T Cell–mediated Pathology in Two Models of Experimental Colitis Depends Predominantly on the Interleukin 12/Signal Transducer and Activator of Transcription (Stat)-4 Pathway, but Is Not Conditional on Interferon γ Expression by T Cells

By Stephen J. Simpson,* Samir Shah,* Martina Comiskey,* Ype P. de Jong,* Baoping Wang,* Emiko Mizoguchi,‡ Atul K. Bhan,‡ and Coxt Terhorst*

From the *Division of Immunology, Beth Israel Deaconess Medical Center and the ‡Department of Pathology, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02115

Summary
The requirements for interleukin (IL)-12/signal transducer and activator of transcription (Stat)-4 signaling and induction of T cell–specific interferon (IFN)-γ expression in the development of T helper cell (Th)1-type pathology were examined in two different models of experimental colitis. In each model, abnormal reconstitution of the T cell compartment in immunodeficient mice by adoptive cell transfer leads to a wasting syndrome and inflammation of the colon, induced by IFN-γ and tumor necrosis factor (TNF)-α–producing T cells. We show here that treatment with anti–IL-12 antibodies in one of the models, or reconstitution with T cells from Stat-4–deficient (Stat-4 null) mice in both models resulted in a milder disease in the majority of recipient animals, compared with those that were left untreated or that had been reconstituted with wt cells. Protected mice in each group also harbored lower frequencies of IFN-γ–producing T cells, suggesting that effects on wasting and colitis resulted from the attenuation of IFN-γ expression by T cells. To test whether the development of pathogenic T cells in the two colitis models was directly dependent on T cell–specific IFN-γ expression, IFN-γ null donors were used for T cell reconstitution in each system. Surprisingly, large numbers of IFN-γ null–reconstituted mice developed wasting and colitis, which in many cases was of comparable severity to that seen in animals reconstituted with wt cells. Furthermore, T cells from these animals expressed TNF-α, demonstrating that they had retained the ability to produce another proinflammatory cytokine. Taken together, these results demonstrate that in some forms of chronic experimental colitis the development of pathogenic T cells is influenced predominantly, though not exclusively, by IL-12 via the actions of Stat-4 proteins. Furthermore, our data suggest that in the models of colitis studied here the effects of IL-12/Stat-4 or other Th1 promoting pathways are not limited to the induction of IFN-γ gene expression in T lymphocytes.

Several rodent models of chronic intestinal inflammation share features of immunopathology with human inflammatory bowel disease (IBD), which exists in the two distinct forms of Crohn’s disease and ulcerative colitis (1–6). In particular, the finding that human and many animal forms of IBD show evidence of aberrant Th1 responses has come under close scrutiny (5, 7–12). Most recently, it has been proposed that mucosal inflammation, such as that found in IBD, emerges from an alteration in the normal balance between the effects of proinflammatory cytokines such as IFN-γ and regulatory cytokines such as transforming growth factor β (TGF-β; reference 13). The observation that TGF-β–deficient mice develop inflammation of various tissues, including the intestine, provides strong evidence in support for this (14). IL-12, a cytokine produced by activated macrophages and dendritic cells, plays a central role in the generation of Th1-type responses, characterized predominantly by the induction of IFN-γ production in T cells (15–20). In light of this, elucidation of the mechanisms...
by which IL-12 might promote mucosal inflammation is of
clear importance for understanding the pathogenesis of IBD.

Evidence that IL-12 is important in intestinal inflamma-
tion has recently been provided in an experimental model
of acute granulomatous colitis, induced by administration
of trinitrobenzene sulfonic acid (TNBS), which could be
prevented by treatment with an anti-IL-12 antibody (21).

The absence of IFN-γ expression in protected animals in
these experiments was consistent with other data that has
shown that IL-12 activity correlates strongly with IFN-γ
expression by T cells (19, 22, 23). Recent studies have ex-
tended these observations by demonstrating that induction
of IFN-γ expression in Th1 cells depends largely upon the
activity of intracellular signal transducer and activator of
transcription 4 (Stat-4) proteins, which mediate signals via
the IL-12 receptor (24–28). Although IL-12 activates other
members of the Stat protein family (including Stat-3; refer-
ence 27), the effects of this cytokine on Th1 cell develop-
ment have been shown to depend specifically on expres-
sion of Stat-4. Consistent with this, T cells from Stat-4null
mice are almost completely unable to produce IFN-γ in
response to IL-12 and show reduced Th1 responses equiva-
lent to those in IL-12null mice (28–30).

