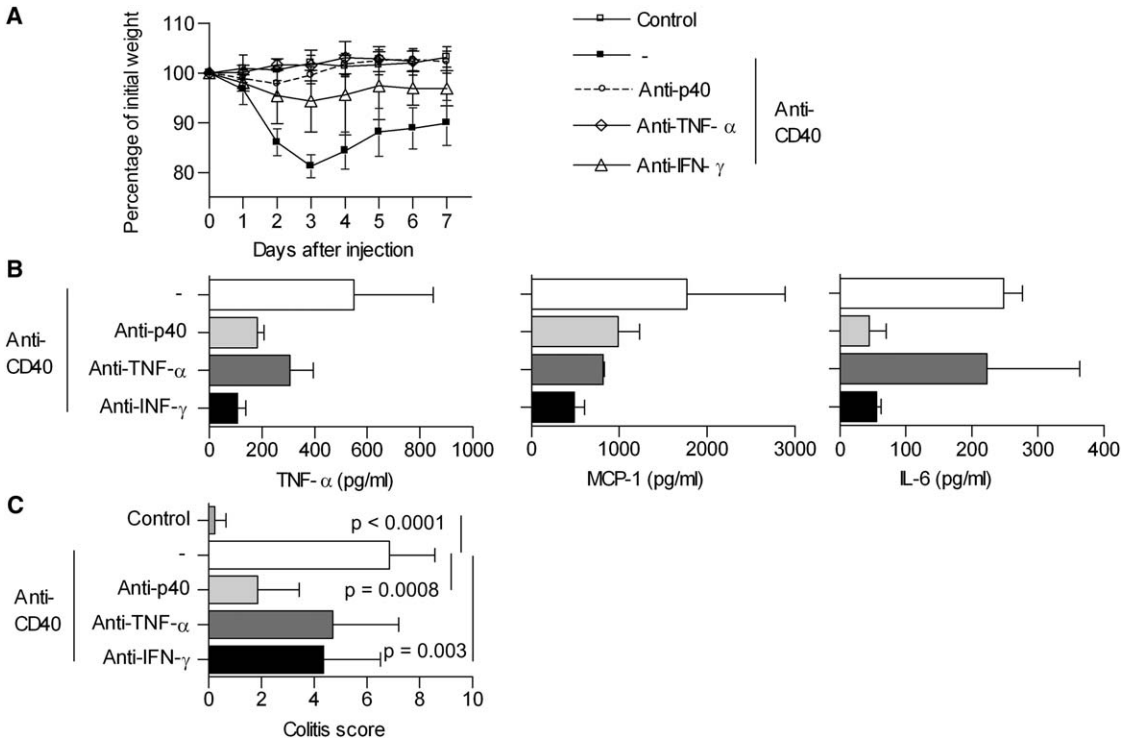

Figure 3. Anti-CD40-Induced Immunopathology Depends Functionally on TNF-α, IL-12 and IL-23p40 as well as IFN-γ

Rag1 KO mice recieved 200 μg CD40 mAb i.p. or isotype antibody. In some groups, anti-IL-12/23p40 (500 μg), anti-TNF-α (1 mg), or anti-IFN-γ (1–2 × 2 mg) were coadministered together with anti-CD40 and analyzed for weight, serum cytokine concentration, and development of colitis. Data represent mean ± SD. Significance was tested by the Mann and Whitney U test.

(A) Weight as a percentage of the initial weight. Data from ≥2 experiments were pooled.

(B) TNF-α, IL-6, and MCP-1 serum concentration at day 7 (n = 3 per group).

(C) Colitis score (n = 7–15 mice per group).

