The transcription factor IFN regulatory factor–4 controls experimental colitis in mice via T cell–derived IL-6

Jonas Mutter,1 Lioubov Amoussina,1,2 Mirjam Schenk,3 Jingling Yu,1,2 Anne Brüstle,4 Benno Weigmann,2 Raja Atreya,2 Stefan Wirtz,2 Christoph Becker,2 Arthur Hoffman,1 Imke Atreya,2 Stefan Biesterfeld,5 Peter R. Galle,1 Hans A. Lehr,5 Stefan Rose-John,7 Christoph Mueller,3 Michael Lohoff,4 and Markus F. Neurath1,2

11st Medical Clinic and 1Institute of Molecular Medicine, University of Mainz, Mainz, Germany.
2Institute of Pathology, University of Bern, Bern, Switzerland. 4Institute of Medical Microbiology, University of Marburg, Marburg, Germany.
3Department of Pathology, University of Mainz, Mainz, Germany. 4Institut Universitaire de Pathologie, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland. 7Department of Biochemistry, University of Kiel, Kiel, Germany.

The proinflammatory cytokine IL-6 seems to have an important role in the intestinal inflammation that characterizes inflammatory bowel diseases (IBDs) such as Crohn disease and ulcerative colitis. However, little is known about the molecular mechanisms regulating IL-6 production in IBD. Here, we assessed the role of the transcriptional regulator IFN regulatory factor–4 (IRF4) in this process. Patients with either Crohn disease or ulcerative colitis exhibited increased IRF4 expression in lamina propria CD3+ T cells as compared with control patients. Consistent with IRF4 having a regulatory function in T cells, in a mouse model of IBD whereby colitis is induced in Rag-deficient mice by transplantation with CD4+CD45RB60 T cells, adoptive transfer of wild-type but not IRF4-deficient T cells resulted in severe colitis. Furthermore, IRF4-deficient mice were protected from T cell–dependent chronic intestinal inflammation in trinitrobenzene sulfonic acid– and oxazolone-induced colitis. In addition, IRF4-deficient mice with induced colitis had reduced mucosal IL-6 production, and IRF4 was required for IL-6 production by mucosal CD90+ T cells, which it protected from apoptosis. Finally, the protective effect of IRF4 deficiency could be abrogated by systemic administration of either recombinant IL-6 or a combination of soluble IL-6 receptor (sIL-6R) plus IL-6 (hyper–IL-6). Taken together, our data identify IRF4 as a key regulator of mucosal IL-6 production in T cell–dependent experimental colitis and suggest that IRF4 might provide a therapeutic target for IBDs.

Introduction

Inflammatory bowel diseases (IBDs) are characterized by chronic relapsing inflammations of the gastrointestinal tract that are not caused by specific pathogens. Based on recent studies, the pathogenesis of IBD seems to involve a complex interplay between certain genetic, environmental, and immunological factors. In particular, an unbalanced activation of the mucosal immune system driven by the commensal flora in a genetically susceptible host appears to cause intestinal inflammation in IBD patients (1–6). The activation of the mucosal immune system is characterized by production of proinflammatory cytokines (7–9). In particular, IL-6 and IL-6 signaling appear to play a pivotal role in IBDs. IL-6 is produced by both macrophages and T cells in IBD patients and mediates T cell resistance against apoptosis in chronic intestinal inflammation. The key functional role of IL-6 signaling was underlined by the observation that neutralizing anti–IL-6R antibodies led to suppression of established intestinal inflammation in animal models of IBD (10–13). Moreover, treatment of patients with Crohn disease (CD) with a humanized, neutralizing anti–IL-6R antibody resulted in significantly higher response rates than placebo therapy, suggesting a therapeutic benefit of anti–IL-6 signaling strategies (14). However, the molecular events that drive IL-6 production in IBD are poorly understood.

The IFN regulatory factor–4 (IRF4, also called PIP, LSIRF, and ICSAT) is a member of the IRF family of transcriptional regulators (15–19). It was originally discovered as a new IRF protein that acts as a binding factor to the Ig light chain enhancer in association with the transcription factor PU.1. IRF4 is expressed in B cells, mature T cells, dendritic cells, and macrophages, and its expression is upregulated by IgM or TCR cross-linking as well as costimulation. As the expression of IRF4 is primarily confined to lymphocytes, it has been proposed that IRF4 controls lymphocyte responses. Indeed, IRF4 together with NFATc2 has been shown to induce IL-4 gene transcription (20). In addition, studies in IRF4-deficient mice have shown profound defects in mature B and T cell function. Subsequent in vivo studies of IRF4 function identified its critical role in helper T cell differentiation (16): in Leishmania major infection, lymph node cells from IRF4–/– mice showed significantly higher IL-4 and IFN-γ secretion than cells from IRF4+/– mice. Furthermore, in vitro differentiation studies showed strongly impaired Th1 and Th2 cytokine responses of IRF4−/− T cells. The defect in Th4–dependent Th2 differentiation was due to the inability of IRF4−/− lymphocytes to upregulate expression of the master transcription factor GATA-3. In turn, retroviral overexpression of GATA-3 restored the capacity to undergo Th2 differentiation.

