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IL-13 Orchestrates Resolution of Chronic Intestinal Inflammation via Phosphorylation of Glycogen Synthase Kinase-3β

Stefan Fichtner-Feigl,*+‡ Rebecca Kesselring,* Maria Martin,* Florian Obermeier,§ Petra Ruemmele,¶ Atsushi Kitani,† Stefan M. Brunner,* Michael Haimerl,* Edward K. Geissler,* Warren Strober,† and Hans J. Schlitt*

Spontaneous amelioration of inflammation (often accompanied by fibrosis) is a well-known, but poorly understood, outcome of many chronic inflammatory processes. We studied this phenomenon in a chronic trinitrobenzene sulfonic acid–induced colitis model, an experimental colitis in mice that we showed to ultimately undergo spontaneous resolution, despite continued trinitrobenzene sulfonic acid stimulation. Analysis of the mechanism of this resolution revealed that it was critically dependent on IL-13 activation of STAT6, followed by phosphorylation (inactivation) of glycogen synthase kinase-3β, at least in part via STAT6 induction of p38 MAPK. Such glycogen synthase kinase-3β inactivation causes changes in CREB and p65 DNA-binding activity that favors decreased proinflammatory IL-17 production and increased anti-inflammatory IL-10 production. Thus, in this case, IL-13 acts as a molecular switch that leads to resolution of inflammation. The Journal of Immunology, 2014, 192: 3969–3980.

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chronic inflammatory responses often result in subsidence of inflammation and an accompanying tissue fibrosis that sometimes causes irreversible organ dysfunction (1–6). This progression can be seen in Crohn’s disease (CD), in which areas of transmural, granulomatous inflammation frequently resolve in association with the formation of stricture-causing segmental fibrosis (3, 5). Recognizing that an understanding of such spontaneous resolution of inflammation could be applied to the prevention or resolution of CD during its more robust inflammatory phase, we turned to an experimental model of colitis, chronic hapten-induced (trinitrobenzene sulfonic acid [TNBS]-induced) colitis, which we (7–9) previously showed exhibits at least one aspect of the resolved CD lesion, fibrosis. In these previous studies we showed that, in chronic TNBS colitis, an initial acute and severe inflammation that is characterized by the presence of Th1 cytokines, IL-12 and IFN-γ, is followed by a more chronic and more sedate inflammation accompanied by the presence of IL-23 and IL-17 (8). During this second phase, a fibrotic program is initiated that is characterized by the production of IL-13 and subsequent IL-13 signaling via a unique IL-13R, IL-13Rα2; the latter, in turn, induces the production of profibrotic TGF-β1 via a non-STAT6 pathway involving AP-1 (8). In the current study, we found that mice subjected to chronic TNBS colitis eventually enter a third phase manifesting by mild and decreasing colitis associated with continuation of the fibrosis. Thus, inflammation in this model undergoes spontaneous resolution, and this model can be used to provide insight into the resolution of inflammation occurring in human disease.

In studies of the mechanism of this spontaneous resolution, we investigated the role of IL-13 in bringing about the end-stage of chronic TNBS colitis, because IL-13 production is a major component of the cytokine response during this stage. We found that IL-13 signaling via IL-13Rα1 induces inactivation (phosphorylation) of glycogen synthase kinase-3β (GSK-3β), a molecule previously shown to be involved in the downregulation of inflammation caused by the innate immune system (10–13). In addition, we found that the induction of phosphorylated GSK-3β led to the decreased IL-23/IL-17 and increased IL-10 production characterizing end-stage and resolving chronic TNBS colitis.

Materials and Methods

Mice

Female BALB/c mice (8–10 wk old) and STAT6-deficient mice were obtained from The Jackson Laboratory (Bar Harbor, ME) or from Charles River (Sulzbach, Germany). The animals were maintained in the holding facilities of the National Institute of Allergy and Infectious Diseases and the University of Regensburg. Animal use adhered to the Laboratory Animal Care Guidelines of the National Institutes of Health and of the University of Regensburg.

Induction of TNBS colitis

Chronic TNBS colitis was induced by weekly administration of increasing doses of TNBS (1.5–2.5 mg in 45% ethanol; Sigma-Aldrich, St. Louis, MO) (7, 8). Mice were lightly anesthetized with isoflurane and then administered TNBS/ethanol per rectum via a 3.5 F catheter equipped with a 1-ml syringe; the catheter was advanced into the rectum until the tip was 4 cm proximal to the anal verge, at which time the TNBS was administered in a total volume of 150 μl. To ensure distribution of TNBS within the entire colon and cecum, mice were held in a vertical position for 30 s after the intrarectal injection.
Collagen assay
Colons of TNBS-treated mice were harvested and homogenized in 0.5 M acetic acid containing pepsin (at a concentration of 10 mg tissue/10 ml acetic acid solution). The resulting mixture was incubated and stirred for 24 h at 4°C. Total soluble collagen content of the mixture was determined with a Sircol Collagen Assay Kit (Biocolor, Carrickfergus, U.K.). Acid soluble type I collagen supplied with the kit was used to generate a standard curve.

Construction and administration of decoy oligonucleotides
Decoy oligonucleotides (ODNs) targeting STAT6 or STAT3 transcription sites and scrambled control ODNs were prepared from complementary single-stranded ODNs obtained from QIAGEN (Hilden, Germany) (8, 14, 15). A total of 75 μg the decoy ODNs was administered to each mouse under study by intrarectal injection following encapsulation in HVJ Envelope (HVJ-E) (16).

Construction and administration of a vector expressing soluble IL-13Rα2–Fc
A vector expressing soluble (s)IL-13Rα2–Fc (pCI-sIL-13Rα2–Fc plasmid) was constructed, as previously described, by first inserting IL-13Rα2 cDNA and then cDNA encoding the Fc segment of human IgG into pCI-Mammalian Expression Vector (Promega, Madison, WI) (8). In various studies, 100 μg plasmid DNA in 20 μl PBS was administered intranasally to mice lightly anesthetized with isoflurane. The quality of plasmid DNA was verified by electrophoresis on 1% agarose gel just before administration.