T cell-dependent and -independent mechanisms of intestinal inflammation (Strober et al., 2002). In IBD, there is evidence that alterations in the innate immune response contribute to disease development because mutations in the *NOD2* gene, which lead to abnormal function of this innate pathogen recognition receptor, confer susceptibility to CD in some patients (Eckmann and Karin, 2005). In IBD patients as well as in many model situations, the CD40L-CD40 pathway plays an important role in the crosstalk between the innate and adaptive immune response. We therefore developed a model where CD40L-expressing activated T cells were replaced with an agonist CD40 mAb and demonstrate that CD40-mediated effector function, in the absence of further T cell help, is sufficient to induce a pathogenic systemic and local inflammatory response. Anti-CD40-induced disease is accompanied by accumulation of myeloid cells including activated cytokine-producing DC in spleen and colon. Production of the inflammatory cytokine IL-12 and IL-23p40 subunit plays a pivotal role in the pathogenesis of systemic and mucosal disease. To date, the functions of IL-23 have only been described in models that involve T cells (Cua et al., 2003; Murphy et al., 2003). However, our data reveal distinct roles for IL-12 and IL-23 within the innate immune system. Thus, IL-12 is a key molecule for systemic immune activation whereas IL-23 drives local intestinal inflammation.

With our model of innate immune activation, we show that a number of proinflammatory cytokines are associated with weight loss and colon pathology. Inhibition of TNF-α and IFN-γ had some protective effect on the systemic immune pathology and colitis, but only blockade of the p40 subunit of IL-12 and IL-23 completely prevented wasting and colitis. These results suggest that IL-12 and IL-23p40 is a key factor within the proinflammatory cytokine cascade. There is considerable evidence that early expression of IL-12 and/or IL-23 directs a variety of downstream effectors of inflammation (for reviews, see Hunter, 2005; Langrish et al., 2004; Trinchieri et al., 2003; McKenzie et al., 2005). Thus, it is likely that the inflammatory cascade induced by CD40 ligation triggers the production of IL-12 and/or IL-23, which induces the release of other proinflammatory cytokines such as TNF-α, IFN-γ, and IL-6 by DC and macrophages as well as IFN-γ by NK cells (Ma, 2001; Mason et al., 2002). Indeed, we found that myeloid cells, in particular CD40-expressing $CD80^+CD86^+MHC\text{-}II^+CD11c^+$ DC become activated after anti-CD40 stimulation and that IL-12 and IL-23 mRNA-producing monocytic cells and DCs accumulate within the colon and spleen. The presence of IFN-γ acts synergistically with CD40 stimulation (Cua et al., 2003; Isler et al., 1999), making it likely that, after anti-CD40 stimulation, significant synergistic interactions between different cytokines and cell types contribute to immunopathology.

Our dissection of the role of IL-23 and IL-12 in anti-CD40-induced pathology revealed a surprising distinction in the regulation of systemic and tissue-specific

