STAT4 Isoforms Differentially Regulate Th1 Cytokine Production and the Severity of Inflammatory Bowel Disease

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STAT4 Isoforms Differentially Regulate Th1 Cytokine Production and the Severity of Inflammatory Bowel Disease

John T. O’Malley,* Rajaraman D. Eri,† Greta L. Stritesky,* Anubhav N. Mathur,* Hua-Chen Chang,* Harm HogenEsch,‡ Mythily Srinivasan,† and Mark H. Kaplan2* STAT4, a critical regulator of inflammation in vivo, can be expressed as two alternative splice forms, a full-length STAT4α, and a STAT4β isoform lacking a C-terminal transactivation domain. Each isoform is sufficient to program Th1 development through both common and distinct subsets of target genes. However, the ability of these isoforms to mediate inflammation in vivo has not been examined. Using a model of colitis that develops following transfer of CD4+ CD45RBhigh T cells expressing either the STAT4α or STAT4β isoform into SCID mice, we determined that although both isoforms mediate inflammation and weight loss, STAT4β promotes greater colonic inflammation and tissue destruction. This correlates with STAT4 isoform-dependent expression of TNF-α and GM-CSF in vitro and in vivo, but not Th1 expression of IFN-γ or Th17 expression of IL-17, which were similar in STAT4α- and STAT4β-expressing T cells. Thus, higher expression of a subset of inflammatory cytokines from STAT4β-expressing T cells correlates with the ability of STAT4β-expressing T cells to mediate more severe inflammatory disease. The Journal of Immunology, 2008, 181: S062–S070.

STAT4 is an important determinant of effector T cell responses. The activation of STAT4 by IL-12 in naive CD4+ T cells is essential for their ability to develop into Th1 cells, characterized by their secretion of IFN-γ but not IL-4 or IL-17 upon TCR stimulation (1, 2). Acute activation of STAT4 by IL-12 and IL-23 leads to the production of IFN-γ and IL-17, respectively (3–5). In addition to IFN-γ, Th1 cells also preferentially secrete other proinflammatory cytokines including TNF-α, GM-CSF, and IL-2 (2). Although the STAT4-dependence of IFN-γ gene expression has been well characterized (6–9), STAT4-dependent regulation of other Th1 and Th17 proinflammatory cytokines, such as TNF-α and GM-CSF is less well defined (10–13). The requirement for STAT4-dependent cytokine regulation in the development of inflammatory immune responses including experimental autoimmune encephalomyelitis, arthritis, and inflammatory bowel disease (IBD)3 highlights the critical role STAT4 in autoimmune diseases (14). For example, STAT4-deficient mice developed significantly milder inflammation of the colon compared with wild-type mice (13). Furthermore, mice that constitutively express STAT4 developed chronic transmural colitis characterized by massive influxes of CD4+ T cells of the Th1 phenotype (15). In humans, there is evidence that STAT4 is also a pathogenic factor in IBD, because STAT4 is constitutively activated in patients with ulcerative colitis and IL-12Rβ2 is markedly up-regulated with increased STAT4 activation in patients with Crohn’s Disease (16, 17). Higher levels of the instructive cytokines IL-12 and IL-23 and the Th1 and Th17 produced cytokines IFN-γ, IL-17, IL-21, IL-6, and GM-CSF correlate with more severe pathologies in these diseases both in humans and in mice (16, 18–23). In addition, TNF-α is a pathogenic factor in Crohn’s Disease and anti-TNF-α therapies have shown impressive clinical efficacy in these patients (24, 25).

We recently described alternatively spliced STAT4 transcripts, a full-length STAT4α and a STAT4β that lacks a C-terminal transactivation domain (26). Primary T cells expressing either STAT4α or STAT4β were able to promote Th1 development in vitro. However, there were some differences in isoform function. IL-12 stimulation of STAT4α-expressing Th1 cells induced more IFN-γ production than T cells expressing STAT4β, while STAT4β-expressing T cells proliferated more vigorously in response to IL-12 stimulation (26). Microarray analysis further demonstrated that the STAT4 isoforms regulated many similar genes but each isoform targeted a unique set of genes. The ability of these isoforms to mediate inflammatory disease in vivo has not been examined.