Small interfering RNA
GSK-3β-specific small interfering RNA (siRNA) and control siRNA for use in gene-silencing studies consisted of predesigned SMART pool siRNA obtained from Dharmacon. For high-efficiency in vivo delivery of siRNA, it was encapsulated in HVJ-E, as described previously (8, 14, 17). The encapsulated siRNA was administered by intrarectal instillation every other day.

HVJ-E transfection
Suspended Sendai virus (25600 hemagglutinating units; AnGes MG) was inactivated by β-propiolactone, followed by UV irradiation and purification by column chromatography. The HVJ-E obtained was mixed with protamine sulfate and incubated on ice. Insertion of ODNs, plasmid DNA, or siRNA into the transfection vector was accomplished using a packaging technique that allowed direct insertion of DNA into the viral envelope. This consisted of mixing DNA and Triton X-100 with the HVJ-E and incubating the resultant mixture on ice. Finally, the HVJ-E–DNA package was centrifuged at 15000 × g and resuspended in PBS containing protamine sulfate.

Inhibitor studies
GSK-3β inhibition. The synthetic GSK-3β inhibitors SB216763 (Sigma-Aldrich) and C799021 (Axon Medchem, Groningen, The Netherlands) were administered i.p. at a concentration of 500 μg/mouse every other day. DMSO, at a final concentration of 0.1%, was used as a vehicle.

PI3K inhibition. The synthetic PI3K inhibitor LY294002 (EMD Chemicals, Gibbstown, NJ) was administered i.p. at a concentration of 15 mg/kg in 50% DMSO every other day.

MAPK inhibition. The synthetic MAPK inhibitor SB203580 (EMD Chemicals) was administered i.p. at a concentration of 12.5 mg/kg in 50% DMSO every other day.

Cell isolation and culture
Colonic lamina propria mononuclear cells (LPMCs) were isolated from colonic tissues, as previously described, and cultured for 48 h in the presence of various stimulants (7, 8, 18). The capacity of cultured LPMCs to secrete IFN-γ, IL-13, and IL-17 was determined by stimulation with plate-bound anti-CD3 Abs (10 μg/ml) and soluble anti-CD28 Abs (1 μg/ml) (BD Biosciences, San Jose, CA). The capacity of cultured LPMCs to secrete IL-12p70 was determined by stimulation with Staphylococcus aureus Cowan 1 (1:10,000 dilution of Pansorbin; EMD Biosciences, Darmstadt, Germany) and IFN-γ (1,000 U/ml; R&D Systems, Minneapolis, MN), their capacity to secrete IL-23 or IL-10 was determined by stimulation with peptidoglycan (100 μg/ml; Sigma-Aldrich), and their capacity to secrete TGF-β was determined by stimulation with IL-13 (20 μg/ml) plus TNF-α (20 μg/ml) (PeproTech, Rocky Hill, NJ).

ELISAs
Cytokine concentrations were measured by ELISA, according to manufacturer’s instructions. ELISA kits for IFN-γ, IL-12p70, TNF-α, and IL-10 were purchased from BD Biosciences, the kit for TGF-β1 was from Invitrogen (Carlsbad, CA), the kit for IL-23 was from eBioscience (San Diego, CA), and the kit for IL-13 was from R&D Systems. TGF-β1 was measured in medium containing TGF-β1-depleted human serum. Measurement of p–GSK-3β at Ser9 was performed using a DuoSet IC Phospho-GSK-3β ELISA kit (R&D Systems), according to the manufacturer’s instructions. Phosphorylation at Ser21/22 was performed in a similar fashion using p–GSK-3β (Ser21/22) Ab from Millipore (Billerica, MA) and GSK-3β (Ser9) Ab from Santa Cruz Biotechnology (Santa Cruz, CA).

Western blot analysis
Western blot analysis was performed as described previously. p–GSK-3β and GSK-3β were detected by incubation with a monoclonal rabbit antibody p–GSK-3β or GSK-3β Ab (Cell Signaling, Boston, MA), followed by incubation with HRP-conjugated anti-rabbit IgG Ab (Cell Signaling).

Transcription factor activation assay
Nuclear extracts from bone marrow–derived dendritic cells (BMDCs) or isolated colonic LPMCs were obtained using a TransFactor Extract Kit (Active Motif, Carlsbad, CA) and tested for DNA-binding activity using a CREB and NF-κB p65 TransFactor Kit (BD Biosciences), according to the manufacturer’s instructions.

Flow cytometry
Colonic LPMCs were isolated and subjected to flow cytometric analysis. For staining of CD4 and Foxp3, cells were treated with mAb to mouse CD4 and mAb to Foxp3, respectively (eBioscience). Nonspecific binding of Abs was blocked by preincubation with Fcy block. Cells were analyzed using a Becton Dickinson FACSscan and FlowJo software.

Histological examination of colonic tissue and scoring of inflammation
Colonic tissues were fixed and embedded for staining. For calculation of inflammation indices or for assessment of fibrosis in treated and control group mice, the sections were read blindly and evaluated according to a previously described scoring system (19).

Statistical analyses
For calculation of differences in body weight at each time point, multivariate analysis (if significant), followed by a Mann–Whitney U test, was used. For calculation of differences in histology score, a Kruskal–Wallace test with Dunn multiple comparison test was used. For calculation of differences in cytokine levels, a two-way ANOVA test with Bonferroni posttest was used. Statistics were computed using GraphPad Prism 4 to evaluate the significance of the differences. A value of p < 0.05 was considered statistically significant.