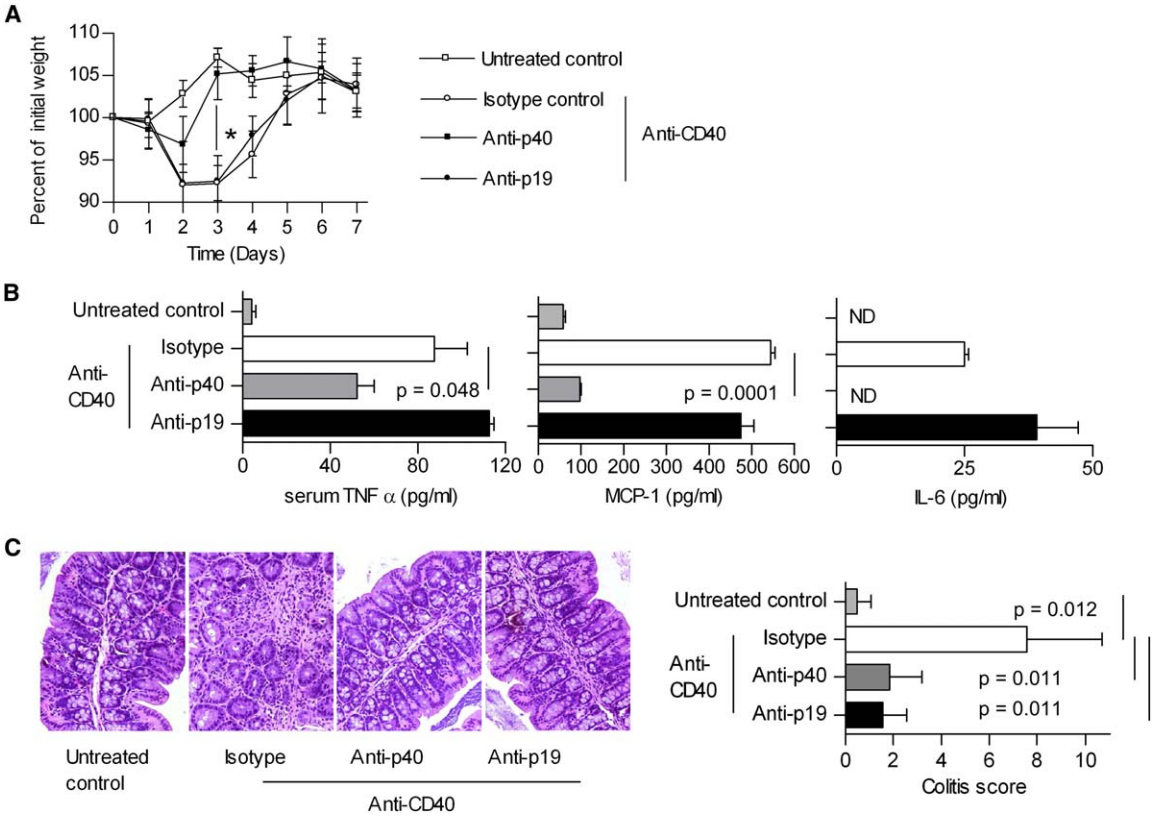

Figure 4. CD40-Induced Intestinal Immunopathology Depends Functionally on IL-23p19

Rag1 KO mice were either left untreated or injected with 125 μg of anti-CD40. In some groups, IL-12 and IL-23p40 (anti-p40; 500 μg), IL-23p19 (anti-p19, 1 mg), or isotype control (1 mg) mAbs were coinjected together with anti-CD40 at day 0 and 3 and analyzed for weight, serum cytokine concentration, and development of colitis. Data are representative of three independent experiments. Mean value is shown ± SD, and significance was tested by the Mann and Whitney U test.

(A) Weight as a percentage of the initial weight (n = 7 per group); *untreated versus isotype control $p < 0.001$ and isotype control versus anti-p19 $p < 0.001$.

(B) TNF-α, IL-6, and MCP-1 serum concentration at day 3 (n = 7 per group). ND, not determined.

(C) H&E staining of proximal colon at day 7. Anti-CD40-mediated pathology including epithelial hyperplasia, lamina propria infiltration, and a reduction of goblet cells was completely inhibited by blocking with anti-p40 or anti-p19. Colitis score (n = 4–7 mice per group).

inflammation. Where IL-23 was essential for local innate inflammation in the colon, it played no role in systemic inflammatory changes such as weight loss, splenomegaly, and elevated serum cytokines. On the other hand, animals with specific IL-12 deficiency (p35 KO) still developed colitis but were protected from acute systemic inflammation, including rises in serum proinflammatory cytokines, indicating that IL-12 is not required for innate mucosal pathology but is required for systemic responses induced by anti-CD40 treatment. These results suggest that IL-12 and IL-23 define an axis of innate regulation that differentially governs systemic and local inflammatory responses.

Precisely how IL-12 controls the systemic inflammatory response has not been investigated here. However, it seems likely that anti-CD40-induced myeloid cell activation is dependent on an autocrine activation loop driven by IL-12. Thus, after CD40 ligation, myeloid cells produce a burst of inflammatory cytokines including IL-12, which leads to IFN-γ production by NK cells and other cells. This in turn can feed back to induce further myeloid cell activation, resulting in increased IL-12 as well as proinflammatory TNF-α, MCP-1, and IL-6.

In mice with anti-CD40-induced colitis, there was a marked increase in IL-23p19 mRNA produced by $CD11c^{hi}$ DCs, indicating that these cells not only are involved in the initiation of the IL-23-mediated cascade but also contribute to IL-23 production under pathogenic conditions. Consistent with these results, increased IL-23 production by mouse DC has been reported after in vitro anti-CD40 stimulation (Krajina et al., 2003; Morelli et al., 2001). Furthermore, the production of IL-23 but not IL-12 has been found in $CD11c^{+}$ cells of the terminal ileum as a consequence of bacterial stimulation (Becker et al., 2003). The ability of anti-IL-23p19 to inhibit local but not systemic immune responses suggests that IL-23 may be an important mediator of tissue inflammation where it may amplify the inflammatory response via effects on activated macrophages or dendritic cells (Cua et al., 2003). Indeed, not only the production of IL-23 but also expression of the IL-23 receptor and functional stimulation via IL-23 have been shown in murine and human dendritic cells and macrophages (Krajina et al., 2003; Morelli et al., 2001; Oppmann et al., 2000; Parham et al., 2002), which supports the basis of an autocrine mode of amplification within the innate immune