Nonstandard abbreviations used: CD, Crohn disease; hyper–IL-6, sIL-6R plus IL-6; HPF, high-power field; IBD, inflammatory bowel disease; IRF4, IFN regulatory factor–4; LP, lamina propria; LPMC, lamina propria mononuclear cell; sIL-6R, soluble IL-6 receptor; TNBS, trinitrobenzene sulfonic acid; UC, ulcerative colitis.

Conflict of interest: The authors have declared that no conflict of interest exists.

Based on the above data, we have analyzed in the present study the role of IRF4 in experimental colitis in mice. We found that IRF4 plays a key regulatory role for IL-6 production and consecutive activation of T lymphocytes in experimental colitis.

**Results**

*Increased expression of IRF4 in mucosal T cells in IBD.* In an initial series of studies, we aimed at analyzing the expression of IRF4 in the mucosa of patients with IBD. To assess expression of IRF4 in CD and ulcerative colitis (UC), we used colonic mucosa derived from surgical resections. Cryosections from colonic samples were analyzed for IRF4 expression by immunofluorescence staining. As shown in Figure 1A, many IRF4-positive mononuclear cells were detected in the lamina propria of IBD patients, whereas relatively few IRF4-positive cells were found in control patients. Quantitative assessment showed a significantly increased number of IRF4-expressing cells in both CD and UC as compared with controls (Figure 1B). Furthermore, IRF4 mRNA expression in the mucosa of IBD patients correlated well with mucosal IL-6 mRNA levels ($r = 0.81$) (Figure 1C). Although this finding does not directly prove that IRF4 regulates IL-6 gene transcription in human lamina propria T cells, our results thus suggested that IRF4 might play a role in regulating mucosal inflammation in IBD.

As IRF4 is expressed in APCs and T cells, we next determined whether CD3-positive lamina propria T cells in IBD would express IRF4. Indeed, we found by double-staining analysis that a large number of CD3-bearing cells coexpress CD3 on their surface. As IRF4 double-staining was performed using antibodies against either CD11c, CD8, or CD4. The number of positive cells was analyzed in 7 HPFs per patient ($n = 6$). CD11c, CD8, and CD4 single-positive cells and the number of double-positive cells were counted. Data represent mean values ± SD.

*Attenuation of inducible, T cell–dependent experimental colitis in IRF4-knockout mice.* To determine the functional role of IRF4 in...
The gut inflammation, we took advantage of IRF4-deficient mice (15). In these studies, we subjected IRF4-deficient mice and WT control mice to T cell–dependent, inducible models of experimental colitis caused by hapten reagents (oxazolone, trinitrobenzene sulfonic acid [TNBS]) that exhibit some similarities to CD and UC in humans.

**Figure 2**

Abrogation of oxazolone-induced colitis in IRF4-deficient mice. (A) IRF4 expression was analyzed by immunofluorescence (red) in oxazolone colitis. IRF4−/− mice served as negative control (upper panel). Colitic WT mice had many IRF4-positive cells in the lamina propria (lower panel) as compared with untreated WT mice (middle panel). Original magnification, ×400. *P < 0.05. (B) WT (n = 8) and IRF4-deficient mice (n = 11) were treated by rectal administration of oxazolone following prior sensitization. WT (IRF4+/+) mice lost significantly more weight as compared with oxazolone-treated IRF4−/− mice, untreated WT mice (n = 5), or untreated knockout mice (n = 3). **P < 0.01 on days 3 and 4. (C) IRF4-deficient mice showed a significantly reduced endoscopic score. (D) Representative endoscopic pictures from each group are shown. (E) Histological scoring revealed a significantly higher degree of inflammation in oxazolone-treated WT mice as compared with IRF4−/− mice. Data are given as mean values ± SEM (IRF4+/+ plus oxazolone, n = 8; IRF4−/− plus oxazolone, n = 11; IRF4+/+, n = 5; IRF4−/−, n = 3). (F) Histological findings showed moderate to severe inflammation in WT mice, whereas little or no inflammation was noted in IRF4−/− mice. (G) In further studies, chronic oxazolone colitis was evaluated. Mice were treated 3 times by intrarectal application of oxazolone and evaluated on day 21. A more severe colitis activity was induced in WT mice (n = 5) as compared with IRF4−/− mice (n = 5) (F). This was underlined by differences (***P < 0.01) in the histologic score between both groups (G). (H) Representative histologic pictures from each group are shown. Original magnification, ×100.
In addition, we performed endoscopic analysis of colitis severity (Figure 2, C and D). Whereas the colonic mucosa of IRF4−/− mice was unaffected, WT mice showed severe inflammation of the mucosa. Consistently, histologic assessment of colitis showed a significantly higher inflammation score in WT mice than in IRF4-deficient mice, and the mean score in the latter mice was similar to that in healthy, untreated control mice (Figure 2, E and F). To assess the role of IRF4 in a more intense form of oxazolone colitis, WT and IRF4-deficient mice were sensitized to oxazolone followed by intrarectal application of oxazolone after 7, 12, and 17 days and final analysis after 21 days. A severe colitis with erosions and ulcer formations was noted in WT mice, whereas IRF4-deficient mice were almost completely protected from colitis. Consistently, histologic scoring revealed a significantly higher colitis activity in WT mice as compared with IRF4-deficient mice (Figure 2, G and H).