Although the IL-12/Stat-4 pathway clearly predomi-
nates in the development of Th1 type T cells, Stat-4-defi-
cient T cells are nevertheless capable of limited IFN-γ pro-
duction in response to IL-12-independent stimuli, such as
CD3–IL-2 costimulation (28, 29). Additionally, IFN-γ has
been shown to efficiently augment IL-12–mediated differ-
entiation of Th1 cells (31). These data leave open the pos-
sibility that pathways other than that provided by IL-12
and Stat-4 contribute to the expression of IFN-γ and develop-
ment of Th1-mediated inflammation. Furthermore, such
experiments beg the question as to whether induction of
IFN-γ gene expression in T cells does in fact represent the
critical step in the development and action of pathogenic
Th1 type cells. For example, it is possible that T cells could
be induced into a Th1-like state in the absence of autocrine
IFN-γ expression, and induce pathology through expres-
sion of other proinflammatory mediators. In this study we
sought to examine these questions by testing the extent to
which Stat-4 signaling and the resulting IFN-γ production
by T cells contribute to pathology in two distinct Th1
models of colitis.

In each colitis model, wasting and intestinal pathol-
ogy has been shown to be generated by the abnormal recon-
stitution of the T cell compartment in nonallogeneic,
immunodeficient mice. In the first system, we have previ-
ously shown that disease develops after bone marrow cell
(BM) reconstitution of T cell and NK cell–deficient Tg26
(C57BL/6 × CBA/J)F1 mice, using (C57BL/6 × CBA/J)F1
donor animals (BM→Tg26) (32–34). In the second sys-
tem, IBD was originally shown to be induced upon recon-
stitution of C57Sd mice with CD45R Bnull CD4+ T cells
from wt Balb/c or C57.17 animals (35–39). In both models,
strong evidence exists to suggest that pathology develops
through a lack of normal T cell regulation. In the case of
the CD45R Bnull transfer model, this occurs due to the ab-
essence of CD45R Bnull CD4+ T cells, which exert their regu-
laratory effects through the expression of IL-10 and TGF-β (38).

Similarly, we have provided evidence in the BM→Tg26
model that abnormal T cell regulation, resulting from aber-
rent development of thymus-derived T cells, is causal in the
development of pathology in these mice (34). Central to
our hypothesis is the observation that the thymi of Tg26
mice lack a normal stromal architecture and thus are unable
to support normal ontogeny of wt donor–derived thymocytes
supplied by bone marrow inoculation. Consequently, we
have suggested that this defect in thymocyte development
prevents the establishment of a regulated T cell repertoire
in BM→Tg26 mice.

In both the BM→Tg26 and CD45R Bnull transfer mod-
els, the notion that T cell dysregulation leads to aberrant
Th1 responses is underscored by the presence of very large
numbers of activated IFN-γ+ and TNF-α–secreting T cells
in the peripheral lymphoid tissue and the colon (38, 40).

However, despite the similarity of the T cell phenotype in
each model, the resulting pathology in each case is clearly
distinct. Thus, in the BM→Tg26 mice, inflammation is
limited to the mucosa of the colon and shares some features
of UC, including crypt abscess formation, crypt cell prolif-
eration, and extensive mononuclear cell infiltration. Al-
though these are also characteristic of the CD45R Bnull
transfer model, colitis in these mice displays additional features
resembling some of those observed in Crohn’s disease, in-
cluding transmural inflammation, formation of granulomas,
and occasional involvement of the distal small intestine.

These distinctions provide a useful means by which to
compare mechanisms important in alternative forms of
chronic intestinal inflammation. Furthermore, since each
system uses adoptive transfer of cells into T cell deficient
host animals, both are amenable to a novel experimental
design whereby alternative chimeras can be generated using
donors genetically deficient in specific proteins of interest.

Using this as one of our approaches, we investigated the re-
quirements for IL-12/Stat-4 signaling and induction of T
null gene expression in the development of
pathology in each form of colitis.