Results
Spontaneous resolution of chronic TNBS-induced colitis in BALB/c mice
Previous studies (7–9, 20) established that a chronic form of TNBS colitis in BALB/c mice, induced by weekly intrarectal administration of TNBS, enters a peak phase of Th17-mediated inflammation in weeks 6–7 (days 42–49) of colitis, corresponding to the appearance of subepithelial fibrosis (Supplemental Fig. 1A, 1B). However, after week 7, IL-23/17 and TNF-α levels gradually decline, so that by weeks 10–12 of colitis, they have fallen to near baseline levels (Fig. 1A); additionally, the histologic picture returns to a near normal state, except for the persistence of fibrosis (Fig. 1B, Supplemental Fig. 1B). In some contrast, IL-13 and TGF-β1 levels increase early during the course of disease and remain elevated until week 8 of colitis, after which they decline somewhat, as well (but never to baseline levels) (Fig. 1A).

Blockade of IL-13 results in restoration of inflammatory cytokine secretion
Because, as shown above, resolution of intestinal inflammation occurring during the late phase of chronic TNBS colitis was accompanied by stable IL-13 levels in the face of decreasing IL-23 and IL-17 levels, we began our investigation of the mechanism of
colitis resolution by assessing the role of IL-13 in this process. To this end, we blocked IL-13 and its downstream signaling in mice with chronic TNBS colitis starting on day 56 of colitis by intranasal administration (every other day) of a plasmid encoding an sIL-13Rα2–Fc fusion protein (pCI–sIL-13Rα2–Fc). Administration of this plasmid results in colonic expression of sIL-13Rα2–Fc (Supplemental Fig. 1C) and, as shown in prior studies (8), reduction in IL-13 levels and blockade of IL-13 signaling in colonic tissue. As demonstrated in Fig. 2A, blockade of IL-13 prevented the decrease in the levels of inflammatory cytokines (IL-17, IL-23, and TNF-α) ordinarily observed on day 70 of chronic TNBS colitis (Fig. 1A). In addition, IL-13 blockade led to a marked decrease in body weight and an increase in histologic signs of inflammation, determined on day 70, compared with untreated mice (Fig. 2B, 2C). To confirm the above-described effects of IL-13 blockade by pCI–sIL-13Rα2–Fc on the resolution of inflammation, we administered inhibitory anti–IL-13 Ab or control IgG to mice with chronic TNBS colitis, beginning on day 56 of disease, at biweekly intervals (500 μg/wk). In this case, we again observed that IL-13 blockade resulted in failure to resolve the inflammation, as indicated by the persistence of increased proinflammatory cytokine production and increased microscopic evidence of inflammation in mice administered anti–IL-13 Ab compared with mice administered control IgG (Supplemental Fig. 1D, 1E).

We next determined whether the above inhibitory effect of IL-13 on inflammation involves IL-13 signaling via the IL-13Rα1 receptor and the activation of STAT6, the major pathway of IL-13 signal transduction. In initial studies addressing this question, we blocked STAT6 function by intrarectal administration of decoy STAT6 ODNs encapsulated in a viral envelope (HVJ-E) that facilitate entry into intestinal cells and blockade of IL-13 signaling in colonic tissue. As demonstrated in Fig. 2A, blockade of IL-13 prevented the decrease in the levels of inflammatory cytokines (IL-17, IL-23, and TNF-α) ordinarily observed on day 70 of chronic TNBS colitis (Fig. 1A). In addition, IL-13 blockade led to a marked decrease in body weight and an increase in histologic signs of inflammation, determined on day 70, compared with untreated mice (Fig. 2B, 2C). To confirm the above-described effects of IL-13 blockade by pCI–sIL-13Rα2–Fc on the resolution of inflammation, we administered inhibitory anti–IL-13 Ab or control IgG to mice with chronic TNBS colitis, beginning on day 56 of disease, at biweekly intervals (500 μg/wk). In this case, we again observed that IL-13 blockade resulted in failure to resolve the inflammation, as indicated by the persistence of increased proinflammatory cytokine production and increased microscopic evidence of inflammation in mice administered anti–IL-13 Ab compared with mice administered control IgG (Supplemental Fig. 1D, 1E).

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Resolution of inflammation correlates with the appearance of GSK-3β inactivation (phosphorylation) in LPMCs

In further studies, we focused on the possibility that IL-13 was facilitating resolution of inflammation by inducing phosphorylation (inactivation) of GSK-3β, a serine/threonine kinase known to downmodulate proinflammatory immune responses when present in a phosphorylated (kinase-inactive) state (10–13, 21). In initial studies exploring whether this is the case, we determined the phosphorylation status of GSK-3β during the course of chronic TNBS colitis by assessment of p–GSK-3β by Western blot and quantitatve ELISA in cellular extracts of LPMCs isolated from the colons of mice at weekly intervals. With the onset of inflammation, GSK-3β detection in LPMCs was present in a nonphosphorylated state and remained in that state throughout the study (i.e., during the period of intense Th1-mediated inflammation previously shown to characterize this phase of colitis) (Fig. 4A) (19, 22–24).
22–24). However, from day 49 through day 63, LPMCs displayed increased levels of p–GSK-3β, which increased further from day 70 through day 91 to the level observed at day 0 before onset of inflammation. It should be noted that this pattern of GSK-3β phosphorylation was noted with an Ab nominally recognizing Ser9 phosphorylation (Western blot or ELISA studies) or with an Ab recognizing Ser389 phosphorylation (ELISA study). However, the Ser389 phosphorylation site is likely to be the dominant phosphorylation site, because the Ser9 phosphorylation signal detected with the anti-Ser9 Ab was relatively weak, and Ser389 phosphorylation is the main phosphorylation site of IL-13 (see below). In additional studies to fine-tune these results, we determined changes in GSK-3β phosphorylation in purified lamina propria cell populations on days 7, 49, and 70 of chronic TNBS colitis. Although CD4+ (T cells) and B220+ (mainly B cells) cells displayed low and unchanging levels of p–GSK-3β at these time points, CD11c+ cells and, to a lesser degree, F4/80+ cells exhibited a pattern of GSK-3β phosphorylation similar to that of unpurified LPMC populations (Fig. 4B). These studies indicated that increased levels of p–GSK-3β characterize the period of decreased inflammation and persistently high IL-13 levels in chronic TNBS colitis, suggesting that IL-13 modulation of inflammation involves regulation of GSK-3β phosphorylation.

