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Enhanced Th1 Activity and Development of Chronic Enterocolitis in Mice Devoid of Stat3 in Macrophages and Neutrophils

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Summary

We have generated mice with a cell type-specific disruption of the Stat3 gene in macrophages and neutrophils. The mutant mice are highly susceptible to endotoxin shock with increased production of inflammatory cytokines such as TNFα, IL-1, IFNγ, and IL-6. Endotoxin-induced production of inflammatory cytokines is augmented because the suppressive effects of IL-10 on inflammatory cytokine production from macrophages and neutrophils are completely abolished. The mice show a polarized immune response toward the Th1 type and develop chronic enterocolitis with age. Taken together, Stat3 plays a critical role in deactivation of macrophages and neutrophils mainly exerted by IL-10.

Introduction

Binding of cytokines to their cognate receptors on the cell surface activates a family of cytoplasmic proteins, designated STATs (signal transducers and activators of transcription), through tyrosine phosphorylation by the receptor-associated Jak kinases. Once phosphorylated and activated, STAT proteins form homo- or heterodimers, rapidly translocate into the nucleus, and induce gene expression (Schindler and Darnell, 1995; Ihle, 1996; Darnell, 1997). Until now, seven mammalian STAT proteins have been identified. Recent studies using knockout mice of various STAT proteins have revealed their important role in biological functions induced by cytokines. For example, Stat1-deficient mice are defective in IFN-mediated functions and show compromised innate immune responses (Durbin et al., 1996; Meraz et al., 1996). Stat4- and Stat6-deficient mice are impaired in IL-12- and IL-4-mediated actions and exhibit defective Th1 and Th2 responses, respectively (Kaplan et al., 1996a, 1996b; Shimoda et al., 1996; Takeda et al., 1996a; Thierfelder et al., 1996).

Stat3 was first identified as a member of the STAT family that was activated by IL-6 family cytokines (Akira et al., 1994; Zhong et al., 1994). Subsequently, Stat3 has been shown to be tyrosine phosphorylated in response to a variety of other stimuli, such as granulocyte colony-stimulating factor, epidermal growth factor, leptin, and IL-10 (reviewed by Akira, 1997). Unlike knockout mice of other STAT proteins, Stat3-deficient mice died during their early embryogenesis (Takeda et al., 1997). Therefore, to assess the role of Stat3 in vivo more precisely, we have disrupted the *Stat3* gene in a tissue- or cell-specific manner, utilizing the Cre-*loxP* recombination system (Gu et al., 1994). In Stat3-deficient T cells, IL-6-induced T cell proliferation was severely impaired due to the lack of IL-6-mediated prevention of apoptosis (Takeda et al., 1998). In addition, IL-2-induced T cell proliferation was partially impaired due to defective IL-2-mediated IL-2 receptor α chain expression, like in Stat5a-deficient mice (Nakajima et al., 1997; Akaishi et al., 1998). Thus, conditional gene targeting of the *Stat3* gene in T cells has revealed a pivotal role for Stat3 in actions mediated by IL-6 and IL-2. However, the biological importance of Stat3 activation in response to many other cytokines such as leptin, granulocyte-colony stimulating factor, IL-10, and IFNγ is still not clarified.

In the present study, we generated mice in which Stat3 is disrupted in a macrophage- and neutrophil-specific fashion. This was achieved by breeding of mice carrying a *loxP*-flanked Stat3 allele (Takeda et al., 1998) with LysMcre mice in which the Cre-recombinase is expressed under control of the murine lysozyme M gene regulatory region. Upon breeding of LysMcre mice to other mouse strains harboring *loxP*-flanked target genes in their genome, these animals were found to efficiently undergo Cre-*loxP*-mediated recombination in macrophages and neutrophils, but not in B and T cells nor the majority of dendritic cells (B. E. C., W. Reith, R. Renkawitz, and I. F., unpublished data). Analysis of LysMcre/Stat3$^{flox/-}$ double mutant mice revealed that macrophages and neutrophils are abnormally activated and display severe impairment in IL-10-mediated functions. Furthermore, these mice develop chronic enterocolitis and show enhanced Th1 cell activity as is the case in IL-10-deficient mice (Kühn et al., 1993; Berg et al., 1996). These findings demonstrate that Stat3 activation in myeloid cells is essential for antiinflammatory reactions mediated by IL-10.

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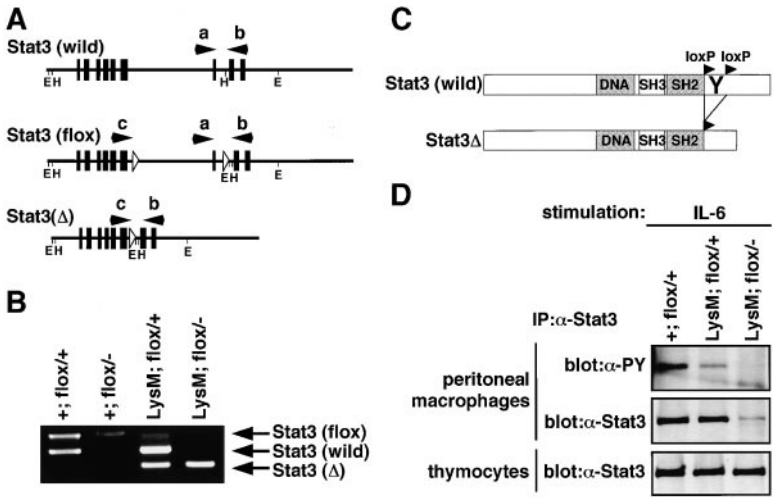

Figure 1. Disruption of Stat3 in Macrophages and Neutrophils

(A) Maps of the wild-type Stat3 locus, the targeted Stat3 locus of Stat3$^{flox/flox}$ mice, and the Stat3 locus of macrophages and neutrophils from LysMcre/Stat3$^{flox/-}$ mice. Closed boxes represent coding exons. Open triangles indicate *loxP* sites. E, EcoRI; H, HindIII.

(B) PCR analysis of genomic DNA from peritoneal macrophages using primers a, b, and c as indicated in Figure 1A. Note that a novel 135 bp band was observed after Cre-mediated deletion in LysMcre/Stat3$^{flox/+}$ and LysMcre/Stat3$^{flox/-}$ mice.

(C) Predicted structure of Stat3Δ protein in macrophages of LysMcre/Stat3$^{flox/-}$ mice. The Stat3Δ protein lacks the tyrosine residue and serine residue critical for STAT activation as described previously (Takeda et al., 1998). Closed triangles represent *loxP* sites. Y, tyrosine residue; DNA, DNA binding domain; SH3, SH3-like domain; SH2, SH2 domain.

(D) Western blot analysis of peritoneal macrophages and thymocytes. Cells were stimulated with IL-6 for 30 min and lysed. Cell lysates were immunoprecipitated with anti-Stat3 and blotted with anti-phosphotyrosine (α-PY) or anti-Stat3 antibodies.