Materials and Methods

Mice. Donor (C57BL/6 × CBA/J)F1, C57BL/6, C57BL/6/
Sd, and C57BL/6/Ifn-γnull mice were purchased from The
Jackson Laboratory (Bar Harbor, ME). The Stat-4null mice
were a gift from Dr. J.N. Ihle (St. Jude's Children's Hospital, Memphis,
T. N.). 129/SvEv RAG-2null and wt mice were purchased from
Taconic Farms (Germantown, N. Y.). The Tg26 recipient mice
were generated as previously described by transgenic overexpres-
sion of a full-length human CD3ε gene (32) and bred on the
original C57BL/6 × CBA/J background in the Beth Israel Dea-
coness Medical Center animal facility. All mice were kept under
standard conditions in microisolator cages with autoclaved food,
water, and bedding. In RAGnull and Sd null reconstitution exper-
iments, recipients were between 4 and 6 wk of age. Tg26 recipi-
ent mice were between 5 and 10 wk of age.
CD4+ T cells obtained from the spleens of donor animals and were enriched by magnetic sorting. For cell purification, the following biotinylated anti–mouse antibodies were used to label non-CD4+ T cells isolated from the spleens of donor mice: B220 (RA3-6B2), M ac-1 (M 1/70), Gr-1 (RB6-8C5), and CD8a (53-6.7). Magnetically labeled streptavidin beads (Miltenyi Biotec Inc., Sunnyvale, CA) were used to bind the biotinylated antibodies. All antibodies were obtained from PharMingen (San Diego, CA). Negative selection was accomplished using a MACS magnetic cell sorter (Miltenyi Biotec Inc.).

The enriched CD4+ cells were then labeled for cell sorting with FITC-conjugated CD45RB (16A) and PE-conjugated CD4 (Becton Dickinson, Mountain View, CA). Subsequently, cells were sorted under sterile conditions by flow cytometry for CD4+ CD45RBhi cells, using criteria similar to that described in previous studies (37). This resulted in a >98% pure population of T cells. Harvested cells were resuspended at 10/9400 ml PBS (no FC5) and injected into the tail veins of recipient mice.

For the BM→Tg26 model, bone marrow was prepared as previously described (34). In brief, BMCs were depleted of T cells using two rounds of treatment with anti-Thy1.2 antibody (30-H12) followed by rabbit complement lysis (Cedarlane Labs, Westbury, NY). This procedure resulted in <0.1% CD4+ and CD8+ cells within the inoculum, as determined by flow cytometry. Bone marrow recipients were total body irradiated with a sublethal dose (400 rads) 6-8 h before transplant or were treated with 5-Fluorouracil (75 mg/kg) 48 h before transplantation. 5 × 107 cells were resuspended in 400 ml PBS (no FC5) and transferred by tail vein injection.

To reduce the possibility of graft versus host disease (GVHD) in the CD45RBhi model, cell transfers were made, where possible, between mice of the same backgrounds. Stat-4null mice were of the mixed background to the Stat-4null donor animals (see Materials and Methods). RAGnull mice were thus reconstituted with 129/SvEv RAGnull. Control RAG null mice in these experiments were transplanted with wt 129/SvEv RAGnull mice, rather than CB.17/SCID mice, as recipients, allowing us to maintain an equivalent genetic background.

Results

The IL-12/Stat-4 Pathway Predominates in Development of Wasting and Intestinal Pathology in Two Models of Colitis. To examine the role of IL-12 in the BM→Tg26 colitis model, Tg26 mice were transplanted with BMCs from C57BL/6 × CBA.F1 donors as previously described (34) and given a weekly dose of anti–IL-12 antibody, or vehicle control (PBS). The requirement for Stat-4 expression in T cells was tested by comparing disease in Tg26 mice transplanted with BMCs from Stat-4null mice or wt control animals of the same genetic background (129/SvEv). Using a similar approach in the CD45RBhi model, we used 129/SvEv RAGnull mice, rather than C.B.17/SCID mice, as recipients, allowing us to maintain an equivalent genetic background to the Stat-4null donor animals (see Materials and Methods). RAGnull mice were thus reconstituted with FACS-sorted splenic CD4+ CD45RBhi T cells from Stat-4null mice (Stat-4null→RAGnull). Control RAGnull mice in these experiments were transplanted with wt 129/SvEv CD4+ CD45RBhi T cells (129/wt→RAGnull).