IL-13 regulates the phosphorylation of GSK-3β during chronic TNBS colitis

To more clearly establish the latter possibility, we next conducted studies to examine whether IL-13 or IL-13-signaling blockade diminishes GSK-3β phosphorylation during the resolution phase of chronic TNBS colitis. Accordingly, we administered sIL-13Rα2–Fc (every other day, i.p.) to mice with chronic TNBS colitis starting on day 56 of disease and measured GSK-3β phosphorylation in nuclear extracts of LPMCs isolated on day 70 of colitis. Using conventional methods, we found that IL-13 treatment significantly reduced GSK-3β phosphorylation, whereas IL-13 signaling blockade (using STAT6 decoy ODNs) did not significantly alter GSK-3β phosphorylation levels. These results are consistent with the hypothesis that IL-13-mediated suppression of inflammation involves regulation of GSK-3β phosphorylation.

**FIGURE 2.** Inhibition of IL-13 restores expression of inflammatory cytokines. (A, B, and C) pCI–sIL-13Rα2–Fc was administered every other day starting on day 56, whereas STAT6 decoy ODNs were administered on days 56 and 63. Cells were extracted from the lamina propria on day 70 and cultured for 48 h under stimulation conditions described in Materials and Methods. Cytokine concentrations were determined in culture supernatants by ELISA. Data shown are mean ± SD from individual cultures of cells derived from mice in two separate experiments, each containing at least five mice/group. (A) LPMC cytokine production on day 70 of chronic TNBS colitis after treatment with pCI–sIL-13Rα2–Fc. (B) Body weight as a percentage of starting weight measured at indicated time points after treatment with pCI–sIL-13Rα2–Fc. Data shown are mean ± SD derived from at least five mice/group. (C) Histological scores on day 70 of chronic TNBS colitis after treatment with pCI–sIL-13Rα2–Fc. Scores shown are mean ± SD from at least five mice/group. (D) LPMC cytokine production on day 70 of chronic TNBS colitis after treatment with STAT6 decoy ODNs. (E) Body weight as a percentage of starting weight measured at indicated time points after treatment with STAT6 decoy ODNs. Data shown are mean values ± SD derived from at least five mice/group. (F) Histological scores on day 70 of chronic TNBS colitis after treatment with STAT6 decoy ODNs. Scores shown are mean ± SD from at least five mice/group. *p ≤ 0.05.
developed with anti–GSK-3β and ELISA developed with anti–GSK-3β (p-Ser389), we found that such inhibition of IL-13 diminishes GSK-3β phosphorylation in the day-70 extracts (Fig. 4C). In addition, administration of Stat6 decoy ODNs using the same regimen as that used with sIL-13Rα2–Fc yielded an identical result (Fig. 4C). Finally, mice with STAT6 deficiency also displayed decreased GSK-3β phosphorylation during the late stage of chronic TNBS colitis (Fig. 4D). Taken together, these studies provided strong in vivo evidence that the decreased level of GSK-3β phosphorylation accompanying the resolution phase of chronic TNBS colitis is causally linked to the persistent elevation of IL-13.

**IL-13 induces GSK-3β phosphorylation via p38 MAPK**

In further exploration of the relationship of IL-13 to GSK-3β phosphorylation in the context of colitis, we next conducted studies to identify the mechanism of IL-13–induced phosphorylation. In an earlier report (25), it was shown that TLR signaling can induce phosphorylation of GSK-3β through the PI3K-Akt–signaling pathway. To determine whether IL-13 signaling also induces GSK-3β phosphorylation by this pathway, we stimulated BMDCs with IL-13 or peptidoglycan (PGN) in the presence of LY294002, a specific inhibitor of the PI3K-Akt pathway and then analyzed cell extracts for the presence of p–GSK-3β with conventional Western blotting developed with anti–GSK-3β (p-Ser389) or with ELISA developed with anti–GSK-3β (p-Ser389). Although LY294002 at least partially inhibited PGN-induced phosphorylation (Fig. 5A), it had no effect on IL-13–induced phosphorylation; similarly, LY294002 did not inhibit such IL-13–induced GSK-3β phosphorylation (Fig. 5A).

The lack of effect of LY294002 on IL-13–induced GSK-3β phosphorylation was examined further in vivo with studies in which the ability of LY294002 to inhibit IL-13’s effects on chronic TNBS colitis was tested. Intraperitoneal administration (every other day) of LY294002 or Stat6 decoy ODNs to mice with chronic TNBS colitis from days 56–70 was followed by studies of GSK-3β phosphorylation and parameters of inflammation on day 70. LY294002 administration did not result in inhibition of GSK-3β phosphorylation, whereas Stat6 decoy resulted in such inhibition (as evaluated with anti–Ser9 in conventional Western blot or anti–Ser389 in ELISA) (Fig. 5B). Similarly, Ly294002 did not affect IL-13’s amelioration of either the colitis intensity or inflammatory cytokine secretion, whereas Stat6 decoy reversed such amelioration (Fig. 5C, 5D). Thus, these in vitro and in vivo findings support the idea that the capacity of IL-13 to induce GSK-3β phosphorylation is channeled through a signaling pathway independent of PI3K-Akt.