IRF4 controls IL-6 production in T cell–dependent experimental colitis.

To characterize the mechanisms for the observed differences in inflammatory activity between WT and IRF4-deficient mice, we next analyzed cytokine expression. Interestingly, whereas equal mRNA levels of the proinflammatory cytokine TNF were detected in the colons of WT and knockout mice, IL-6 mRNA levels in the lamina propria were higher in oxazolone-treated WT mice as compared with oxazolone-treated IRF4−/− mice (Figure 4A). In TNBS-treated IRF4−/− mice, IL-6 mRNA was also strikingly decreased compared with WT mice. In contrast, levels of the proinflammatory cytokine TNF were similar in both groups (Figure 4B). IFN-γ production was reduced on average in the absence of IRF4, although this was not statistically significant (Figure 4B).

As T cells and APCs can produce IL-6, we next determined the ability of lamina propria mononuclear cells (LPMCs) from untreated mice to respond to T cell–specific stimulation (Figure 5A). Upon T cell–specific stimulation with anti-CD3/CD28 antibodies, IRF4−/− LPMCs produced significantly lower amounts of IL-6 protein as compared with WT LPMCs. In contrast, under unstimulated conditions, IL-6 production was comparable between IRF4−/− and WT LPMCs (Figure 5A), suggesting that T cell–dependent IL-6 production is reduced in the absence of IRF4. Indeed, IL-6 production...
by T cell–enriched LPMCs and purified CD90+ lamina propria T cells from TNBS-treated IRF4-deficient mice was significantly reduced as compared with WT mice (Figure 5, B and C). However, IL-6 production by splenic T cells was not different between WT and knockout cells (Figure 5D), suggesting that mucosal rather than systemic IL-6 levels are controlled by IRF4. Consistently, the number of IL-6–expressing cells in the lamina propria of TNBS-treated IRF4-deficient mice was significantly lower than in control mice (Figure 5, E and F).

As mucosal IL-6 production is a key regulator of T cell resistance against apoptosis in colitis (15), we next determined whether the reduced mucosal IL-6 levels in IRF4−/− mice would affect apoptosis of lamina propria cells in experimental colitis (Figure 5G). Interestingly, more apoptotic mononuclear cells were found in the mucosa of IRF4-knockout mice as compared with WT controls, suggesting that IRF4 regulates mucosal IL-6 levels and T cell apoptosis in TNBS colitis. Indeed, double-staining analysis revealed that apoptosis was induced in CD3-positive lamina propria T cells from IRF4−/− but not WT mice (Figure 5H).