Treatment of BM→Tg26 mice with anti–IL-12 antibody resulted in a significant reduction in disease compared with control (untreated) mice. A gross clinical disease activ-
milder disease compared with control animals that had received wt BMC (Fig. 1). 129/wt→RAGnull mice developed wasting and colitis 7–10 wk after cell transfer. In contrast, Statnull→RAGnull retained a healthy appearance over the same period of time and showed mild colitis at necropsy, similar to that seen in RAGnull mice that had received sorted CD4+ CD45R Bhi T cells (36, 37).

The reduced severity of disease found in each experimental group of mice was reflected both in the extent of loss of body mass (Fig. 2 A) and for the most part in the level of mucosal inflammation in the large bowel, determined by examination of histological tissue sections (Fig. 2 B). Thus, the extent of mucosal inflammation in all anti–IL-12–treated F1→Tg e26 mice, and the majority of Stat-4null reconstituted mice, was lower than in control animals. Nevertheless, significant histological colitis could be detected in a proportion of Stat-4null recipient animals, even in cases where severe gross colitis (extensive colon thickening and diarrhea) was not apparent. In Stat-4null→RAGnull experiments, a large majority of animals showed considerably lower grades of colitis than did control mice. Representative histological sections from colons of CD45R Bhi→RAGnull and BM→Tg e26 mice from different groups are shown in Fig. 3, illustrating the principal differences between mild and severe forms of colitis in each model. The limited extent of mucosal inflammation in most of the Stat-4null recipients and in those subjected to anti–IL-12 antibody treatment was evident from the nominal level of cellular infiltration, crypt cell hyperplasia, and the paucity of crypt abscesses formation. However, it was noted, in the colons of some Stat-4null→RAGnull mice, that even in the absence of continuous mucosal inflammation there was a moderate frequency of focal inflammatory involvement. In some cases this was accompanied by the appearance of granulomas, a characteristic feature of the CD45R Bhi transfer model (Fig. 3 D). Collectively, the use of anti–IL-12 antibodies and Stat-4null mutant mice as donors revealed a
predominant role for IL-12 and Stat-4 proteins in the development of both the wasting syndrome and colon inflammation in two distinct models of IBD.

IL-12 and Stat-4 Cooperate in the Expression of IFN-γ by Th1 Cells. To examine whether disease correlated directly with the level of Th1 involvement in the two colitis models, we examined the development of Th1-type T cells in each system. Since previous studies have shown a direct effect of IL-12 and Stat-4 on the production of IFN-γ by T cells, we used cytoplasmic staining of IFN-γ and FACS analysis to determine the frequencies of T cells capable of IFN-γ expression. As shown in Fig. 4, the frequency of IFN-γ-producing CD4+ T cells in the mesenteric lymph nodes (MLN) in anti–IL-12–treated F1→Tg e26 mice and in Stat-4null→Tg e26 and Stat-4null→RAGnull mice was 60–80% lower than in control animals. Additionally, numbers of colon lamina propria CD4+ T cells capable of IFN-γ production were also markedly reduced in protected animals (data not shown). Previous reports have suggested that in the absence of Stat-4 signaling T cells develop a Th2-like phenotype (29). Examination of IL-4 and IL-10 expression by MLN T cells in Stat-4null recipients revealed no detectable expression of either cytokine (data not shown).

IFN-γ-deficient T cells mediate disease and produce TNF-α. Together, the above data implicate a causal relationship between the numbers of T cells capable of IFN-γ production and the extent of wasting and colitis. We reasoned that if induction of IFN-γ gene expression did in fact represent a critical step in the development of pathogenic Th1 cells, then the ablation of this specifically in T cells should be sufficient to prevent disease. To test this, we reconstituted the T cell compartment of Tg e26 and Scid or RAGnull mice using IFN-γnull mice (reference 41).