An alternative pathway of GSK-3β phosphorylation that might underlie IL-13–induced GSK-3β phosphorylation is suggested by recent studies (26) showing that p38 MAPK also phosphorylates (and inactivates) GSK-3β; however, in this case, the phosphorylation (in mice) occurs mainly at Ser389. This is relevant to IL-13–induced phosphorylation of GSK-3β, because p38 MAPK is induced by IL-13 via STAT6 activation (27). Initial support for this idea came from the studies described above showing that IL-13 induction of GSK-3β phosphorylation can be detected both in vivo and in vitro with an Ab recognizing Ser389 phosphorylation. To verify that this Ab has specificity for the Ser389 site, we conducted studies showing that phosphorylation of GSK-3β by IL-13 is inhibited by a pSer389 peptide mimicking the S389 phosphorylation site in GSK-3β, but not by control unphosphorylated Ser389 peptide, and that this peptide does not inhibit anti-Ser9 (data not shown). With this information in hand, we determined whether Ser389 phosphorylation in IL-13–stimulated BMDCs is downregulated by p38 MAPK–specific siRNA. In vitro IL-13 induction of GSK-3β phosphorylation in BMDCs is inhibited by p38 MAPK–specific siRNA, whereas PGN induction of GSK-3β phosphorylation is less inhibited (Fig. 5E). Thus, these data led to the conclusion that IL-13 phosphorylation of GSK-3β is mediated by p38 MAPK.

**IL-13 versus PGN induction of GSK-3β phosphorylation and effects on downstream GSK-3β function**

Because IL-13 stimulation is in potential competition with TLR stimulation for GSK-3β phosphorylation, we next conducted studies of IL-13–induced and TLR–induced phosphorylation of GSK-3β, both with respect to persistence of phosphorylation and downstream effects of phosphorylation on NF-κB activation. In
initial studies, we stimulated BMDCs in vitro with IL-13 or a TLR ligand (PGN) alone or in combination (again using both anti–GSK-3β[p-Ser9]– and anti–GSK-3β[p-Ser389]–detection Abs). We found that cellular extracts of BMDCs stimulated by IL-13 alone expressed p–GSK-3β after exposure to IL-13 at 30 min, as well as at 60 and 120 min (Supplemental Fig. 2A, 2B). Further, cellular extracts of BMDCs stimulated by PGN alone also expressed p–GSK-3β; however, such expression was relatively transient and disappeared by 60 min after stimulation. In addition, PGN plus IL-13 stimulation also led to persistent GSK-3β phosphorylation, whereas PGN plus IL-17 or TGF-β1 stimulation (i.e., stimulation with other cytokines expressed during chronic TNBS colitis) led to short-lived GSK-3β phosphorylation (Supplemental Fig. 2B, 2C). Finally, similar results were obtained in studies in which the TLR4 ligand, LPS, was evaluated with respect to GSK-3β phosphorylation (Supplemental Fig. 2D). Taken together, these studies established that IL-13–induced phosphorylation of GSK-3β is more persistent than that induced by TLR ligand.

In further studies we explored the possible functional consequences of persistent IL-13–induced GSK-3β phosphorylation. It was shown previously that the anti-inflammatory effect of phosphorylated (inactive) GSK-3β is due, at least in part, to the fact that it enhances the activation of CREB and, thus, the latter’s translocation to the nucleus, where it competes with NF-κB for binding to CREB-binding protein (CBP). This, in turn, leads to reduced formation of the CBP/NF-κB p65 DNA-binding complex that is necessary for NF-κB p65 activation of proinflammatory cytokine promoters and, thus, results in inhibition of NF-κB function (11, 28). Therefore, we assessed downstream effects of IL-13–, PGN–, or IL-13/PGN–induced phosphorylation of GSK-3β by measuring the level of activated (presumably CBP-complexed) CREB and NF-κB p65 in nuclear extracts of induced cells (BMDCs). This was accomplished using a TransFactor Assay (see Materials and Methods) that quantitates binding of CREB and NF-κB p65 to consensus CREB and NF-κB p65 promoter target sequences, respectively.
We found that IL-13 stimulation of BMDCs led to increases in nuclear CREB binding after both 30 and 120 min, but these increases were significantly lower than those elicited by PGN stimulation of cells at 30 min; however, this large PGN increase was transient in that it was not observed after 120 min (Supplemental Fig. 3A). In contrast, IL-13 plus PGN stimulation of cells led to increased nuclear CREB binding at both time points. These data show that, although IL-13 has a relatively modest effect on nuclear CREB binding, it has the capacity to sustain the induction of activated CREB by PGN, in keeping with its more prolonged effect on GSK-3β inactivation. IL-13 and PGN have reciprocal effects on NF-κB p65 binding (Supplemental Fig. 3A). Thus, although PGN, but not IL-13, enhances the level of NF-κB p65 binding, particularly at 30 min in the presence of IL-13, such enhancement by PGN is diminished, presumably due to the effect of IL-13 on GSK-3β inactivation and induction of activated CREB. Thus, in vitro studies show that IL-13 and TLR ligand have different effects on GSK-3β downstream function: IL-13–induced GSK-3β phosphorylation favors changes in CREB binding that are associated with decreased PGN-induced NF-κB p65 binding to NF-κB binding sites and decreased proinflammatory cytokine production.

We found that IL-13 stimulation of BMDCs led to increases in nuclear CREB binding after both 30 and 120 min, but these increases were significantly lower than those elicited by PGN stimulation of cells at 30 min; however, this large PGN increase was transient in that it was not observed after 120 min (Supplemental Fig. 3A). In contrast, IL-13 plus PGN stimulation of cells led to increased nuclear CREB binding at both time points. These data show that, although IL-13 has a relatively modest effect on nuclear CREB binding, it has the capacity to sustain the induction of activated CREB by PGN, in keeping with its more prolonged effect on GSK-3β inactivation. IL-13 and PGN have reciprocal effects on NF-κB p65 binding (Supplemental Fig. 3A). Thus, although PGN, but not IL-13, enhances the level of NF-κB p65 binding, particularly at 30 min in the presence of IL-13, such enhancement by PGN is diminished, presumably due to the effect of IL-13 on GSK-3β inactivation and induction of activated CREB. Thus, in vitro studies show that IL-13 and TLR ligand have different effects on GSK-3β downstream function: IL-13–induced GSK-3β phosphorylation favors changes in CREB binding that are associated with decreased PGN-induced NF-κB p65 binding to NF-κB binding sites and decreased proinflammatory cytokine production.