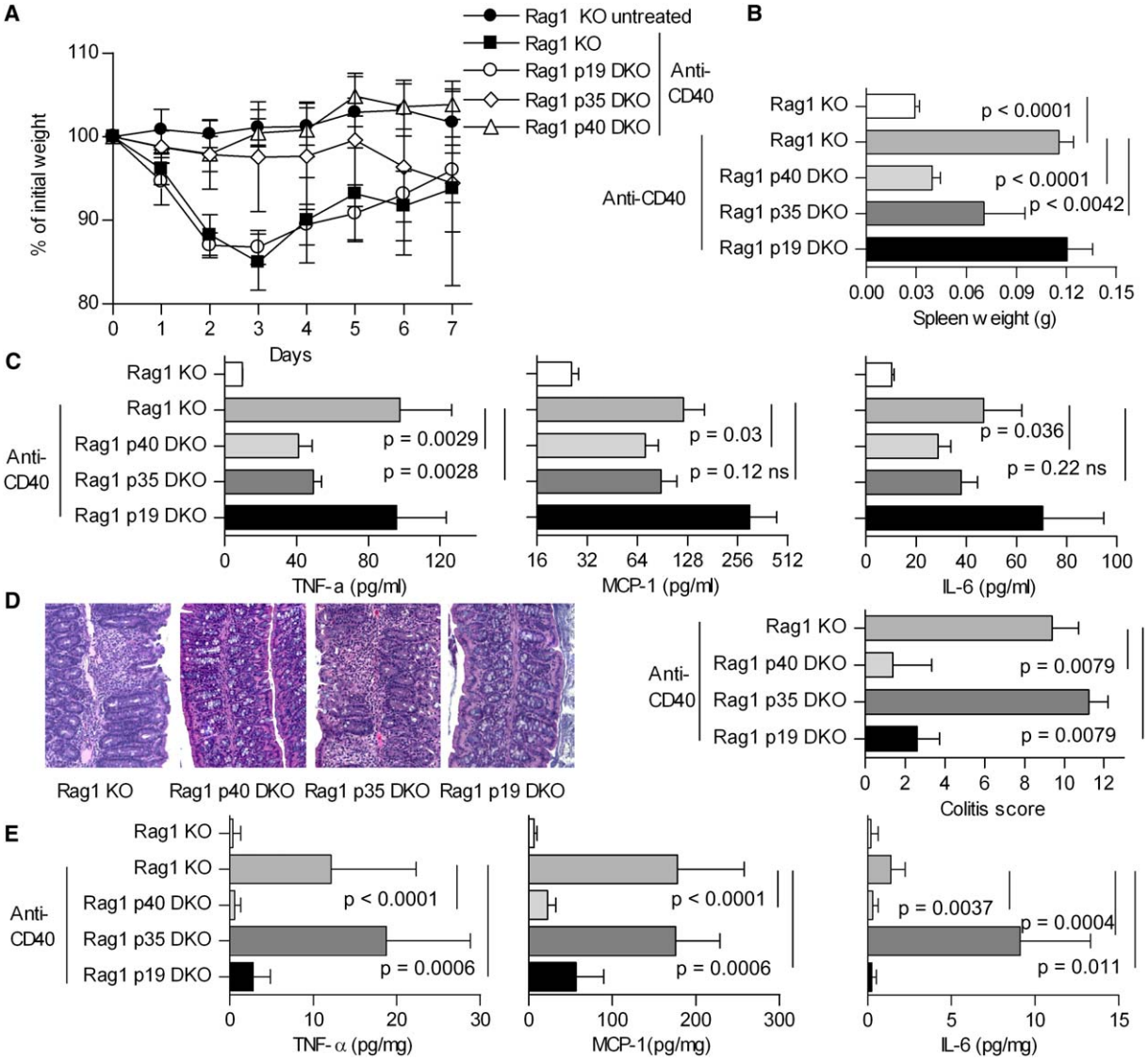

Figure 5. Differential Role of IL-12 and IL-23 in Systemic and Mucosal Innate Immunity