The protective phenotype of IRF4−/− mice is abrogated by administration of hyper–IL-6. To assess whether the reduced mucosal IL-6 production in IRF4−/− mice was relevant in vivo, we next injected recombinant IL-6 or the designer protein hyper–IL-6 (IL-6 plus soluble IL-6 receptor: IL-6/sIL-6R) intraperitoneally into TNBS-treated IRF4-knockout mice (Figure 6A). While such treatment had little or no effects in WT mice, both treatment modalities abrogated the protective phenotype of IRF4-deficient mice in TNBS colitis, and such mice developed severe colitis with weight loss and mucosal ulcerations as well as histologic evidence of colitis (Supplemental Figure 1; available online with this article; doi:10.1172/JCI33227DS1). As these data suggested that IL-6 signaling is important to suppressing the protective effects caused by IRF4 deficiency, we next tested the effects of hyper–IL-6 in oxazolone-treated IRF4−/− mice (Figure 6B). Administration of hyper–IL-6 completely blocked the protective effect of IRF4−/− deficiency in oxazolone-treated IRF4−/− mice (Figure 6B). In fact, endoscopic images and histologic analysis showed severe mucosal inflammation upon hyper–IL-6 administration. **P < 0.01. (B) The mRNA expression pattern of several cytokines was also determined in TNBS colitis (n = 8 per group). Whereas no significant differences in the expression of the proinflammatory cytokines TNF and IFN-γ were noted between the groups, IL-6 mRNA expression was strongly induced in WT but not IRF4-deficient mice upon oxazolone administration. *P < 0.05.
The protective effect of IRF4 deficiency is mediated via T cells. As the above colitis models are mediated by T cells, we next tested the possibility that the protective effect of IRF4 deficiency in experimental colitis is mediated via T cell–derived IL-6. Accordingly, we chose to determine the effects of IRF4 in a transfer colitis model in which lator of mucosal IL-6 levels. In addition, induction of IL-6 levels upon hyper–IL-6 administration in IRF4-deficient mice was associated with a suppression of mucosal mononuclear cell apoptosis (Figure 7, B–D), indicating that such treatment prevents apoptosis in experimental colitis.
The protective effect of IRF4 deficiency is abrogated in TNBS and oxazolone colitis by administration of recombiant IL-6 or hyper-IL-6. (A) To determine the functional role of IL-6 signaling for the effects of IRF4 on TNBS colitis activity in vivo, recombiant IL-6 or the designer fusion protein hyper-IL-6 was intraperitoneally administered to TNBS-treated IRF4-deficient mice. TNBS-treated IRF4–/– mice exhibited severe inflammation and weight loss upon IL-6 or hyper-IL-6 treatment that was indistinguishable from colitis in WT control mice (TNBS-treated IRF4–/– plus hyper-IL-6 versus TNBS-treated IRF4–/– without hyper-IL-6, *P < 0.05). Data are given as mean values ± SEM (IRF4–/– plus TNBS, n = 6; IRF4–/– plus TNBS and hyper-IL-6, n = 5; IRF4–/– plus TNBS and hyper-IL-6, n = 4; IRF4–/– plus TNBS and hyper-IL-6, n = 5, in 2 independent experiments). (B) To determine the functional role of IL-6 signaling for the effects of IRF4 on oxazolone colitis activity in vivo, hyper-IL-6 was intraperitoneally administered to oxazolone-treated IRF4-deficient mice. Oxazolone-treated IRF4–/– mice exhibited severe inflammation and weight loss upon hyper-IL-6 treatment that was indistinguishable from colitis in WT control mice (oxazolone-treated IRF4–/– plus hyper-IL-6 versus oxazolone-treated IRF4–/– without hyper-IL-6). Data are given as mean values ± SEM (IRF4–/– plus oxazolone [oxa], n = 12; IRF4–/– plus oxazolone, n = 13; IRF4–/– plus oxazolone plus hyper-IL-6, n = 7; IRF4–/– plus oxazolone plus hyper-IL-6, n = 6, in 3 independent experiments).

Discussion
IRF4 is a lymphoid- and myeloid-restricted member of the IRF family of transcription factors that binds to IFN sequence response elements present within the promoters of IRF-regulated genes (15, 18, 21). In the present study, we have identified a key regulatory role of IRF4 in controlling IL-6 production by mucosal T cells and subsequently T cell apoptosis. Studies in 3 different models of chronic intestinal inflammation revealed that IRF4 plays a pathogenic role in colitis by regulating mucosal IL-6 production. Importantly, administration of IL-6 or hyper-IL-6 prevented mucosal cell apoptosis and abrogated the protective effect of IRF4 deficiency, suggesting that IRF4 exerts its effects by modulating IL-6 production. These data provide what we believe are novel insights into the pathogenesis of IBDs and suggest that targeting of IRF4 may be beneficial for therapy of intestinal inflammation.

Although an important role of IRF4 in T cell development has been described (15, 16, 19, 20), its regulatory role in chronic intestinal inflammation was poorly understood. Here, we have analyzed the role of IRF4 in intestinal inflammation using IRF4-deficient mice. Our data reveal that IRF4 plays an important role in several T cell–mediated models of experimental colitis. Specifically, IRF4 deficiency suppressed both oxazolone- and TNBS-induced colitis. Furthermore, we addressed the role of IRF4 in T cells, as recent data have shown that IRF4 is also expressed in B cells and APCs (22–24). Interestingly, we found that IRF4-deficient T cells fail to induce colitis in adoptive transfer experiments in RAG-knockout mice, suggesting that the protective capacity of IRF4 deficiency is at least partially due to effects in T lymphocytes.