Results

Generation of Mice in which Stat3 Is Deficient in Macrophages and Neutrophils

To assess the role for Stat3 in inflammatory responses of mice, we utilized the Cre-*loxP* recombination system. The bacteriophage-derived Cre-recombinase recognizes *loxP* sequences and deletes DNA fragments flanked by two *loxP* sites in the same orientation (reviewed by Kühn and Schwenk, 1997). To disrupt the *Stat3* gene specifically in macrophages and neutrophils, we crossed two types of mice: one is a mouse in which the *Stat3* gene is flanked by two loxP sites (Stat3$^{flox/flox}$) (Takeda et al., 1998) and the other a mouse in which the *cre* cDNA is inserted into the mouse *lysozyme M* gene by a knockin approach (LysMcre mice) (B. E. C., W. Reith, R. Renkawitz, and I. F., unpublished data). Lysozyme M has been shown to be exclusively expressed in cells of the monocyte/macrophage and granulocyte lineages of hematopoietic differentiation (Cross et al., 1988; Bonifer et al., 1994). In LysMcre/Stat3$^{flox/flox}$ mice, Cre-mediated deletion of the *Stat3* gene was therefore expected to take place only in these cell types. For more efficient elimination of the Stat3 protein, we performed the experiments described below with hemizygous mice, bearing one floxed Stat3 allele and one already disrupted Stat3 allele (LysMcre/Stat3$^{flox/-}$). As reported previously, mice heterozygous for the Stat3 null mutation (Stat3$^{+/-}$) did not show any obvious abnormality (Takeda et al., 1997), and the population of macrophages and granulocytes in the peritoneal cavity of these mice was not altered as determined by surface expression of Mac-1 and Gr-1 (data not shown). First, we evaluated the efficiency of Cre-mediated deletion of the floxed Stat3 gene in LysMcre/Stat3$^{flox/-}$ mice. Genomic DNA was extracted from peritoneal macrophages and subjected to polymerase chain reaction (PCR) analysis using three primers as indicated in Figure 1A. In peritoneal macrophages of LysMcre/Stat3$^{flox/-}$ or LysMcre/Stat3$^{flox/+}$ mice, Cre-mediated deletion resulted in the appearance of a novel 135 bp band corresponding to the Stat3Δ gene. Due to differences in the targeting strategy, this Stat3Δ PCR product cannot be amplified from the Stat3^{-} allele (Takeda et al., 1997). Despite appearance of the Stat3Δ-specific band in LysMcre/Stat3$^{flox/+}$ and LysMcre/Stat3$^{flox/-}$ macrophages, a 283 bp band corresponding to the floxed Stat3 gene was still detectable in these cells, indicating that Cre-mediated deletion did not occur completely at the DNA level (Figure 1B). The efficiency of Cre-mediated deletion of the floxed Stat3 gene was about 97%, as determined with Perkin Elmer Sequence Detection System (data not shown). This is in line with a deletion efficiency of 75%–99% in various macrophage or granulocyte preparations of LysMcre mice harboring other loxP-flanked target genes (B. E. C., W. Reith, R. Renkawitz, and I. F., unpublished data). Western blot analysis of peritoneal macrophages also detected a small amount of residual wild-type Stat3 protein in LysMcre/Stat3$^{flox/-}$ mice. However, IL-6-induced tyrosine phosphorylation of Stat3 in macrophages from LysMcre/Stat3$^{flox/-}$ mice was greatly reduced (Figure 1D). This may be in part because Cre-mediated deletion of the floxed Stat3 gene results in the production of a truncated form of the Stat3 protein that acts as a dominant negative protein for Stat3 activation (Figure 1C) (Takeda et al., 1998). Thus, cytokine-induced Stat3 activation was almost completely abrogated in spite of the residual wild-type Stat3 protein in peritoneal macrophages of LysMcre/Stat3$^{flox/-}$ mice. In neutrophils, IL-6-induced Stat3 activation was also completely abolished in LysMcre/Stat3$^{flox/-}$ mice (data not shown). However, expression of Stat3 was not disturbed in thymocytes of LysMcre/Stat3$^{flox/-}$ mice, indicating that Cre-mediated Stat3 deletion indeed occurred in a macrophage- and neutrophil-specific fashion.

LysMcre/Stat3$^{flox/-}$ Mice Are Highly Susceptible to LPS-Induced Endotoxin Shock

Macrophages have been shown to be activated and to secrete a variety of inflammatory cytokines and mediators in response to lipopolysaccharide (LPS), an endotoxin derived from Gram-negative bacteria. In addition,

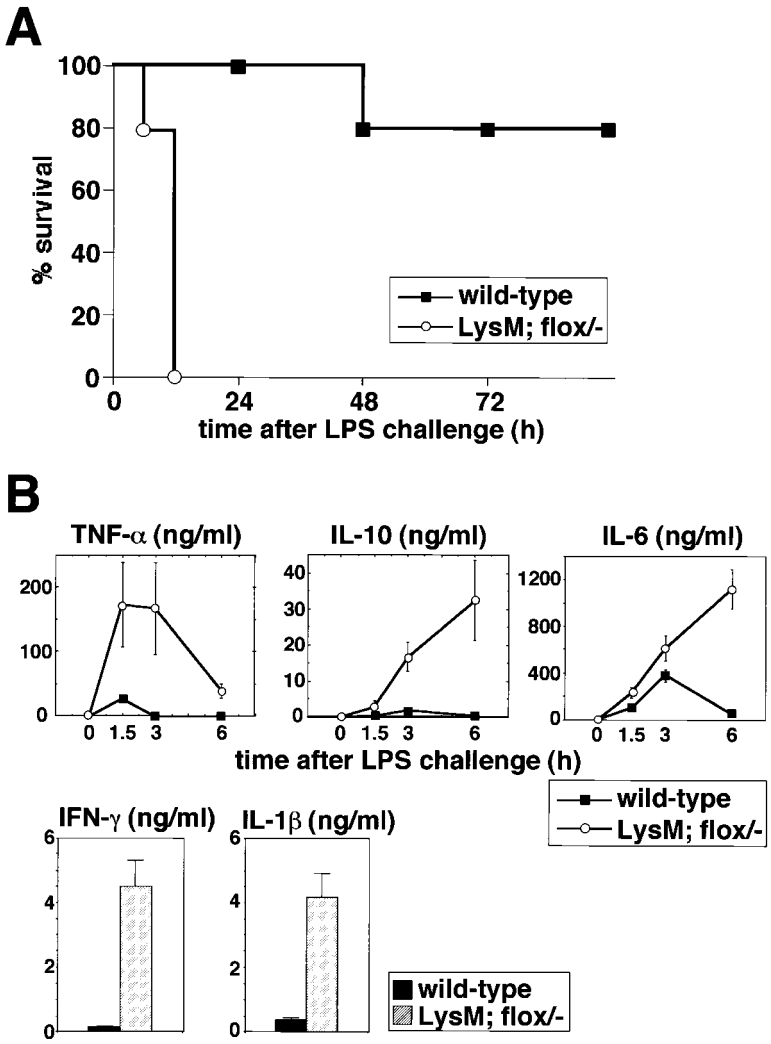

Figure 2. LysMcre/Stat3$^{flox/-}$ Mice Are Highly Susceptible to LPS-Induced Endotoxin Shock

(A) Age-matched mice were intravenously injected with 20 μg LPS. Survival of mice was checked every 12 hr. Ten wild-type control and 10 LysMcre/Stat3$^{flox/-}$ mice were used for the experiments. Note that LysMcre/Stat3$^{flox/-}$ mice died within 24 hr.