Rag1 KO mice as well as double knockout mice (Rag1 p40 DKO, Rag1 p35 DKO, and Rag1 p19 DKO) were injected with 125 μg anti-CD40. Data are representative of three independent experiments. Mean value is shown ± SD.

(A) Weight as a percentage of the initial weight (n = 6 per group).

(B) Spleen weight at day 7 (n = 6 per group).

(C) TNF-α, IL-6, and MCP-1 serum concentration at day 3 (n = 6 per group).

(D) H&E staining of proximal colon at day 7. Anti-CD40-mediated pathology including epithelial hyperplasia, lamina propria infiltration, and a reduction of goblet cells was completely prevented in Rag1 p40 DKO and Rag1 p19 DKO but not Rag1 p35 DKO mice. Colitis score (n = 4–5 per group).

(E) Cytokine concentrations in colon homogenates at day 7 were measured by CBA and normalized to total protein content for each sample. Results represent mean cytokine levels from two experiments (n = 6–9 mice per group).

system. Thus, IL-23 production is likely to be a primary step in the mucosal inflammatory autocrine cascade that drives local expression of proinflammatory mediators. In support of this, there was increased production of IL-23 in the colon compared to the spleen after anti-CD40 stimulation, and increases in colonic inflammatory cytokines such as TNF-α and IL-6 were dependent on IL-23. However, since IL-23 production in our model is not restricted to myeloid cells within the colon, it seems unlikely that the tissue-specific activity of IL-23 is solely a consequence of increased IL-23 production.

Investigation of IL-23-mediated effects within different T cell-dependent models has revealed that IL-23 promotes the development of autoreactive T cells that mediate destruction via release of the proinflammatory cytokines IL-17, IL-6, and TNF-α (Langrish et al., 2005). On the basis of their unique development and function, these IL-17-producing T cells have been grouped into their own lineage, now termed Th17 (Harrington et al., 2005; Langrish et al., 2005; Park et al., 2005; McKenzie et al., 2005). Through neutralizing antibody and gene ablation studies, we now show that IL-23 is the key driver

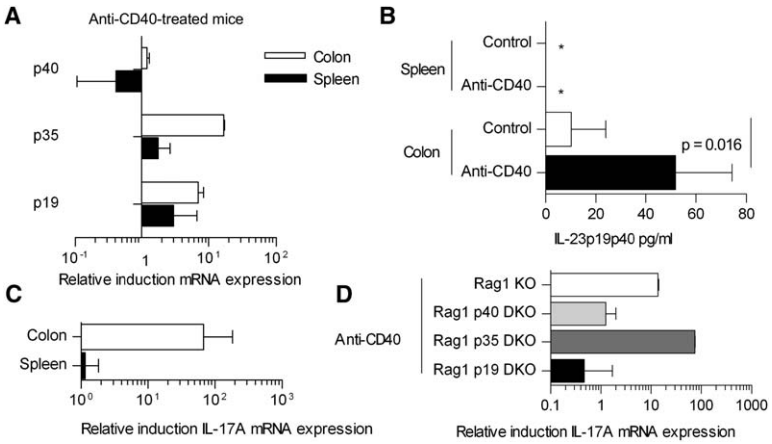

Figure 6. Induction of IL-23 and IL-17 after Stimulation with Anti-CD40 In Vivo

Rag1 KO mice were injected with anti-CD40 i.p. At day 7, spleen and colon homogenate mRNA were assayed via real-time RT-PCR. Mean ± SD are presented.

(A) Induction of IL-23 p19 mRNA expression in the colon. IL-23 p19, IL-12 p35, and IL-12 and IL-23 p40 mRNA expression was determined in isotype or anti-CD40-treated mice at day 7 (n = 2–6 per group). Data are normalized for HPRT mRNA content and expressed as fold change over untreated spleen or over untreated colon.