In further mechanistic studies, we analyzed the cytokine production in experimental colitis. In TNBS-induced colitis, we found that IL-6 expression was strongly upregulated in the inflamed colon of WT but not IRF4-deficient mice. Interestingly, IL-6 production of splenic cells in colitis was not different between the WT and IRF4–/– groups, suggesting that IRF4 regulates mucosal rather than systemic IL-6 levels in experimental colitis. Moreover, we recently found that, similarly to peripheral T cells (25), the mucosal production of IL-17 was strikingly reduced in the absence of IRF4, possibly at least partially due to the reduced expression of the IL-6 inducible transcription factor RORγt (J. Mudter et al., unpublished observations). The latter transcription factor plays a fundamental role in the development of Th17 cells, a T cell subset that can be induced by stimulation of T cells with IL-6 plus TGF-β and activated with IL-23 (26–29). Thus, our data suggest a model in which IRF4 regulates mucosal IL-6 production, RORγt levels, and Th17 development. However, IL-17A–deficient mice develop normal TNBS and oxazolone-induced colitis, and T cells derived from such mice are fully capable of inducing colitis in reconstituted RAG-knockout mice (B. Weigmann et al., unpublished observations). Thus, factors other than IL-17A play an important role in mediating the effects of IRF4 in T cell–mediated colitis. As Th17 cells are known to produce IL-17F and IL-22 in addition to IL-17A, it is possible that these cytokines rather

CD4+CD45RBhi T cells are adoptively transferred into immunocompromised hosts. Splenic CD45RBhiCD4– T cells were isolated either from WT or IRF4–/– mice. Cells were then intraperitoneally injected in immunodeficient RAG mice. Within 15 days, RAG mice reconstituted with CD45RBhiCD4– T cells from WT mice showed diarrhea and lost body weight (Figure 8A). In contrast, RAG mice reconstituted with CD45RBhiCD4– T cells from IRF4–/– mice gained weight (Figure 8A). Consistently, colonoscopy of mice in the latter group revealed no signs of mucosal inflammation, whereas RAG-deficient mice reconstituted with WT CD45RBhiCD4– T cells exhibited severe mucosal inflammation (Figure 8, B and D). Moreover, the latter mice showed a significantly higher histologic score of colitis activity than the former mice (Figure 8C). Reduced IL-6 production in RAG-knockout mice transferred with IRF4–/– T cells. We next analyzed cytokine expression in RAG-recipient mice reconstituted with CD45RBhiCD4– cells from WT or IRF4–/– mice. Whereas the mRNA expression of IL-6 was significantly reduced in the latter as compared with the former mice, little or no significant changes in the expression of TNF and TGF-β were noted (Figure 9A). Furthermore, there was a higher induction of mucosal mononuclear cell apoptosis in RAG-knockout mice reconstituted with IRF4-deficient cells as compared with RAG-knockout mice given WT cells (Figure 9B), consistent with the idea that IRF4 regulates survival of mucosal T cells.
than IL-17A control T cell–dependent colitis. Alternatively, other proinflammatory cytokines released by mucosal T cells in an IRF4-dependent fashion might play a key functional role.

The cytokine IL-6 is known to induce STAT-3 expression in T cells and thereby control T cell resistance against apoptosis in experimental colitis (11–13). Here, we identified IRF4 as a key regulator of mucosal IL-6 production and T cell apoptosis in experimental colitis. The functional relevance of this finding was highlighted by the observation that treatment of IRF4-deficient mice with IL-6 or IL-6 plus the soluble IL-6R (hyper–IL-6) (30) abrogates the protective phenotype of these mice. More importantly, hyper–IL-6 treatment prevented the induction of mononuclear cell apoptosis in the colon of IRF4-deficient mice. One representative experiment is shown (n = 4 per group). Original magnification, ×300.