(B) Serum concentrations of several inflammatory cytokines after LPS injection. Mice were bled 1.5, 3, and 6 hr after LPS (20 μg) challenge, and concentrations of TNFα, IL-6, and IL-10 were measured by ELISA. As for IL-1β and IFNγ, sera were analysed 6 hr after LPS challenge. Results are mean values ± S.D. of sera samples from five mice.

overactivation of macrophages by large amounts of LPS can result in lethal endotoxin shock in mice. We analyzed the effect of Stat3 deficiency on LPS responsiveness in vivo. A small amount of LPS (20 μg) was intravenously injected into both wild-type and LysMcre/Stat3$^{flox/-}$ mice. Almost all of the wild-type control mice survived over 4 days after injection of 20 μg LPS, while all of the LysMcre/Stat3$^{flox/-}$ mice died within 24 hr (Figure 2A). Thus, LysMcre/Stat3$^{flox/-}$ mice were highly susceptible to LPS-induced endotoxin shock. We next analyzed serum concentrations of several inflammatory cytokines after LPS challenge. In control mice, serum concentrations of tumor necrosis factor-α (TNFα) were increased at 1.5 hr after LPS challenge and decreased to undetectable levels by 3 hr. In contrast, serum TNFα levels of LysMcre/Stat3$^{flox/-}$ mice at 1.5 hr were about 5-fold elevated compared to those of control mice. In addition, high serum TNFα levels in LysMcre/Stat3$^{flox/-}$ mice sustained even at 3 and 6 hr after LPS challenge (Figure 2B). In the case of serum concentrations of IL-6 and IL-10, peak levels were observed at 3 hr after LPS challenge in control mice. In LysMcre/Stat3$^{flox/-}$ mice, serum IL-6 and IL-10 levels at 3 hr were significantly higher than those of control mice. Furthermore, both levels were increased even at 6 hr after LPS challenge in LysMcre/Stat3$^{flox/-}$ mice (Figure 2B). Serum IL-1β and IFNγ concentrations of LysMcre/Stat3$^{flox/-}$ mice at 6 hr after LPS challenge were also increased relative to control mice (Figure 2B). Thus, serum concentrations of several inflammatory cytokines were significantly elevated after LPS challenge in LysMcre/Stat3$^{flox/-}$ mice, accounting for the high susceptibility of LysMcre/Stat3$^{flox/-}$ mice to endotoxin shock.

Constitutive Activation of Macrophages in LysMcre/Stat3$^{flox/-}$ Mice

We analyzed the activation status of peritoneal macrophages in LysMcre/Stat3$^{flox/-}$ mice on the basis of surface phenotype and production of inflammatory mediators. Peritoneal macrophages were isolated from nontreated mice and analyzed for surface expression of MHC class II and B7-1, both of which are upregulated in activated macrophages. In LysMcre/Stat3$^{flox/-}$ mice, expression of both MHC class II and B7-1 were augmented relative to control macrophages (Figure 3A). We next examined production of inflammatory mediators from thioglycollate-elicited peritoneal macrophages (PEC) in response to LPS or IFNγ plus LPS. Control PEC secreted elevated levels of TNFα, IL-6, and nitric oxide (NO) in response

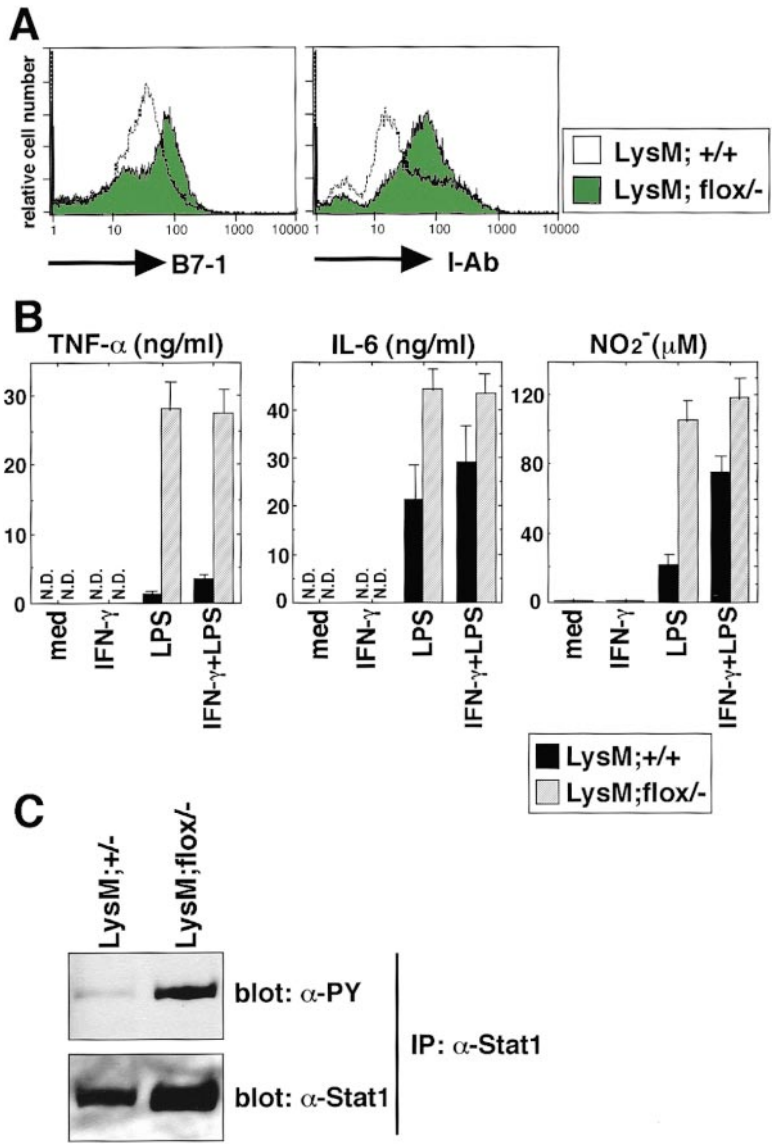

Figure 3. Peritoneal Macrophages of LysMcre/Stat3$^{flox/-}$ Mice Are Abnormally Activated

(A) Peritoneal macrophages from nontreated, 5-week-old mice were stained with anti-B7-1 and I-Ab and analyzed by flow cytometry.

(B) Mice were intraperitoneally injected with thioglycollate, and 3 days later peritoneal macrophages were isolated. Macrophages were stimulated with 30 U/ml IFNγ and/or 2 μg/ml LPS for 24 hr. Concentrations of TNFα, IL-6, and NO in the culture supernatants were measured. N.D.; not detected.

(C) Peritoneal macrophages from nontreated, 5-week-old mice were lysed, immunoprecipitated with anti-Stat1 antibody, and blotted with anti-phosphotyrosine antibody (α-PY) or anti-Stat1 antibody.

to LPS, and the synthesis of these substances was enhanced when stimulated with IFNγ plus LPS. In contrast, PEC from LysMcre/Stat3$^{flox/-}$ mice produced significantly higher levels of TNFα, IL-6, and NO in response to LPS relative to control PEC (Figure 3B). Synthesis of TNFα, IL-6, and NO seemed to already have reached peak levels in response to LPS alone because costimulation with IFNγ plus LPS did not lead to further elevation. We also analyzed expression and phosphorylation of Stat1, which is induced by IFNγ and essential for macrophage activation. Interestingly, Stat1 protein in peritoneal macrophages from nontreated LysMcre/Stat3$^{flox/-}$ mice was increased in amounts and already tyrosine phosphorylated, indicating that the Stat1 protein was constitutively activated (Figure 3C). Taken together, these results demonstrate that macrophages of LysMcre/Stat3$^{flox/-}$ mice are in a constitutively activated state.