(B) Induction of IL-23 protein (IL-23p19p40) in colon after anti-CD40 stimulation. Spleen and colon pieces from anti-CD40 and control mice were cultured for 24 hr. IL-23 protein in the supernatant was measured by ELISA (n = 4–5 per group). Significance was tested by the Mann and Whitney U test. *not detectable.

(C) Expression of IL-17-A mRNA is induced in colon after anti-CD40 stimulation (n = 6 per group). Data are normalized for HPRT mRNA content and plotted as fold change over untreated spleen or over untreated colon. Data represent the mean ± SD and are pooled from two experiments.

(D) Expression of IL-17-A mRNA induced after anti-CD40 stimulation depends on IL-23.

Rag1 KO, Rag1 p40 DKO, Rag1 p35 DKO, and Rag1 p19 DKO mice were injected with 200 μg anti-CD40 (n = 4–6 mice per group). Expression of IL-17-A mRNA was assayed in colon via real-time RT-PCR. Data are normalized for HPRT mRNA content and plotted as fold change over untreated colon.

of T cell-independent mucosal inflammation. Thus, in this setting, IL-23 promotes tissue destruction that is not mediated via induction of IL-17-producing T cells. However, somewhat unexpectedly we found that anti-CD40 stimulation in Rag1 KO mice led to an increase in IL-17 mRNA expression, indicating T cell-independent sources of IL-17. Strikingly, IL-17 induction was evident in the colon but not the spleen, providing a potential mechanism for our findings that IL-23 drives intestinal but not systemic immune pathology. Like other inflammatory cytokines, induction of IL-17 mRNA in the colon was dependent on IL-23 and not IL-12. Further experiments are required to identify the innate source of IL-17 and to test its functional role. In addition to effects on IL-17, we have previously shown that IL-23 synergises with anti-CD40 to induce TNF-α, IL-1β, and IFN-γ production by macrophages (Cua et al., 2003), suggesting that IL-23-mediated macrophage/myeloid cell activation may also contribute to intestinal pathology.

In conclusion, the experiments presented here support a role for the innate immune system as a potent effector arm involved in intestinal tissue pathology. We uncover a striking dichotomy in the regulation of systemic and local inflammation, where IL-12 promotes innate systemic responses and IL-23 directs local inflammation. These experiments raise the possibility that some of the therapeutic effects of anti-IL-12/23p40 mAbs in colitis models (Neurath et al., 1995; Schmidt et al., 2002) and in CD (Mannon et al., 2004) may be mediated via effects on IL-23. Our results suggest that IL-23 and activated IL-23-secreting DC/myeloid cells may be effective targets for therapeutic strategies in tissue-specific inflammatory conditions such as IBD. Blocking IL-23 may inhibit not only maintenance of pathogenic Th17 responses but also local myeloid effector cell responses. Importantly, our results suggest that targeting IL-23 may be effective for the treatment of tissue-restricted inflammatory conditions such as IBD while maintaining the ability to mount protective immune responses.

Experimental Procedures

Mice

CB-17 SCID (SCID), C57BL/6 recombinase-activating gene 1-deficient (Rag1 KO), and double deficient for recombinase-activating gene 1 as well as IL-12 or IL-23 or IL-12 and IL-23 (Rag1 p35 DKO, Rag1 p19 DKO, Rag1 p40 DKO) mice were bred under specific pathogen-free conditions and kept in microisolators with filtered air at the Pathology Support Building of the Sir William Dunn School of Pathology (Oxford, UK) and the DNAX Research Institute (Palo Alto, CA). Sentinel animals from the SCID and Rag1 KO mouse colonies were tested to be free from the intestinal pathogens *Helicobacter spp.* and *Citrobacter rodentium*. Mice were used at 7–12 week of age. Experiments were performed according to the UK Scientific Procedures Act 1986.