To test the ability of STAT4 isoforms to mediate inflammatory disease, we used a model wherein CD4+ CD45RBhigh T cells expressing either STAT4α or STAT4β were transferred into SCID recipients to induce colitis. We observed that STAT4β mediated more severe inflammation, and this correlated with the ability of STAT4β-expressing T cells to secrete higher levels of a subset of Th1 inflammatory cytokines in vitro and in vivo. Thus, STAT4β, an isoform that lacks the C-terminal transactivation domain, is more efficient than STAT4α in promoting inflammation in vivo.

Materials and Methods
Mice

The generation of B6.129S2-Stat4tm1Gr (Stat4−/−) mice was previously described (27). Stat4−/− mice were maintained on a C57BL/6 background and strain matched C57BL/6 wild type (WT) control mice were purchased from Harlan (Indianapolis, IN). B6.CB17-Prkdcs1Shi/SzJ (B6 SCID) mice

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3 Abbreviations used in this paper: IBD, inflammatory bowel disease; WT, wild type; MLN, mesenteric lymph node; pSTAT4, phospho-STAT4.

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were purchased from The Jackson Laboratory (Bar Harbor, ME). The STAT4α and STAT4β transgenic mice were maintained on a Stat4<sup>−/−</sup>-C57BL/6 background. All experiments were approved by the IACUC. The mice were maintained in an SPF barrier facility. Eight- to 14-wk-old female mice were used in the experiments.

**Isolation of CD45RB<sup>high</sup> and CD45RB<sup>low</sup>-CD4<sup>+</sup> T cells and induction of colitis by cell transfer**

Spleen and lymph node cells were used as a source of CD4<sup>+</sup> cells for reconstitution of B6 SCID recipient mice. CD4<sup>+</sup> T cells were isolated as previously described (28). The enriched CD4<sup>+</sup> T cells were then labeled for cell sorting with FITC-conjugated CD4 and PE-conjugated CD45RB (BD Pharmingen). Subsequently, cells were sorted under sterile conditions by flow cytometry for CD4<sup>+</sup>CD45RB<sup>high</sup> on a FACSVantage machine (BD Biosciences). The CD45RB<sup>high</sup> and CD45RB<sup>low</sup> populations were defined as the brightest staining 10–15% and the dullest staining 15–20% CD4<sup>+</sup> T cells, respectively. Intermediate staining populations were discarded. All populations were >99% pure on re-analysis. The purified CD45RB<sup>high</sup> and CD45RB<sup>low</sup> (4 × 10<sup>5</sup>) cells diluted in 200 μl of PBS were injected i.p. into B6 SCID recipient mice. A separate group of B6SCID mice received CD45RB<sup>low</sup>-CD4<sup>+</sup> (4 × 10<sup>5</sup>) cells as a negative control. The recipient mice were weighed initially, then weekly thereafter. The animals were sacrificed 14 wk after transfer.

**Macroscopic and microscopic assessment of colon appearance**

Once the animals were sacrificed, tissue samples were taken from each segment of the colon (cecum, ascending, transverse, and descending colon and rectum) and fixed in 10% neutral buffered formalin. The samples were routinely processed, sectioned at 5-μm thickness, and stained with H&E for light microscopic examination. The slides were evaluated by light microscopy in a blind fashion using a semi-quantitative scoring system (29). In brief, four general criteria were evaluated in all sections: 1) severity, 2) degree of mucosal hyperplasia, 3) degree of ulceration, if present, and 4) percentage of area involved. The score was then determined from each slide by the following mathematical formula: (inflammation score + ulceration score + hyperplasia score) × (Area involved score) for a score range of 0–27. Scores from each section of the colon were averaged to determine the overall histological score per experimental group. Histological grades were assigned in a blinded fashion. For scoring the lamina propria neutrophils, the following scoring system was used and scores were averaged from 5 to 10 high-powered fields: 0: 0–5 PMNs, 1: 6–10 PMNs, 2: 11–20 PMNs, 3: 21–40 PMNs, and above.