Impaired IL-10-Mediated Functions in Macrophages of LysMcre/Stat3$^{flox/-}$ Mice

As shown in Figure 4A, Stat3 is tyrosine phosphorylated in response to several cytokines in normal peritoneal macrophages. These include IL-6 family members, IFNγ, IL-10, and granulocyte-colony stimulating factor (G-CSF). We first studied whether the function mediated by IFNγ is defective in macrophages of LysMcre/Stat3$^{flox/-}$ mice. MHC class II expression on peritoneal macrophages was further enhanced in response to IFNγ even in LysMcre/Stat3$^{flox/-}$ mice, in which MHC class II expression was already augmented (Figure 4B). This indicates that IFNγ-mediated signaling in peritoneal macrophages of LysMcre/Stat3$^{flox/-}$ mice was not impaired.

We next analyzed IL-10-mediated macrophage functions. When control PEC were pretreated with IL-10, these cells showed reduced production of TNFα in a dose-dependent manner. In contrast, production of TNFα from PEC of LysMcre/Stat3$^{flox/-}$ mice did not decrease even when pretreated with high concentrations of IL-10 (Figure 5A). Similarly, IL-10 pretreatment of PEC from LysMcre/Stat3$^{flox/-}$ mice did not reduce IL-6 and NO production in response to LPS (Figure 5B). We also examined IL-10-mediated functions in bone marrow-derived macrophages (BMM) generated in the presence of macrophage-colony stimulating factor (M-CSF) or

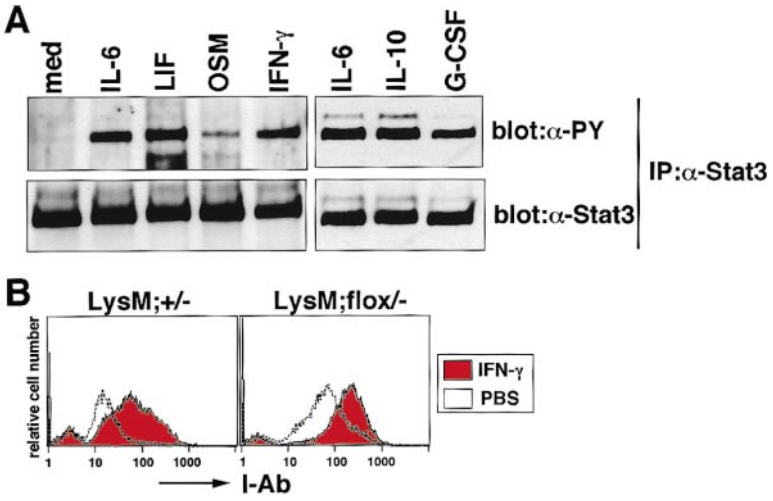

Figure 4. Stat3 Is Activated by Various Cytokines in Macrophages

(A) Peritoneal macrophages from wild-type mice were stimulated with the indicated cytokines for 30 min. Cells were lysed, immunoprecipitated with anti-Stat3 antibody, and blotted with anti-phosphotyrosine antibody (α-PY). LIF, leukemia inhibitory factor; OSM, oncostatin M; G-CSF, granulocyte colony stimulating factor.

(B) Mice were intraperitoneally injected with 300 U of IFNγ or PBS. Peritoneal macrophages were isolated 3 days later and stained with anti-I-Ab antibody.

granulocyte-macrophage-colony stimulating factor (GM-CSF). Pretreatment with IL-10 reduced LPS-induced production of TNFα and IL-6 in both GM-CSF- and M-CSF-induced BMM from normal mice but not from LysMcre/Stat3$^{flox/-}$ mice (data not shown).

Proliferation of wild-type BMM can be enhanced in response to M-CSF in a dose-dependent manner and pretreatment with IL-10 or IL-6 inhibits the growth of these cells. In comparison, BMM from LysMcre/Stat3$^{flox/-}$ mice displayed a very similar proliferative response to M-CSF but neither IL-10 nor IL-6 inhibited their growth (Figure 5C). Growth inhibition of BMM from LysMcre/Stat3$^{flox/-}$ mice was not observed even when cells were cultured with higher concentrations of IL-10 (Figure 5D). If anything, IL-6 rather enhanced the proliferation of BMM from LysMcre/Stat3$^{flox/-}$ mice when cultured with low concentrations of M-CSF (Figure 5C).

IL-10 has been shown to act on neutrophils as well as on macrophages (Nikolaus et al., 1998). To assess the responsiveness of neutrophils from control or mutant mice to IL-10, neutrophils were isolated from the peritoneal cavity of mice injected with thioglycollate 4 hr before, and pretreated with IL-10. Then, we measured production of H_2O_2 and TNFα in response to phorbol-12 myristate 13 acetate (PMA) and LPS, respectively (Figure 5E). In control neutrophils, IL-10 pretreatment reduced H_2O_2 and TNFα production. In contrast, IL-10 did not have any effect on production of H_2O_2 and TNFα from neutrophils of LysMcre/Stat3$^{flox/-}$ mice. Thus, IL-10-mediated suppressive functions were completely abolished in both macrophages and neutrophils of LysMcre/Stat3$^{flox/-}$ mice.

As shown in Figure 5F, both Jak1 and Tyk2 were normally tyrosine phosphorylated in PEC of LysMcre/Stat3$^{flox/-}$ mice in response to IL-10, demonstrating that defective IL-10-mediated functions were not due to decreased expression of the IL-10 receptor and/or associated tyrosine kinases.

Taken together, these results demonstrate that Stat3 activation is indispensable for IL-10-mediated antiinflammatory and antiproliferative responses in macrophages and neutrophils.

LysMcre/Stat3$^{flox/-}$ Mice Develop Enterocolitis

LysMcre/Stat3$^{flox/-}$ mice appeared to thrive and grow normally after birth, but some of them died at the age of 20–24 weeks. In addition, LysMcre/Stat3$^{flox/-}$ mice at the age of 20 weeks were found to develop leukocytosis and anemia (data not shown). When we performed histological analysis on 20-week-old LysMcre/Stat3$^{flox/-}$ mice, pathological changes were observed in the colon. Transverse sections of colon in LysMcre/Stat3$^{flox/-}$ mice revealed thickening of the colonic wall and a reduced gland number. Glands in the colon of LysMcre/Stat3$^{flox/-}$ mice were characterized by depletion of the mucin-producing goblet cells, and the columnar epithelium contained markedly hyperchromatic nuclei, indicating the regeneration of epithelium. In addition, a marked infiltration of inflammatory cells, consisting of neutrophils, lymphocytes, and plasma cells, was observed in the lamina propria. Crypt abscesses were frequently found (Figures 6A and 6B). Occasionally, mucosal ulcerations were observed, but there was no evidence of granuloma formation. Immunohistological staining revealed expression of MHC class II molecules on the colonic epithelium of LysMcre/Stat3$^{flox/-}$ mice, but not on that of wild-type control mice (Figures 6C and 6D). In the lamina propria of LysMcre/Stat3$^{flox/-}$ mice, an increased number of plasma cells were stained for immunoglobulin A when compared with wild-type controls (Figures 6E and 6F). Furthermore, numerous CD4-positive T cells were present in the lamina propria of LysMcre/Stat3$^{flox/-}$ mice (Figures 6G and 6H). These findings demonstrate that aging LysMcre/Stat3$^{flox/-}$ mice developed chronic enterocolitis.