In parallel studies, we determined whether the above pattern of downstream effects of IL-13–induced GSK-3β phosphorylation (i.e., changes in CREB and NF-κB p65 binding to DNA binding sites obtained during chronic TNBS colitis). To this end, we first assessed CREB and NF-κB p65 binding activity in nuclear extracts of colonic LPMCs at various stages of chronic TNBS colitis. We found that NF-κB p65 DNA–binding activity was greatly increased on day 7 of chronic TNBS colitis (at a time of intense inflammation), whereas it was reduced on day 49 (at a time of moderate inflammation) and decreased to basal levels on day 70 (at a time of minimal inflammation) (Supplemental Fig. 3B). In contrast, CREB DNA–binding activity was only marginally elevated on day 7 of chronic TNBS colitis, more elevated on day 49, and greatly elevated on day 70. Thus, the patterns of CREB and

**FIGURE 5.** IL-13 induction of p–GSK-3β is dependent on p38 MAPK. (A) Nuclear extracts derived from BMDCs stimulated with PGN and IL-13 under PI3K inhibition were subjected to Western blots for p–GSK-3β, GSK-3β, and β-actin. Data shown are representative of three independent experiments. (B) ELISA for detection of p–GSK-3β in nuclear extracts of LPMCs from the colons of mice on day 70 of chronic TNBS colitis after administration of Ly294002 or STAT6 decoy ODN. Data shown are representative of three independent experiments involving groups of mice containing at least five individuals/group. (C) Histological scores and DNA-binding activity of LPMCs on day 70 of chronic TNBS colitis after treatment with Ly294002 or STAT6 decoy ODN. (D) LPMC cytokine production on day 70 of chronic TNBS colitis after treatment with Ly294002 or STAT6 decoy ODN. (E) Nuclear extracts derived from BMDCs stimulated with IL-13 and PGN were subjected to p–Ser389–GSK-3β ELISA (see Materials and Methods) in the presence or absence of p38-specific siRNA. Data shown are mean ± SD from three independent experiments. *p ≤ 0.05.
NF-κB p65 binding to DNA during the course of TNBS colitis were reciprocal and correlated with the level of GSK-3β phosphorylation. Finally, to determine whether these binding patterns are dependent on IL-13 secretion, we examined CREB and NF-κB p65 binding to DNA in mice with late-stage TNBS colitis, in whom IL-13 signaling was inhibited as a result of administration of sIL-13Rβ2-Fc (as described above), or in STAT6 deficiency. Inhibition of IL-13 signaling led to reduced CREB binding and increased NF-κB p65 binding to DNA, consistent with the presence of decreased IL-13–induced GSK-3β phosphorylation (Supplemental Fig. 3C, 3D).

Overall, this series of studies suggests that IL-13 induction of GSK-3β phosphorylation has a more anti-inflammatory outcome than does PGN (i.e., TLR) induction of GSK-3β phosphorylation. Furthermore, these studies show that the downstream effects of GSK-3β phosphorylation during the course of chronic TNBS colitis reflected a pattern predicted by IL-13 induction of such phosphorylation.

Inhibition of active GSK-3β during the late phase of chronic TNBS colitis restores effector cytokine secretion in the face of IL-13 blockade

If IL-13 contributes to the resolution of inflammation during the late stage of chronic TNBS colitis through its effect on the phosphorylation of GSK-3β, then one should be able to inhibit effector cytokine secretion by administration of a specific inhibitor of GSK-3β (CT99021) that maintains GSK-3β in a phosphorylated state, even when IL-13 induction of GSK-3β phosphorylation is blocked (29). Accordingly, we administered a plasmid expressing sIL-13Rβ2-Fc, every other day starting on day 56 of chronic TNBS colitis (as described above), to block IL-13 induction of GSK-3β phosphorylation. Again, we found that GSK-3β is maintained in a functionally inactive (i.e., phosphorylated) state, as assessed by CREB- and NF-κB p65–binding activity, on day 70 (Fig. 6A). In addition, this is associated with high IL-17 and IL-23 levels at this time point (Fig. 6B). In contrast, when we combined sIL-13Rβ2–Fc administration with CT99021 administration, we again obtained inhibition of IL-17 and IL-23 production, albeit not as profound as that obtained with CT99021 alone. In addition, administration of CT99021 reversed the effect of blockade of IL-13 signaling noted above on CREB and p65 DNA binding, indicating that the effects of GSK-3β inhibition on IL-17 and IL-23 were likely due to underlying effects on transcriptional control of NF-κB (Fig. 6A). Similar data were obtained with the use of another (less-specific) GSK-3β phosphorylation inhibitor, SB216763 (data not shown).

To buttress these findings, we repeated the above studies with a third inhibitor of GSK-3β activation having a different mechanism of action, LiCl (30). Administration of LiCl also was associated with inhibition of effector cytokine secretion and concomitant effects on CREB and NF-κB binding (Fig. 7A, 7B). Finally, in a third variation of this approach, we determined the outcome of preventing the in vivo effects of p–GSK-3β on late-stage inflammation by downregulating GSK-3β protein itself via administration of GSK-3β–specific siRNA using the HVJ-E in vivo–transfection method. Administration of such siRNA during the late stage of chronic TNBS colitis resulted in the downregulation of GSK-3β protein, as well as inhibition of effector cytokine secretion and concomitant effects on CREB and NF-κB binding (Fig. 6C–E).