In Vivo Antibody Treatment

Mice were injected i.p. with the anti-CD40, IgG2a monoclonal antibody FGK45 (Rolink et al., 1996). FGK45 was purified from hybridoma supernatant via affinity chromatography. If not stated differently, 200 μg FGK45 in PBS was used. Isotype control rat IgG2a κ R35-95 (BD Pharmingen) or IgG2a GL117 (Ferlin et al., 1996) as well as PBS injections were used in age- and sex-matched control mice. No differences were observed between isotype control and PBS-treated animals (n > 10 for each group). After antibody injection, mice were observed daily and—if not otherwise stated—sacrificed after 7 days or when they were moribund or had lost up to 20% of their initial weight.

To interfere with specific cytokine activities in vivo, mice received 1 mg TNF-α blocking mAb (clone XT22), 2–4 mg of IFN-γ blocking mAb (clone XMG1.2), 500 μg of IL-12 and IL-23 p40 mAb (anti-p40, clone CB17.8.20), or IL-23 p19 (anti-p19, clone MB490; B.S.M. and D.J.C., unpublished data) i.p. together with anti-CD40 (clone FGK45). Control mice received IgG2a (clone GL117), IgG1 (clone 27F11), or PBS together with FGK45.

The amount of lipopolysacharide (LPS, endotoxin) in all FGK45 antibody preparations was less than 1 endotoxin unit (limulus amebocyte lysate assay) per dose and mouse. The antibody preparations that were used in the germ-free experiment were screened for sterility and low endotoxin content.

FACS

The following antibodies were used for flow cytometry: anti-mouse MHC class II I-Ad/I-Ed FITC (2G9), CD11c PE (HL3), CD40 FITC (HM40-3), CD80 FITC (16-10A1), and CD86 FITC (GL1), as well as isotype control antibodies for activation markers (all PharMingen).

Stained cells were analyzed with BD FACS Calibur or FACS Sort and CellQuest Software (Becton Dickinson, San Jose, CA).

Tissue Preparation

Cells of spleen and MLN were prepared essentially as described previously (Malmstrom et al., 2001). Spleen and MLN were cut into pieces and incubated for 25 min under agitation at 37°C in the presence of 1 mg/ml collagenase/dispase (Sigma, St. Louis, MO) and 100 U/ml DNase (Sigma) before 5 min of deaggregation in the presence of 10 mM EDTA. The tissue was then passed through a 70 μm membrane to generate single-cell suspensions.

Lamina propria (LP) lymphocytes were purified as described (Powrie et al., 1994). In brief, colon tissue was cut into 0.5 cm pieces and incubated in Ca- and Mg-free PBS containing 10% heat-inactivated FCS (GIBCO-BRL) and 5 mM EDTA to release intraepithelial lymphocytes. The remaining tissue was further digested with collagenase/dispase (100 U/ml; Sigma Chemical Co.), and the LP cells were then layered on a 30%/40%/75% Percoll gradient (Amersham Pharmacia Biotech). Cells were recovered after centrifugation (600 × g, 20 min) at the 40%/75% Percoll interface.

Histology

For H&E histology, tissue samples were fixed in 3.7% formalin. Paraffin-embedded sections were cut and stained with haematoxylin and eosin. 5 μm colon sections were analyzed. By adding the individual parameter epithelial hyperplasia, goblet cell depletion, lamina propria infiltrate, and epithelial cell damage (0 no pathology, 1 mild changes, 2 intermediate, 3 severe changes), a colitis score (0–12) was calculated. The degree of epithelial hyperplasia and the lamina propria infiltrate were measured quantitatively.

For immunohistochemistry, tissue samples were snap frozen in OTC medium (Sakura, Zoeterwude, The Netherlands). After sectioning, the 6 μm slides were acetone fixed and processed including blocking steps with 10% normal goat serum and blocking of endogenous peroxidase with H_2O_2 as well as glucose oxidase and sodium azide. The primary monoclonal antibodies against mouse CD11c (HL3 or N418) were detected with POD-labeled donkey anti-hamster antibodies (Jackson ImmunoResearch, West Grove, PA) and the tyramide amplification method with Cy5-tyramide (NEN, Boston, MA). Cell nuclei in the tissue were counterstained with DAPI (Sigma). Zeiss Axioplan microscopes were used including a Kodak Spot-2 digital camera system and image software (Diagnostic Instruments Inc., MI).