Imbalanced T Helper Cell Development in LysMcre/Stat3$^{flox/-}$ Mice

Chronic enterocolitis has been shown to be associated with a predominant T helper type 1 (Th1) phenotype in the damaged tissues. In addition, IL-10, which is a designated Th2 type cytokine, has been shown to inhibit Th1 cell development. Therefore, we analyzed whether T cells from LysMcre/Stat3$^{flox/-}$ mice manifest skewed Th1 cell responses. Splenic T cells were purified from 12-week-old mice, stimulated with immobilized anti-CD3

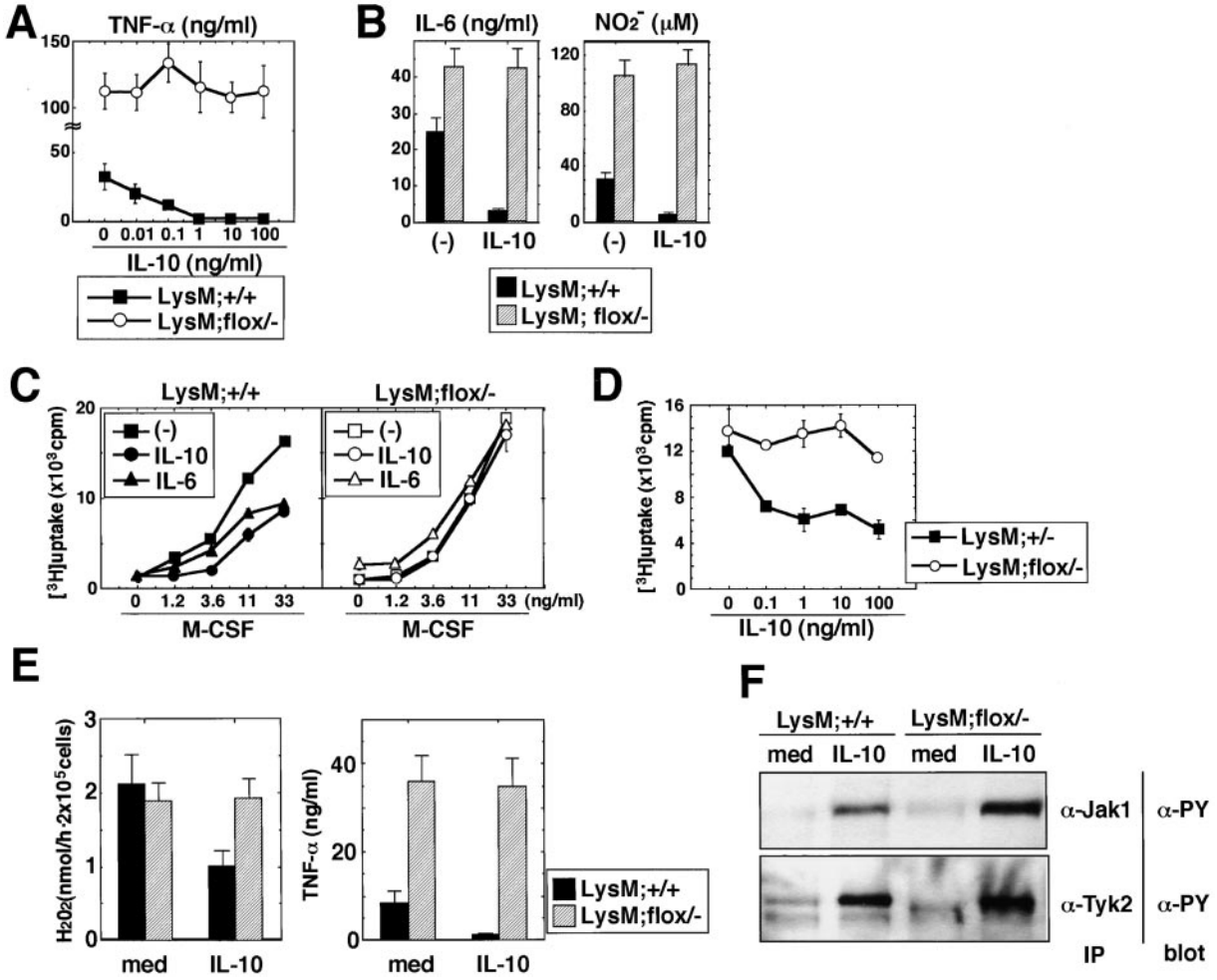

Figure 5. Impaired IL-10-Mediated Functions in Macrophages and Neutrophils of LysMcre/Stat3$^{flox/-}$ Mice

(A) Mice were intraperitoneally injected with thioglycollate, and peritoneal macrophages (PEC) were isolated 3 days later. PEC were cultured with the indicated concentrations of IL-10. After 12 hr culture, LPS was added to the final concentration of 2 μg/ml, and the cells were cultured for an additional 24 hr. Concentrations of TNFα in the culture supernatants were measured by ELISA.

(B) PEC from wild-type and LysMcre/Stat3$^{flox/-}$ mice were precultured with or without 10 ng/ml IL-10 for 12 hr and then incubated in the presence of 2 μg/ml LPS for 24 hr. Concentrations of IL-6 and NO in the culture supernatants were measured.

(C) BMM were precultured with 10 ng/ml IL-6 or IL-10 for 12 hr and then cultured in the presence of the indicated concentration of M-CSF for 48 hr. Proliferation was measured by [^{3}H]-thymidine incorporation.

(D) BMM were pretreated with the indicated concentration of IL-10 for 12 hr, and then cells were cultured in the presence of 10 ng/ml M-CSF for 48 hr. [^{3}H] incorporation was measured.

(E) Peritoneal cells from mice injected with thioglycollate 4 hr prior to cell harvest were cultured with or without 10 ng/ml IL-10 for 12 hr. Nonadherent neutrophils were collected and stimulated with PMA for 1 hr or with LPS for 24 hr. After PMA stimulation, H_2O_2 production was determined based on reduction of ferricytochrome C. After LPS stimulation, TNFα production was measured by ELISA.

(F) Peritoneal macrophages from wild-type and LysMcre/Stat3$^{flox/-}$ mice were cultured with or without IL-10 for 15 min. Cells were lysed, immunoprecipitated with anti-Jak1 or anti-Tyk2 antibodies, and blotted with anti-phosphotyrosine antibody (α-PY).

antibody for 24 hr, and IFNγ concentrations in the culture supernatants were measured. Splenic T cells from LysMcre/Stat3$^{flox/-}$ mice produced about 4-fold higher concentrations of IFNγ when compared with control T cells (Figure 7A). Furthermore, these T cells produced increased levels of IFNγ in response to IL-12 or IL-18, both of which enhance IFNγ production from Th1 cells. We further analyzed IFNγ production from T cells of 5-week-old mice, at a time, when LysMcre/Stat3$^{flox/-}$ mice had not yet developed severe enterocolitis. T cells from 5-week-old LysMcre/Stat3$^{flox/-}$ mice also produced increased levels of IFNγ when compared with wild-type control cells and likewise showed enhanced IFNγ production in response to IL-12 and IL-18 (Figure 7A). Thus, splenic T cells from LysMcre/Stat3$^{flox/-}$ mice already displayed a Th1 cell phenotype prior to chronic inflammation in the gut. Th1 cell development has been shown to be induced by IL-12 that is secreted from activated macrophages (Trinchieri, 1995). Therefore, we examined IL-12 production from PEC. PEC were stimulated with LPS or a combination of IFNγ and LPS, and concentrations of IL-12 in the culture supernatants were measured. Wild-type control PEC produced only a small amount of IL-12 following LPS stimulation. In contrast,