Figure 7
Hyper–IL-6 treatment induces IL-6 but not TNF production in IRF4–/– mice. (A) WT and IRF4-knockout mice were treated with TNBS, and some mice received hyper–IL-6. Relative expression levels of IL-6, TNF, and TGF-β mRNA were measured by quantitative real-time RT-PCR on day 4. Values were normalized to β-actin expression levels. TNBS-treated IRF4–/– mice (n = 8) showed low mucosal expression of IL-6 mRNA. Hyper–IL-6 application induced a 14-fold increase of IL-6 production in TNBS-treated IRF4–/– mice (n = 6) (** P < 0.01). The expression levels of the proinflammatory cytokine TNF as well as levels of TGF-β remained unaffected, however. Treatment of WT mice with hyper–IL-6 (n = 5; WT mice, n = 4) did not lead to a further significant increase of IL-6, TNF, or TGF-β. Data are shown as mean values ± SEM from 3 experiments. (B and C) IRF4-knockout mice and WT mice were treated with TNBS, and the presence of apoptosis in gut mononuclear cells was determined by propidium iodide and annexin V staining using FACS analysis (n = 6 per group). IRF4-deficient mononuclear cells in the gut showed a significantly higher presence of cell apoptosis in TNBS colitis than in WT cells, and this could be abrogated by hyper–IL-6 administration. One representative experiment is shown. * P < 0.05. (D) Apoptosis of gut mononuclear cells was determined by TUNEL assays. Hyper–IL-6 treatment prevented the induction of mononuclear cell apoptosis in the colon of IRF4-deficient mice. One representative experiment is shown (n = 4 per group).
high amounts of IL-6 and are resistant against T cell apoptosis (11), consistent with the idea of a regulatory role of IRF4 in T cells in IBD. This concept is underlined by the observation that mucosal IRF4 levels in IBD correlate well with local IL-6 mRNA levels. In any case, our data identify what we believe is a novel role for IRF4 as a key regulator of mucosal immune homeostasis via T cell–derived IL-6 and highlight its potential as a therapeutic target in IBDs.

**Methods**

*Human gut tissue.* Colon samples were obtained from resection specimens from patients with CD ($n = 10$) (colonic disease) and UC ($n = 10$). Surgery was performed because of stenoses, fistulas, or therapy-refractory disease. The CD group consisted of 4 male and 6 female patients between 20 and 50 years. At the time of surgery, patients were treated with either 5-ASA, corticosteroids, or anti-TNF antibodies. Five patients were receiving azathioprine. The UC group consisted of 5 male and 5 female patients, 25–60 years of age. At the time of resection, all patients were receiving corticosteroids and 4 were receiving azathioprine. The control group consisted of 10 patients (5 male, 5 female; age, 30–60 years) not receiving corticosteroids or immunosuppressants.

For real-time PCR, mRNA from endoscopic biopsy specimens of 10 control patients and 16 IBD patients was used. Groups were age matched. Among the IBD patients, 10 suffered from CD and 6 from UC. Treatment consisted of systemic corticosteroids, salicylates, and budesonide.

The collection of surgical samples and biopsies was approved by the ethical committee and the institutional review board of the University of Mainz, and each patient gave written informed consent.

**Immunofluorescence staining.** Mucosal samples were snap-frozen and embedded in OCT compound to obtain cryosections. Immunofluorescence was done on gut cryosections. Tissues were fixed in 4% paraformaldehyde in PBS and washed in 0.01 M PBS. Samples were then pretreated with 10% serum in a solution of 0.1% Triton X-100 in PBS and incubated overnight at 4°C with primary antibody (polyclonal rabbit antibody against IRF4; Santa Cruz Biotechnology Inc.) in 0.3% bovine serum albumin and 0.1% Triton X-100 in PBS. Samples without primary antibody served as negative control. The next day, samples were rinsed in PBS and incubated with a biotinylated secondary IgG antibody (1:100–1:200 dilution; Vector Laboratories) followed by incubation with streptavidine-conjugated Cy2 or Cy3 (1:500–1:1,000 dilution; all from Dianova). Samples were subjected to a second cycle of staining by using monoclonal mouse antibodies against human CD3, CD4,
In brief, CD4 cryosections. Paraffin-embedded sections were stained with H&E for his

Transfer colitis was performed as described by Morrissey and Powrie (34, 35).

power fields (HPFs) were counted in all patients for each condition.

have been well established in the laboratory as previously described (32, 33).

reagent (Dako) to eliminate unspecific background staining. Slides were

fixed in ice-cold acetone for 10 minutes followed by sequential incubation

with methanol, avidin/biotin (Vector Laboratories), and protein-blocking

reagents. All animal studies were approved by the Institutional

oxazolone. Final evaluation was performed 21 days after sensitization.

frozen and either homogenized or embedded in OCT compound to obtain

endothelial scoring of colitis severity. In transfer colitis, the murine colon was

divided into a proximal and distal segment and 1 piece of each segment

was used to isolate mRNA.

Histologic analysis of colon cross sections and endoscopy. Tissues were removed

from colitic mice, formalin fixed, and embedded in paraffin, and sections

were made and stained with H&E. The degree of inflammation on micro-

scopic cross sections of the colon was graded semiquantitatively on a scale

of 0–6 or alternatively of 0–14 in the case of transfer colitis.