Detection of Cytokines via a Cytometric Bead Array System and ELISA

25 μl undiluted mouse serum samples were used for the parallel detection of mouse IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12p70 in mouse serum. The Luminex assay system (Luminex Corporation) or the mouse inflammation bead array system (BD Biosciences, UK) was used according to the manufacturer's instructions and analyzed with a FACSCalibur (Becton Dickinson).

Frozen intestinal tissue samples (pooled from 2–3 mice per group) were homogenized in PBS containing a cocktail of protease inhibitors (Roche) via a Polytron homogenizer. After centrifugation at 10,000 × g to pellet debris, concentrations of cytokines in supernatants were measured with the cytometric bead assay (BD Biosciences, UK). Cytokine levels were normalized to the total protein levels present in each sample, which were measured by the Bradford assay (Bio-Rad).

Pieces of colon or spleen were cultured in complete media for 24 hr. Supernatant was collected for IL-23p19p40 quantification by ELISA (eBioscience, Insight Biotechnology Limited, UK). The sensitivity limit of the IL-23 ELISA is 30 pg/ml.

Detection of Cytokines by Quantitative PCR

Total RNA from spleen or colon homogenates was isolated with the RNAeasy mini kit (Quiagen). To analyze mRNA levels in myeloid cells of mice after anti-CD40 or isotype treatment, spleen and colon were removed immediately after sacrifice, digested, and FACS sorted for $CD11c^{high}$ and $CD11c^{low}CD11b^{high}$ cells. RNA was prepared with RNeasy Mini columns (Quiagen) and DNase digestion. After RNA preparation, the cDNA was transcribed with a single reverse transcriptase synthesis step with Superscript reagents (Invitrogen, Paisley, UK). cDNA samples were stored at −20°C. Quantitative PCR primers were designed with Primer Express software from ABI (Applied Biosystems, Foster City, CA). Quantitative PCR was performed with the Taqman Sequence detector 1.6.3 Abi software (Applied Biosystems, UK). For all quantitative PCR amplification reactions, the following parameters were used. After an initial incubation for 2 min at 50°C and 10 min at 95°C, cycles of denaturation at 95°C for 15 s were followed by annealing and elongation for 2 min at 60°C. 40 to 50 cycles were applied. As an alternative approach, samples were detected with SYBR green incorporation into double-stranded PCR products. The specificity of the reaction was tested by product separation on gel electrophoresis or by melting curve analysis in case of SYBR green incorporation. The following reagents were used for cDNA amplification: IL-23 p19 primer AGCGGGAC ATATGAATCTACTAAGAGA, GTCCTAGTAGGGAGGTGTGAAGTTG, and FAM/TAMRA-labeled probe CCAGTTCTGCTTGCAAAGGATCC GC; IL-12 p35 primer TACTAGAGAGACTTCTTCCACAACAAGAG, TCTGGTACATCTTCAAGTCCTCATAGA, and FAM/TAMRA-labeled probe AGACGTCTTTGATGATGACCCTGTGCCT; IL-12 and 23 p40 primer GACCATCACTGTCAAAGAGTTTCTAGAT, AGGAAAGTCTT GTTTTTGAAATTTTTTAA, and FAM/TAMRA-labeled probe CCACT CACATCTGCTGCTCCACAAGAAG; HPRT primer GACCGGTCCC GTCATGC, TCATAACCTGGTTCATCATCGC; VIC/TAMRA-labeled probe ACCCGCAGTCCCAGCGTCGTC; IL17A primer GCTCCA GAAGGCCCTCAG, CTTTCCCTCGCATTGACA; and VIC/TAMRA-labeled probe ACCTCAACCGTTCCACGTCACCCTG.

For each individual sample, cytokine gene expression was compared to expression of the housekeeping gene HPRT. Mean relative expression of cytokine genes were calculated and differences calculated using the $2^{-\Delta C(t)}$ method.

Statistics

The two-tailed Mann and Whitney U Test and Fisher exact test were performed with GraphPad Prism 3.00 (GraphPad Software, San Diego, CA). p values ≤ 0.05 were regarded as significant. Data are presented as mean ± 1 SD.

Supplemental Data

Supplemental Data include three figures and Supplemental Experimental Procedures and can be found with this article online at http://www.immunity.com/cgi/content/full/25/2/309/DC1/.

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