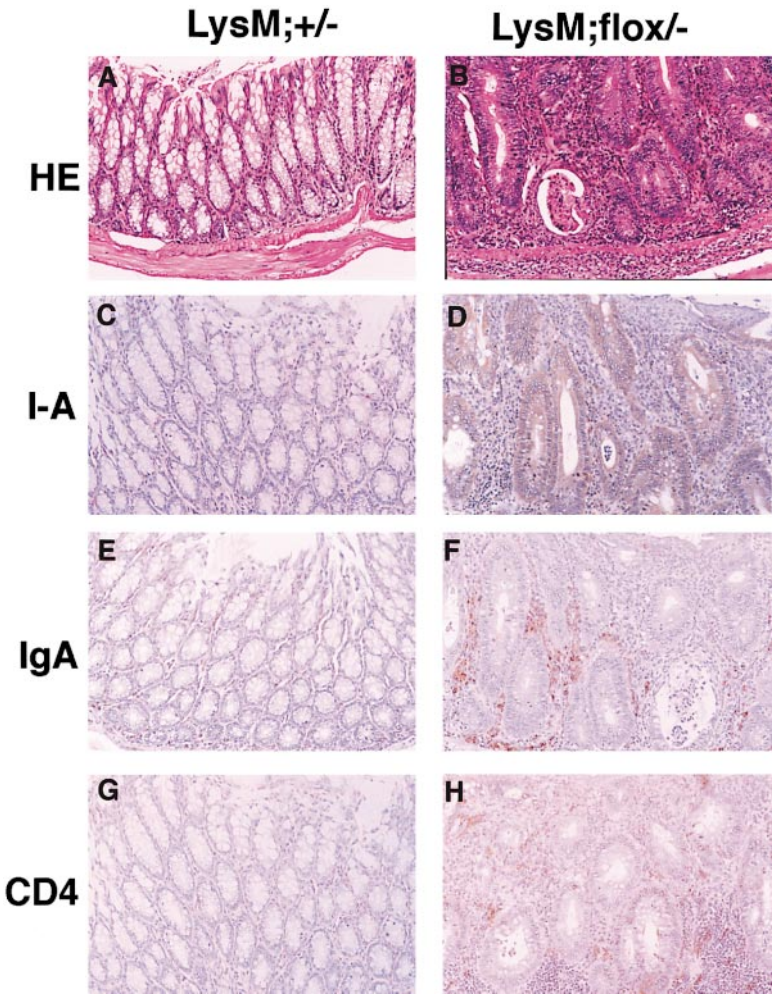

Figure 6. Histopathology of LysMcre/Stat3$^{flox/-}$ Mice

(A) Colon mucosa of LysMcre/Stat3$^{+/-}$ (wild-type control) mice. Hematoxylin-eosin staining. Magnification, 50×. (B) Colon mucosa of LysMcre/Stat3$^{flox/-}$ mice showing goblet cell-depleted epithelium, mucosal inflammatory cell infiltrates, and crypt abscesses. Hematoxylin-eosin staining. Magnification, 50×. (C) Colon mucosa of LysMcre/Stat3$^{+/-}$ (wild-type control) mice immunostained with anti-I-Ab. Magnification, 50×. (D) Colon mucosa of LysMcre/Stat3$^{flox/-}$ mice immunostained with anti-I-Ab, showing the expression of MHC class II molecules on the irregular colonic epithelium. Magnification, 50×. (E) Colon mucosa of LysMcre/Stat3$^{+/-}$ (wild-type control) mice immunostained with anti-IgA. Magnification, 50×. (F) Colon mucosa of LysMcre/Stat3$^{flox/-}$ mice immunostained with anti-IgA, identifying the infiltration of IgA-producing plasma cells in the lamina propria. Magnification, 50×. (G) Colon mucosa of LysMcre/Stat3$^{+/-}$ (wild-type control) mice immunostained with anti-CD4. Magnification, 50×. (H) Colon mucosa of LysMcre/Stat3$^{flox/-}$ mice immunostained with anti-CD4, demonstrating the infiltration of CD4-positive T cells in the lamina propria. Magnification, 50×. All animals were kept under conventional breeding conditions.

PEC from LysMcre/Stat3$^{flox/-}$ mice produced significantly increased levels of IL-12 in response to LPS alone. In addition, costimulation with IFNγ and LPS further enhanced IL-12 production from PEC in LysMcre/Stat3$^{flox/-}$ mice (Figure 7B). Taken together, these results demonstrate that LysMcre/Stat3$^{flox/-}$ mice exhibit excessive Th1 cell responses.

Discussion

In the present study, we have generated and characterized mice lacking Stat3 in a macrophage- and neutrophil-specific fashion. Stat3-deficient macrophages are in a constitutively activated state and secrete large amounts of cytokines including TNFα, IL-1, IL-6, and IL-12 in response to inflammatory stimuli. T cells from the mutant mice display a polarized Th1 response. In addition, these mice develop chronic inflammatory bowel disease with age. These findings show that Stat3 plays a critical role in the physiological suppression of overshooting macrophage and neutrophil functions.

Role for Stat3 in IL-10 Signaling in Macrophages

IL-10 is an antiinflammatory cytokine that suppresses macrophage activity (Moore et al., 1993). It antagonizes the production of inflammatory cytokines such as TNFα, IL-1, and IL-6 by macrophages stimulated with LPS and IFNγ and also inhibits their proliferation. Signal transduction through the IL-10 receptor leads to the activation of both Stat1 and Stat3, and Stat3 docking sites have been identified in the cytoplasmic domain of the IL-10 receptor (Finbloom and Winestock, 1995; Lai et al., 1996; Weber-Nordt et al., 1996; Wehinger et al., 1996). However, the biological importance of Stat1 versus Stat3 activation in IL-10-mediated functions remains unclear. As demonstrated here, the inhibitory activity of IL-10 on LPS-induced production of inflammatory cytokines from Stat3-deficient macrophages was completely blocked. In addition, IL-10-mediated inhibition of M-CSF-induced BMM proliferation was not observed. Thus, in Stat3-deficient macrophages, IL-10-mediated functions were severely impaired, demonstrating that Stat3 activation is essential for exerting IL-10-mediated functions in macrophages.