Histological scoring was performed in a blinded fashion by pathologists

(H.A. Lehr and S. Biesterfeld). A combined score ranging from 0 to 6 was

used to screen TNBS and oxazolone colitis. Inflammatory cell infiltration

was scored from 0–3, and tissue damage was scored from 0–3. Occasion-

al or no inflammatory infiltrate in the lamina propria was scored as 0,

increased numbers of inflammatory cells restricted to the lamina propria

were scored as 1, inflammatory infiltrates reaching the submucosa were

scored as 2, and transmural inflammation was scored as 3. The combined

inflammatory and histological score resulted in the overall score ranging from 0 (no changes)

to 6 (severe inflammatory infiltrate and mucosal damage).

To assess the histopathological alterations of the colon in the CD4 T

cell transfer model, a scoring system ranging from 0–14 was used and the

pathologist (C. Mueller) was blinded to sample identity. The following

parameters were included: (a) mucin depletion/loss of goblet cells

(scores from 0 to 3); (b) crypt abscesses (scores from 0 to 3); (c) epithelial

erosions (scores from 0 to 1); (d) hyperemia (scores from 0 to 2); (e) cellular

infiltration (scores from 0 to 3); and (f) thickness of colonic mucosa

(scores from 0 to 2). These individual scores were added to obtain the

final histopathology score for each sample (score 0, no alterations; score

14, most severe signs of colitis).

Figure 9

Analysis of cytokine expression and apoptosis in the colon of reconstituted mice. (A) Expression of cytokine mRNA levels in the colon of RAG2-knockout mice reconstituted with WT or IRF4-deficient T cells was performed by quantitative PCR. Whereas mucosal IL-6 expression was significantly higher (**P < 0.01) in RAG2-knockout mice reconstituted with WT but not IRF4-deficient cells, no significant differences were noted in TNF and TGF-β1 levels. One representative experiment out of 3 is shown. (B) In addition, TUNEL assays on colonic cryosections were performed to detect apoptotic LPMCs. There was a significantly higher number of apoptotic LPMCs in the colons of mice reconstituted with IRF4-deficient T cells as compared with mice given WT T cells. Original magnification, ×200.

CD8, and CD11c as primary antibodies (all obtained from BD Biosciences — Pharmingen) and streptavidine-conjugated Cy3 as a chromogen. Slides were mounted with mounting medium for fluorescence (Vector Laboratories) and analyzed with an Olympus microscope. Finally, cells in 7 high-power fields (HPFs) were counted in all patients for each condition.

To detect IRF4 and IL-6 in murine colon samples, cryosections were fixed in ice-cold acetone for 10 minutes followed by sequential incubation with methanol, avidin/biotin (Vector Laboratories), and protein-blocking reagent (Dako) to eliminate unspecific background staining. Slides were then incubated overnight with primary antibodies directed against IRF4 and IL-6 (all from Santa Cruz Biotechnology Inc.). Subsequently, the slides were incubated for 30 minutes at room temperature with biotinylated secondary antibodies (Dianova). All samples were finally treated with streptavidine-conjugated Cy3. Before examination, the nuclei were counterstained with Hoechst 3342 (Molecular Probes).

Animals and induction of colitis. The TNBS and oxazolone colitis models have been well established in the laboratory as previously described (32, 33).

Transfer colitis was performed as described by Morrissey and Powrie (34, 35). In brief, CD4+CD45RBhi T cells were sorted by flow cytometry (FACSCalibur; BD), and 5 × 10⁶ cells were intraperitoneally transferred into Rag2−/− mice. T cells were obtained either from IRF4−/− or C57BL/6 donors, as specified below. For modulation of colitis activity, mice were given 1 μg hyper–IL-6 or 5 μg IL-6 by intraperitoneal injection. For examination, the nuclei were counterstained with Hoechst 3342 (Molecular Probes).

TNBS and oxazolone colitis models were performed at days 7, 12, and 17 after initial sensitization to TNF and TGF-β1. These individual scores were added to obtain the final histopathology score for each sample (score 0, no alterations; score 14, most severe signs of colitis).
To perform experimental endoscopy, mice were anesthetized using intraperitoneal injection of Avertine (Sigma-Aldrich). Mouse endoscopy was performed using a miniature endoscope (scope 1.9 mm outer diameter), a xenon light source, a triple-chip camera, and an air pump (all from Karl Storz), and MEICS score was used for assessment as previously described (36).

**Cell isolation and purification of spleen mononuclear cells.** Spleen mononuclear cells were isolated from freshly obtained spleen specimens from healthy B6 mice. Erythrocytes were removed from spleen cell suspension by hypotonic lysis in ammonium chloride and potassium chloride (ACK) buffer. The MACS system (Miltenyi Biotec) was used to isolate CD4+ T cells (purity >95%, as determined by FACS analysis). The cells were cultured at a density of 1 x 10^6/ml in complete RPMI medium in a humidified atmosphere with 5% CO₂ in a 37°C incubator in the presence or absence of antibodies against mouse CD3 and CD28 (CD3, clone 145-2C11, 2 μg/ml; CD28, clone 37.51, 0.5 μg/ml; BD Biosciences – Pharmingen).