Thus, the presence of GSK-3β in an inactive (phosphorylated) state is functionally equivalent to the absence of GSK-3β.

These studies clearly showed that agents that maintain GSK-3β phosphorylation or downregulation of GSK-3β itself mimic the effect of IL-13 signaling. Thus, they provide strong evidence that IL-13 is acting through GSK-3β to promote reduced effector cytokine secretion during the late stage of chronic TNBS colitis.

Specific inhibition of IL-10 results in continued inflammatory cytokine expression in late chronic TNBS colitis

Previous studies (11) showed that LPS-induced immune responses occurring under conditions in which GSK-3β is phosphorylated...
lead to increased IL-10 expression. In view of the association of activated GSK-3β with amelioration of chronic TNBS colitis, we next investigated the possibility that IL-10 is involved in such amelioration. In initial studies along these lines, we determined the expression of IL-10 during the course of chronic TNBS colitis. The levels of IL-10 produced by isolated colonic LPMCs, stimulated ex vivo as described previously, remained rather low until day 42, but they increased substantially by day 49 and remained on a high plateau until day 84, after which they declined to some degree (Fig. 8A). Thus, high levels of IL-10 were produced throughout the late phase of chronic TNBS colitis.

We then conducted experiments to elucidate the possible role of IL-10 in the resolution of the inflammatory response in chronic TNBS colitis. We found that inhibition of IL-10 by i.p. administration of anti–IL-10 Ab and inhibition of STAT3 by administration of STAT3 decoy ODNs on days 56 and 63 of colitis resulted in increased expression of IL-23, IL-17, and TNF-α on day 70 of chronic TNBS colitis (Fig. 8B, Supplemental Fig. 4A). However, in the same study, inhibition of IL-10 and STAT3 did not result in reduced expression of p–GSK-3β (Fig. 8C, Supplemental Fig. 4B). In addition, the DNA-binding activity of CREB, a known inducer of IL-10, was not affected by inhibition of either IL-10 or STAT3, whereas such inhibition led to increased DNA-binding activity of NF-κB p65 (Fig. 8D). The effects of inhibition of IL-10 or STAT3 was also reflected in the weight curves of mice with chronic TNBS colitis, because the treated mice started to lose...
weight and showed increased signs of illness from day 56 on (Supplemental Fig. 4C). Finally, to determine the relationship of IL-13 to IL-10 levels, we also measured IL-10 production by colonic LPMCs (stimulated ex vivo by anti-CD3/anti-CD28) on day 70 following inhibition of either IL-13 by sIL-13Rα2–Fc (every other day) starting on day 56. The inhibition of IL-13 led to significantly reduced IL-10 levels (Fig. 8E).

Taken together, these data provide strong evidence that IL-13, acting through GSK-3β, induces IL-10; the latter, in turn, is an important component in the amelioration of inflammation in the late stage of chronic TNBS colitis.

Discussion

The chronic TNBS colitis model, developed initially by Lawrance et al. (9), has proved to be an excellent vehicle for the investigation of sequential changes in the nature of the inflammation occurring during a chronic intestinal inflammation (7, 8). As shown in this study and previously, the process begins as an acute and severe inflammation dominated by the production of IL-12p70 and IFN-γ. It then morphs into a less intense, but on-going, inflammation characterized by IL-23, IL-25, and IL-17 production (8). This set of cytokines supports the development of IL-13 and TGF-β1 responses that play a major role in the development of a fibrotic program that is quite distinct from the underlying inflammatory program. In the current study, in which chronic colitis was observed over a longer period of time than previously, it was found that the IL-23 and IL-17 responses peaked on day 49 and then underwent a precipitous decline, so that at day 91 they had returned to near-background levels. This drop occurred during a resolution phase of colitis during which inflammation subsided and likely was the cause of such a decrease. In contrast, the IL-13 responses were much more persistent and never dropped to baseline levels. This corresponded to the fact that resolution of the inflammation was dependent on the continued secretion of IL-13,
as evidenced by the fact that maneuvers that blocked such secretion led to continued colitis. Therefore, it was evident that IL-13, in the context of chronic TNBS colitis, was driving a program that ameliorated inflammation. Thus, this conclusion is consonant with a previous study in which IL-13 was found to reduce the level of colitis in mice with IL-10 deficiency (31).

A key to understanding how IL-13 signaling could lead to modulation of the disease comes from studies that showed that GSK-3β, a previously well-studied serine/threonine kinase central to glucose/glycogen metabolism and insulin function, also regulates the reciprocal production of proinflammatory cytokines (e.g., IL-12p70) and anti-inflammatory cytokines (e.g., IL-10) in APCs (10–13, 21, 32, 33). In particular, in an inactive (phosphorylated) state, GSK-3β facilitates the formation of p-CREB that translocates to the nucleus and then competes with NF-κB (p65) for binding to CBP; this has the effect of inhibiting the binding of NF-κB (p65) to its target genes and, thus, the transcription of proinflammatory genes, such as IL-12p70. At the same time, GSK-3β enhances the binding of CREB to its target genes and, thus, the transcription of anti-inflammatory IL-10. In contrast, in an active (unphosphorylated) state, GSK-3β has no effect on CREB translocation and, therefore, does not impede NF-κB (p65) activity and proinflammatory cytokine production (11, 12). In this way, phosphorylation of GSK-3β shifts APCs from a proinflammatory mode to an anti-inflammatory mode.