A recent study using dominant negative Stat3 protein argued that Stat3 induces antiproliferative but not antiinflammatory signals in macrophages (O'Farrell et al., 1998). In the J774 macrophage cell line that expresses the dominant negative Stat3 protein, IL-10-mediated inhibition of cell proliferation could not be detected,

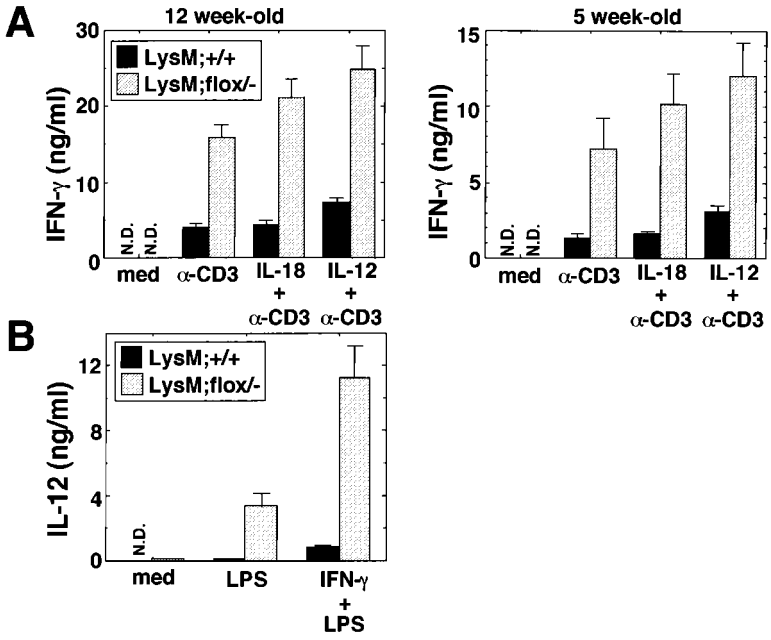

Figure 7. Enhanced Th1 Activity in LysMcre/Stat3$^{flox/-}$ Mice

(A) Splenic CD4$^+$ T cells were purified from 5- and 12-week-old mice, and cultured in anti-CD3 antibody-coated plates for 24 hr in the presence or absence of 2 ng/ml IL-12 and/or 20 ng/ml IL-18. Culture supernatants were analyzed for IFNγ production by ELISA. N.D.; not detected.

(B) Mice were intraperitoneally injected with thioglycollate, and 3 days later peritoneal macrophages were isolated. Macrophages were stimulated with 30 U/ml IFNγ and/or 2 μg/ml LPS for 24 hr. Concentrations of IL-12 in the culture supernatants were determined. N.D.; not detected.

whereas IL-10-mediated inhibition of LPS-induced TNFα, IL-1β, and B7-1 expression was still observed. In their cell line expressing the C-terminal deleted Stat3, IL-10-induced Stat3 tyrosine phosphorylation was still observed, although the level was severely reduced. The remaining Stat3 activation may be responsible for the IL-10-induced antiinflammatory response.

Stat3 Activation Is Critical for Suppression of Macrophage Activation

Macrophages are shown to be activated through a two-stage process; a priming stage and a triggering stage (Adams and Hamilton, 1987). IFNγ is a well characterized cytokine to prime macrophage activation. LPS is a major effector to trigger macrophage activation. In the priming stage, IFNγ induces surface expression of MHC class II and B7–1, 2 on macrophages. These IFNγ-mediated macrophage functions have been shown to be mediated through activation of Stat1, another member of STAT family proteins (Durbin et al., 1996; Meraz et al., 1996). IFNγ-primed macrophages, in turn, are capable of enhanced production of inflammatory cytokines and mediators such as TNFα, IL-6, and NO in response to LPS. Stat3-deficient PEC displayed enhanced expression of MHC class II and B7-1 and increased amounts and constitutive activation of Stat1. Thus, Stat3-deficient macrophages exhibited an activated phenotype as if they were already primed with IFNγ. In fact, Stat3-deficient macrophages secreted large amounts of cytokines and NO in response to LPS alone, and additional treatment with IFNγ did not augment their secretion. The finding that neither Stat3-deficient PEC nor splenocytes showed constitutive expression of IFNγ mRNA (our unpublished data) excludes the possibility that Stat3-deficient PEC may be continuously exposed to IFNγ.

Endogenous IL-10 does not only exert autoregulatory effects on TNFα, IL-1, or IL-6 production by macrophages, but also downregulates its own production and MHC class II expression on monoyctes in an autoregulatory fashion (de Waal Malefyt et al., 1991). Hence, the lack of antiinflammatory effects of IL-10 on activated macrophages further induce the continuous production of low amounts of IL-10 from macrophages. As IL-10 is shown to phosphorylate both Stat1 and Stat3, increased IL-10 production may promote the activation of Stat1 alone in Stat3-deficient macrophages. Alternatively, the deficiency of wild-type Stat3 in the macrophages may be compensated by an increased amount of Stat1 protein as well as tyrosine phosphorylation of Stat1 through an unidentified mechanism. Future investigations will be required to clarify the mechanism of activation of Stat1 in Stat3-deficient macrophages.

Stat3 Deficiency in Macrophages and Neutrophils Leads to Chronic Inflammatory Bowel Disease

Chronic inflammatory bowel disease (IBD) has been shown to be associated with increased Th1 cell activity (Powrie, 1995). LysMcre/Stat3$^{flox/-}$ mice showed polarized Th1 cell development as demonstrated by an increased production of IFNγ from splenic T cells, although these cells themselves have normal Stat3 expression. This fact indicates that Stat3 activation in macrophages plays a critical role in the normalization of the Th1 dominant phenotype. Gut macrophages are expected to be continuously activated by foreign substances such as bacteria and their products present in the mucosa, and secrete several inflammatory cytokines and mediators including TNFα, IL-1, and NO, which may result in tissue damage of the intestinal wall. Activated macrophages also secrete IL-12 and IL-18, which induce development of Th1 cells to produce IFNγ, which, in turn, activates macrophages. In normal mice, IL-10 is simultaneously secreted from activated macrophages and suppresses their activation to maintain the finely regulated homeostasis in vivo. However, in LysMcre/

Stat3$^{flox/-}$ mice, IL-10-induced suppression does not occur, and both macrophages and neutrophils are constitutively activated, resulting in progression to chronic inflammation.

Several evidences indicate that CD4$^+$ T cells play a central role in animal models of IBD (Morrissey et al., 1993; Powrie et al., 1994; Hollander et al., 1995; Davidson et al., 1996). However, the role of macrophages and neutrophils in the initiation and maintenance of IBD in the IL-10-deficient mouse model has not been assessed, although IL-10 is shown to act on both macrophages and neutrophils (Davidson et al., 1996; Nikolaus et al., 1998). In our present study, Stat3-deficient macrophages and neutrophils displayed constitutively activated phenotypes, and the mice developed chronic enterocolitis as in the case of IL-10-deficient mice. Interestingly, blockade of IL-10 signaling in Stat3-deficient macrophages and neutrophils only is sufficient for the development of colitis, although T, B, and epithelial cells have the capacity to both produce and respond to IL-10. This might indicate that the antiinflammatory effects of IL-10 on macrophages and neutrophils are more important than on T cells in the initiation of IBD. This speculation may be further strengthened by the observation that mice lacking Stat3, and as a consequence IL-10-signaling, specifically in T cells, did not develop enterocolitis (our unpublished data). In this respect, it is of note that LysMcre/Stat3$^{flox/-}$ mice develop enterocolitis even though they produce much higher amounts of IL-10 than normal mice in response to inflammatory stimuli, a condition that is normally expected to induce anergy in antigen-specific T cells (Groux et al., 1996).

It is conceivable also that, in the case of our Stat3 mutant mice, the initiation of colitis may already occur without the development of adaptive immunity that is mediated by activation of CD4$^+$ T cells with specific peptide-MHC complex recognition. An interesting future question will be whether or not mice that lack Stat3 in a macrophage- and neutrophil-specific fashion will develop IBD in the absence of a peripheral T cell population.