In the BM→Tg e26 model, animals injected with BM Cs from IFN-γnull donors backcrossed to the C57BL/6 background were compared with controls injected with C57BL/6/wt BM Cs. In the CD45R Bhi→transfer model, CD45R Bhi CD4+ T cells from IFN-γnull mice were transferred into C57BL/6/Sd or RAGnull mice. Sd and RAGnull mice transplanted with C57BL/6/wt CD4+ CD45R Bhi T cells served as controls in these experiments. Fig. 5 shows the cumulative results from these experiments, which compare the mean disease activity scores from each group. In both BM→Tg e26 and CD45R Bhi→Sd RAGnull animals little difference was apparent between the overall gross disease in each group of mice (Fig. 5). However, in some cases, weight loss and/or histological colitis were milder in IFN-γnull→recipient mice (Fig. 6, A and B). A large number of IFN-γnull→Tg e26 mice showed a range of histological scores and in some cases, despite showing clinical evidence of significant colitis,
revealed comparatively mild inflammation of the mucosa by histological examination (Fig. 6B). In CD45R B\(^\text{hi}\)→S\(\text{d}d/\text{RAG}\text{null}\) experiments, most animals showed visible signs of wasting. However, due to their young age at the time of cell transfer, many of these mice increased in body mass due to compensatory growth. The extent of colon involvement observed in the large majority of CD45R B\(^\text{hi}\)→S\(\text{d}d/\text{RAG}\text{null}\) mice was equivalent whether or not animals had received IFN-\(\gamma\)null or C57BL/6\(\text{wt}\) T cells. Representative histological sections from mice that developed colitis in the presence of IFN-\(\gamma\)null T cells are shown in Fig. 7. In each case the salient features of pathology were similar to those in animals reconstituted with wt T cells.

**Discussion**

In our study we used a novel experimental approach to examine the mechanisms leading to development and action of pathogenic Th1-type T cells in colitis. The significant effect of Stat-4 deficiency in limiting progression of disease in both the BM→Tg26 and the CD45R B\(^\text{hi}\) transfer systems establishes a principal role for this pathway in the development of pathogenic Th1-type T cells in colitis. Furthermore, the similar attenuation of both disease and T cell-specific IFN-\(\gamma\) expression seen in anti-IL-12→treated mice...
BM \rightarrow Tg e26 mice and Stat-4null \rightarrow Tg e26 mice underscores the link between the actions of IL-12 and Stat-4. In the two colitis models studied here, inhibition of IL-12 and/or Stat-4 signaling affected two divisible aspects of disease, colitis and wasting, implicating involvement of Th1 cells in both components of pathology.

To an extent, our experimental results appear consistent with those obtained in the more acute TNBS-induced model of colitis (21). However, we observed that although disease was for the most part attenuated in the absence of IL-12/Stat-4 signaling, many animals nevertheless revealed evidence of pathology. Furthermore, this correlated with the continued (although strongly reduced) presence of T cells capable of IFN-γ expression. These data differ from those in the TNBS colitis model, where an almost com-

**Figure 6.** Wasting and colitis in the absence of IFN-γ expression in T cells. (A) Change in body weights and (B) histological colitis are represented as described in Fig. 2 in animals reconstituted with IFN-γnull T cells. In the CD45R Bhi transfer model, closed symbols represent RAGnull, rather than Scid recipients mice.

**Figure 7.** Histological colitis in IFN-γnull T cell–reconstituted mice. Sections taken from distal colon are shown from (A) an IFN-γnull \rightarrow Tg e26 mouse with mild colitis (2+ histological score), (B) IFN-γnull \rightarrow Tg e26 mouse severe colitis (4+ histological score), and (C) IFN-γnull \rightarrow Sod mouse with severe colitis (5+ histological score).

**Figure 8.** T cells from IFN-γnull–recipient mice produce TNF-α. MLN T cells were examined for expression of IFN-γ (hatched bar) and TNF-α (solid bar) using flow cytometry after overnight in vitro stimulation with an anti-CD3 antibody. The mean percentages of cytokine-positive cells in each group are shown \( \pm SD (n = 5–10/\text{group}) \).
plete inhibition of both disease and IFN-γ production were observed after anti-IL-12 treatment. These differences make apparent that the mechanisms of pathology in colitis models studied here and that induced by TNBS are not equivalent, and they suggest that factors other than IL-12 contribute to the development of more chronic forms of intestinal inflammation. In this capacity, the recently described IFN-γ-inducing factor (IL-18) is a potential candidate, since this cytokine has been shown to have a significant effect on the long-term ability of T cells to produce IFN-γ (42–44). By inference it is likely that this could significantly affect the development of pathogenic Th1-like T cells.