The observation in this study that GSK-3β phosphorylation status reflects the pattern of inflammation in chronic TNBS colitis and that IL-13 signaling via STAT6 could result in GSK-3β phosphorylation suggested that IL-13 was driving the resolution of chronic TNBS colitis via its effect on GSK-3β. Several findings reported in this article strongly support this possibility. First, we showed with in vitro studies that IL-13 had an enhanced capacity to induce GSK-3β phosphorylation in that stimulation of cells with TLR ligands in the presence of IL-13 led to more sustained GSK-3β phosphorylation than that obtained with PGN. Such sustained phosphorylation was, in turn, associated with increased CREB DNA binding and decreased p65 DNA binding, a pattern of DNA binding characterizing transcription factors extracted from cells during the resolution phase of chronic TNBS colitis. These in vitro effects of IL-13 on GSK-3β phosphorylation predicted that the onset of IL-13 secretion during chronic TNBS colitis would be accompanied by the appearance of p-GSK-3β. This was indeed the case, although levels of the latter peaked somewhat later, perhaps reflecting the fact that, in vivo, IL-13 induction of GSK-3β phosphorylation must initially overcome the negative influence of inflammatory cytokines on phosphorylation (see later discussion). Second, we tied IL-13 to resolution of inflammation and GSK-3β phosphorylation by showing that in vivo blockade of IL-13 signaling by sIL-13R2–Fc, as well as by STAT6 decoy ODNs or STAT6 deficiency, not only blocked resolution of the inflammation, it also allowed continued p65 DNA binding and inhibited CREB DNA binding. Concomitantly, such blockade prevented GSK-3β phosphorylation normally seen during the resolution phase of chronic TNBS colitis. Third, and perhaps most importantly, we showed that administration of two separate and specific inhibitors of GSK-3β activation, which maintained GSK-3β in a phosphorylated state and, thus, mimicked the effect of IL-13 signaling, throughout the late phase of TNBS colitis led to resolution of the colitis in the face of a block in IL-13 signaling. Similarly, late stage in vivo downregulation of GSK-3β by administration of a GSK-3β–specific siRNA also had the effect of causing resolution of colitis. These data provide strong multifaceted evidence that the locus of control of late-stage inflammation in chronic TNBS colitis was inherent in the activation state of GSK-3β, and the ability of IL-13 to affect such inflammation was dependent on its capacity to regulate this activation state.

In studies of the proximal mechanisms of inflammation resolution, we focused on the role of the anti-inflammatory cytokine IL-10. As already noted, IL-13–induced GSK-3β phosphorylation and CREB activation led to increased IL-10 production, and high-level IL-10 production continued throughout the resolution phase (11, 12). Such IL-10 could be tied to upstream IL-13 by studies showing that blockade of IL-13 signaling by sIL-13R2–Fc administration inhibited IL-10 production. Further evidence of the involvement of IL-10 in the resolution of inflammation came from studies showing that inhibition of IL-10 with anti–IL-10 Ab administration or blockade of the main signaling molecule induced by IL-10, STAT3, by administration of STAT3 decoy ODNs led to continued high production of proinflammatory IL-23 and IL-17. As expected, such blockade had no effect on GSK-3β phosphorylation or CREB DNA binding because it is downstream of these effects; however, it affected p65 DNA binding, presumably because IL-10 participates in inhibition of the Th1 response (34, 35).

As shown in this study and previously, TLR stimulation leads to activation of PI3K/Akt, a kinase that inactivates GSK-3β via phosphorylation at serine 9 (12). Given that TLR stimulation is likely to be constantly present in the gut milieu, this raises the question of why such stimulation itself cannot cause resolution of colitis via effects on GSK-3β and why IL-13 is necessary for this purpose. The answer to this question is likely related to the fact that, as suggested by our in vitro studies, TLR inactivation of GSK-3β is a relatively transient phenomenon that cannot sustain persistent GSK-3β phosphorylation throughout the course of chronic TNBS colitis. This may be the case, because TLR stimulation also upregulates possible inhibitors of Akt or other TLR-dependent kinases involved in GSK-3β phosphorylation. For instance, TLR stimulation induces activation of CYLD, a deubiquitinating enzyme shown to downregulate TLR activation of TRAF6 or Akt via effects on ubiquitination (36, 37). Also relevant is the recent study by Thornton et al. (26), which showed that GSK-3β can be phosphorylated (and inactivated) via phosphorylation at serine 389 by p38 MAPK. This appears to be the main pathway of IL-13 inactivation of GSK-3β, because we present evidence that IL-13 phosphorylation of GSK-3β does not depend on PI3K/Akt; instead, it depends, at least partially, on p38 MAPK, a kinase known to be induced via STAT6 signaling (38). On this basis, a reasonable interpretation of the data is that, although IL-13–induced phosphorylation and inactivation of GSK-3β via p38 MAPK are persistent, TLR activation of GSK-3β via this kinase is transient; thus, IL-13 inactivation of GSK-3β is not redundant with TLR inactivation of GSK-3β in the context of colitis resolution.

In summary, as diagrammed in Fig. 8F, the data presented in this article suggest that IL-13 induced during the course of chronic TNBS colitis functions to bring about the spontaneous resolution of the inflammation that ultimately occurs during the course of this colitis. The key mechanism of such IL-13 activity is the signaling of cells via Stat6/p38 MAPK to cause inactivation (phosphorylation) of GSK-3β and, thus, downregulation of NF-κB proinflammatory activity coupled with upregulation of CREB activity and the production of IL-10, an anti-inflammatory factor. Although not addressed in this study, IL-13 also induces TGF-β during chronic TNBS colitis, so that TGF-β may also be important in the resolution of inflammation, particularly because this cytokine can induce regulatory T cells (39, 40). Finally, the findings presented in this article may have relevance to CD, which, as mentioned above, exhibits a “burned out” stage characterized by fibrotic tissue relatively devoid of inflammatory cells (41). Thus, it
is possible that this stage arises from the secretion of cytokines that lead to formation of inactivated GSK-3β and the latter’s inhibitory effect on inflammation. Further studies of CD and other chronic inflammatory processes are warranted to address this question.

Disclosures
The authors have no financial conflicts of interest.

References