Experimental Procedures

Generation of Mice in which Stat3 Is Deficient Specifically in Neutrophils and Macrophages

Generation of mice in which the Stat3 gene was flanked by two loxP sites (Stat3$^{flox/+}$ mice) was described previously (Takeda et al., 1998). LysMcre mice expressing Cre under the control of the mouse lysozyme M gene regulatory region were generated by targeted insertion of the Cre recombinase cDNA including a nuclear localization sequence into the endogenous lysozyme M gene precisely at its ATG-start codon through homologous recombination in E14.1 embryonic stem cells. Generation and characterization of this mouse strain will be described in detail elsewhere (B. E. C., W. Reith, R. Renkawitz, and I. F., unpublished data). LysMcre mice were on a mixed 129/Ola, C57BL/6 and CB.20 background and were crossed to mice heterozygous for a Stat3 null mutation (Takeda et al., 1997) to decrease the amount of residual Stat3 protein after Cre-mediated deletion. These matings lead to the generation of mice that carried the *cre* gene and the heterozygous Stat3 null mutation (LysMcre/Stat3$^{+/-}$ mice). LysMcre/Stat3$^{+/-}$ mice were further mated with Stat3$^{flox/+}$ mice to generate LysMcre/Stat3$^{flox/-}$ mice. LysMcre/Stat3$^{+/+}$ or LysMcre/Stat3$^{+/-}$ from these crosses were used as wild-type littermate controls in the experiments. The primer sequences indicated in Figure 1A were as follows:

a, 5′-CCTGAAGACCAAGTTCATCTGTGTGAC-3′;
b, 5′-CACACAAGCCATCAAACTCTGGTCTCC-3′;
c, 5′-TTTGGAAAGTACTGTAGGCCCGAG AGC-3′.

Immunoprecipitation and Western Blot Analysis

Cells were isolated from the peritoneal cavity of nontreated mice by washing with Hank's balanced salt solution (HBSS) and incubated on dishes for 2 hr. Cells were washed to remove nonadherent cells. Adherent cells were incubated with the indicated stimuli for 30 min and solubilized with 1 ml of ice-cold lysis buffer containing 0.5% Nonidet P-40, 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4, and 5 μg/ml aprotinin. Whole cell lysates were incubated with antibodies to Stat3, Stat1, Tyk2 (Santa Cruz Biotech), or Jak1 (Upstate Biotechnology), and protein A-Sepharose (Pharmacia) for 4 hr at 4°C. Immunoprecipitates were separated on SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and incubated with anti-phosphotyrosine monoclonal antibody (4G10, Upstate Biotechnology). Bound antibody was visualized with an enhanced chemiluminescence system (DuPont).

Flow Cytometric Analysis

Cells isolated from the peritoneal cavity of nontreated mice were incubated on dishes for 2 hr. Adherent cells were harvested by scraping dishes and stained with biotin-conjugated anti-I-Ab or biotin-conjugated anti-B7-1 antibodies (PharMingen), followed by FITC-conjugated streptavidin. Stained cells were analyzed on a FACS Calibur using CELLQuest software (Becton Dickinson).

Preparation of Peritoneal Macrophages, Neutrophils, and Bone Marrow–Derived Macrophages

For isolation of PEC, mice were intraperitoneally injected with 2 ml of 4% thioglycollate. Peritoneal exudate cells were isolated from the peritoneal cavity 3 days post injection. Cells were incubated for 2 hr and washed three times with HBSS. Remaining adherent cells were used as PEC for the experiments. For peritoneal neutrophils, cells were isolated from the peritoneal cavity after 4 hr of thioglycollate injection and cultured for 18 hr in the presence or absence of 10 ng/ml IL-10. Then, nonadherent cells were harvested and used for further experiments. These cells were >90% positive for Gr-1 as determined by flow cytometry. In the case of more strict experiments, the cells were further enriched by magnetic cell sorting (MACS, Miltenyi Biotec) using biotin-conjugated anti-Gr-1 antibody and streptavidin-microbeads.

Bone marrow cells were isolated from femurs and cultured with RPMI1640 supplemented with 10% fetal bovine serum and 50 ng/ml M-CSF (R&D Systems) or 20 ng/ml GM-CSF (Genzyme). Culture fluid was exchanged to fresh culture medium every 4 days. Under these conditions, adherent macrophage monolayers were obtained within 8 to 10 days. These cells were >99% positive for Mac-1 and Fcγ receptor as determined by flow cytometry, identifying them as macrophages. Cells were harvested by scraping dishes and seeded on 96-well plates. After culture for 6 hr without M-CSF or GM-CSF, the cells were used for the experiments as bone marrow–derived macrophages.

Measurement of Cytokines in Sera and Culture Supernatants

Mice were intravenously injected with 20 μg of LPS and bled at the indicated times. Serum concentrations of TNFα, IL-10, IL-6, IFNγ, and IL-1β were determined by ELISA (Genzyme).

PEC and BMM were cultured with 30 U/ml IFNγ and/or 2 μg/ml LPS for 24 hr. In some experiments, PEC and BMM were precultured with the indicated concentrations of IL-6 and IL-10 for 12 hr. Concentrations of TNFα, IL-6, and IL-12 in the culture supernatants were determined by ELISA. Concentration of NO was measured using Griess reagents as previously described (Takeda et al., 1996b).

Proliferation Assay of Bone Marrow–Derived Macrophages

Bone marrow-derived macrophages (1×10^4) were cultured in 96-well plates in the presence of the indicated concentration of IL-6 or IL-10 (Genzyme). After 12 hr, M-CSF was added to the cultures

and the BMM were incubated for an additional 48 hr. 1 μCi of [^{3}H]-thymidine was pulsed for the last 6 hr of culture. [^{3}H]-thymidine uptake was measured in a beta scintillation counter (Packard).

Histological and Immunohistological Analysis

Tissues were fixed in 10% phosphate-buffered formalin, and paraffin-embedded tissue sections were stained with hematoxylin and eosin using standard techniques. For immunostaining, sections were incubated in 0.3% H_2O_2 in methanol for 30 min to inactivate internal peroxidases, washed with phosphate-buffered saline (PBS; 10 mM sodium phosphate buffer and 150 mM NaCl [pH 7.4]), and incubated with normal horse serum (Vector Laboratories) to block nonspecific binding of antibodies. Sections were then incubated with biotin-conjugated rat anti-mouse I-Ab monoclonal antibody (PharMingen), biotin-conjugated goat anti-mouse IgA polyclonal antibody (Southern Biochemistry Association), or rat anti-mouse CD4 monoclonal antibody (PharMingen) at 4°C overnight. For immunostaining of anti-CD4, sections were incubated with biotin-conjugated rabbit anti-rat IgG (Dako A/S) for 60 min. Immunolabeled cells were visualized with streptavidin-peroxidase (Vecstatin ABC Elite kit; Vector Laboratories) and diaminobenzidine (Sigma). The sections were lightly counterstained with hematoxylin.

Measurement of IFNγ Production from T Cells

Splenic CD4$^+$ T cells were positively enriched by magnetic cell sorting (MACS, Miltenyi Biotec) using anti-CD4 microbeads. Purified T cells were analyzed by PE-conjugated anti-CD4 antibody (PharMingen), and >98% cells were found to be CD4-positive. 2×10^5 CD4$^+$ T cells were stimulated with immobilized anti-CD3 antibody (10 μg/ml, PharMingen) for 24 hr. Concentrations of IFNγ in the culture supernatants were determined by ELISA.

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