**Cytokine measurement.** Cytokine concentration was measured in supernatants of mononuclear spleen cells or isolated splenic CD4+ T cells, LPMCs, and CD90+ lamina propria T cells. Quantification was performed using multiple cytokine measurement kits and flow cytometry or sandwich-ELISA according to the manufacturer’s instructions (Bender MedSystems).

**Real-time PCR.** Total RNA from murine colon biopsy was extracted using RNeasy Mini Kit (QIAGEN). 1 μg of total RNA was reverse transcribed using random hexamer primer (Amersham). Real-time PCR analysis for quantification of expression of IL-6, TNF, TGF-β, IL-4, IFN-γ, and β-actin mRNAs was performed in duplicate on an iCycler (Bio-Rad Laboratories) using the ABSolute SYBR Green Fluorescent Kit (Applied Biosystems). QuantiTect primer sets were used for these experiments (QIAGEN).

**Cell isolation and purification of LPMCs.** LPMCs were isolated from freshly obtained murine colon specimens using a modification of the technique described by van der Heijden and Stok (37). After removal of Peyer patches, the colon was washed in HBSS free of calcium and magnesium and incubated twice in HBSS containing 5 mM EDTA for 15 minutes at 37°C. After incubation, the epithelial cell layer and intraepithelial lymphocytes were removed by intensive vortexing and passing through a 100-μm cell strainer; new EDTA solution was added. Next, the tissue was washed in HBSS, cut in 1-mm² pieces, and placed in 5 ml digestion solution containing 4% fetal calf serum, 0.5 mg/ml collagenase D, DNase I grade 2, and 50 U/ml dispase II (both obtained from Roche). Digestion was performed by incubating the pieces at 37°C for 20 minutes. After the initial 20 minutes, the solution was passed through a 100-μm cell strainer, and the pieces were collected and placed into fresh digestion solution 3 times. Supernatants were washed in cold FACS buffer, resuspended in 10 ml of the 40% solution of a 40:80 Percoll gradient, and overlaid on 5 ml of the 80% fraction in a 15-ml tube. Lamina propria (LP) cells were collected at the interface of the Percoll gradient, washed once, and resuspended in FACS buffer or cell culture medium at a density of 10⁶ cells/ml. In some experiments, the MACS system (Miltenyi Biotec) was subsequently used to isolate CD90+ T cells from the lamina propria (purity >90%, as determined by FACS analysis).

**Detection of apoptosis.** Spleen mononuclear cells and LPMCs were isolated as described above. The cells were cultured at a density of 1 x 10⁶/ml in complete RPMI medium in a humidified atmosphere with 5% CO₂ in a 37°C incubator in the presence of antibodies against mouse CD3 and CD28 (CD3, clone 145-2C11, 2 μg/ml; CD28, clone 37.51, 0.5 μg/ml; BD Biosciences – Pharmingen). Cells were cultured in the presence or absence of hyper–IL-6. For FACS analysis, apoptotic cells were detected by staining with annexin V and propidium iodide using the Annexin V FITC Apoptosis Detection Kit I (BD Biosciences – Pharmingen). In addition, double staining of spleen cells and LPMCs was performed using annexin V (FITC) and anti-CD3 antibodies (PE conjugated), as previously described (10–13).

**Statistics.** Statistical significance of evaluated data was tested using Mann-Whitney U Test and Student’s t test. Results were considered as statistically significant at P < 0.05. Results are expressed as mean values ± SD or ± SEM. The error bars in histogram figures represent SD or SEM as indicated. Relative expression of IRF4 and IL-6 mRNA from each patient was correlated by using the linear correlation coefficient r to measure the strength and the direction of a linear relationship between these 2 variables.

**Acknowledgments**

The work from M.F. Neurath and J. Mudter was supported by a grant from the Stiftung Rheinland-Pfalz für Innovation. M. Lohoff was supported by the DFG grant LO396-1. M.F. Neurath was supported by grants from the SFB548 of the DFG. The technical assistance of Francoise Burri is gratefully acknowledged. The authors thank Katrin Rücknagel for excellent support and technical assistance.

Received for publication July 10, 2007, and accepted in revised form April 30, 2008.

Address correspondence to: Markus F. Neurath, 1st Department of Internal Medicine and Institute of Molecular Medicine, University of Mainz, Langenbeckstrasse 1, 55131 Mainz, Germany. Phone: 49-6131-175740; Fax: 49-6131-175583; E-mail: neurath@1-med.klinik.uni-mainz.de.