Notwithstanding the above arguments, the results from our study suggest a degree of independence between the actions of IL-12 and/or other Th1-promoting cytokines and the specific expression of IFN-γ by T cells. Thus, although the absence of IFN-γ expression by T cells altered the severity of colitis in many cases, the overall effects on disease were less pronounced than might have been anticipated. On first assessment this is surprising, principally for three reasons. First, the dependence of IFN-γ expression on IL-12 signaling suggests that the effects of IL-12 (via Stat-4) result from its ability to induce IFN-γ transcription in T cells (22). Second, IFN-γ has been shown to be required for efficient IL-12 priming of Th1 cells, presumably through autocrine expression (45–47). Principally, this appears to be achieved through the upregulation of the IL-12 receptor (48–49). Third, a general consensus exists that the well-documented proinflammatory effects of IFN-γ are central to pathology in IBD (4, 7, 13). The validity of this assumption in experimental colitis has been strengthened by a previous study by Powrie et al., who demonstrated that anti–IFN-γ treatment was effective in reducing disease in the CD4+ CD45R Bhi transfer model (38). It is possible that in this latter case anti–IFN-γ antibody treatment might block all available IFN-γ. By contrast, in our experiments non-T cells, resident in the host animals, might remain a sufficient source of IFN-γ to promote effects on both T cells and macrophages. However, the development of colitis in the absence of IFN-γ expression is not unprecedented, since IL-2null mice, which succumb to a Th1-type colitis similar to that seen in BM → Tge26 mice, also develop disease after import of the IFN-γnull mutation (Zand, M., C. Stevens, and T. Strom, personal communication). In addition, a recent study by Berg et al. revealed that antibody neutralization of IFN-γ in IL-10null mice was sufficient to prevent disease only when administered to animals at 3 wk of age and not in animals aged 3 mo or older (50). Thus, although IFN-γ was required to establish colitis in IL-10null mice, other inflammatory mediators were clearly sufficient to mediate disease once a pathogenic T cell phenotype had been established.

In two autoimmune models classically associated with Th1-type T cell responses, it has been observed that Th2 cells are also capable of mediating disease (51, 52). These studies support the contention that, in some cases, immunopathology normally associated with Th1-type responses can also be attributable to cells that have undergone immune deviation, leading to the expression of Th2-associated cytokines. However, in our studies we were unable to detect any IL-4 or IL-10 expression in the absence of either Stat-4null or IFN-γ expression by T cells. Although these findings do not unequivocally demonstrate lack of immune deviation, they do argue that this is not predominant in the colitis models studied here.

Collectively, the results offered here lend credence to the argument that Th1-type cells develop pathogenicity via a complex range of mechanisms, not all of which fall under the influence of IL-12 or their ability to produce IFN-γ. Ultimately, this is most likely achieved by the concerted expression of a range of cytokines and cytotoxic molecules which could act directly or indirectly to promote inflammation. TNF-α, which is produced by T cells as well as macrophages, is a cytokine that encompasses these characteristics and is known to induce potent inflammatory effects. Consistent with this, we have shown that in colitic mice T cells expressed TNF-α at a similar frequency whether IFN-γ was expressed by the same cells or not. Furthermore, we have found that anti–TNF-α antibody treatment is highly effective in reducing disease in the BM → Tge26 model.2 Similarly, Powrie et al. demonstrated an effect of anti–TNF-α treatment in the CD45RBhi → Scid model (38). These data are consolidated by the finding that anti–TNF-α antibody therapy has been shown to be efficacious in treating Crohn’s disease (53).

In summary, our study offers insights into the requirements for both the development and pathogenic activity of Th1-type cells in two distinct models of inflammatory colitis. The observations that IL-12 is upregulated in human IBD (7) and that interruption of the TNF-α pathway inhibits Crohn’s disease correlate well with our observations in the two models studied here (38).2 Further dissection of the pathways which lead to aberrant Th1 pathology in animal models of IBD will undoubtedly provide a useful source of information for future therapy in IBD.

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