**Cell preparations and cytokine analysis**

Splenocytes and mesenteric lymph node (MLN) cells were harvested and single cell suspensions were obtained as described previously (30). Viable cells were counted and determined by trypan blue exclusion. Surface and cytoplasmic staining and FACS analysis were performed as previously described (31). One × 10<sup>6</sup> cells/ml were plated on anti-CD3-coated plates (2 μg/ml) or stimulated with IL-12 plus IL-18 (1 ng/ml and 25 ng/ml, respectively), or IL-23 plus IL-18 (4 ng/ml and 25 ng/ml, respectively) for 72 h. Supernatants were collected and assessed for cytokine production using ELISA as previously described (31). For intracellular cytokine staining, cell-free supernatants were collected 24 h after anti-CD3 or cytokine stimulation and assessed for cytokine production using ELISA as previously described (31). For intracellular cytokine staining, cells were stimulated with anti-CD3 or 50 ng/ml PMA and 500 ng/ml ionomycin for 4 h in the presence of GolgiPlug. For mRNA analysis, total RNA isolated from cells activated with anti-CD3 or cytokine stimulation and assessed for cytokine production using ELISA as previously described (31).

**In vitro T cell culture**

For anti-CD3 differentiation with irradiated APCs, naive CD4<sup>+</sup> T cells were isolated by negative selection according to the manufacturer’s

T<sub>GF</sub>6 mRNA and normalized for β<sub>2</sub>m expression. Results are relative to WT cells. E. Cells cultured under Th1 priming conditions for 5 days were stimulated in the indicated condition for 24 h before cell-free supernatants were collected for analysis of TNF-α. F. Cells cultured as in A for 5 days were stimulated for 24 h and cell-free supernatants were analyzed by ELISA for TNF-α and IL-2. Results are represented as mean ± SD and are representative of two to four independent experiments. *p < 0.05 from WT, Stat4α, and Stat4<sup>−/−</sup> Th1 cultured cells using unpaired Student’s t test.

**FIGURE 1.** T cells expressing STAT4 isoforms have differential TNF-α production. A. CD4<sup>+</sup>CD62L<sup>+</sup> T cells from mice of the indicated genotypes were cultured under Th1 priming conditions (IL-12, anti-IL-4, α-CD3, α-CD28) with irradiated APCs (30 Gy) for 5 days. Every 24 h, supernatants of the developing Th1 cells were collected from each genotype. Cell-free supernatants were analyzed for TNF-α using ELISA. Results are represented as mean fluorescence intensity of the x-axis. Results are representative of three independent experiments. D. RNA was isolated from Th1 cells cultured as in A following 4 h of treatment with anti-CD3. Quantitative PCR was performed for TNF-α mRNA and normalized for β<sub>2</sub>m expression. Results are relative to WT cells. C. CD4<sup>+</sup>CD62L<sup>+</sup> T cells were activated with anti-CD3 for 4 h was reverse transcribed and used for quantitative PCR analysis. Raw levels were normalized to β<sub>2</sub>m as an endogenous control.
To obtain APCs, splenocytes were depleted of CD4^-/H11001^ and CD8^-/H11001^ cells by incubating with CD4 and CD8 microbeads (Miltenyi Biotec) and the flow through of cells from LS columns was collected. Cells were irradiated (30 Gy) and used as APCs in T cell cultures. Th1 cells were differentiated with 5 ng/ml IL-12 and anti-IL-4 (11B11 10^-9262^g/ml) and Th17 cells were differentiated with 2 ng/ml TGF-β, 100 ng/ml IL-6, 10 ng/ml IL-23, anti-IFN-γ (XMG 10^-9262^g/ml), and anti-IL-4 (11B11 10^-9262^g/ml) for 5 days. Cells were expanded on day 3 with 20 U/ml IL-2 and half the concentration of cytokines and neutralizing Abs for the final 2 days of culture. For differentiation of cells with IL-23 only, cells were cultured as described (31).

**Western blot analysis and phospho-Stat analysis**

For immunoblot, whole-cell protein lysates (100 μg) from 5-day differentiated Th1 cells were immunoblotted with STAT4-H119 Ab (Santa Cruz Biotechnology) and counterblotted for actin (Calbiochem). Densitometry is presented as arbitrary units normalized to expression of actin. Phospho-STAT4 intracellular staining was performed using 1.5% paraformaldehyde fixing of cells before methanol permeabilization for 10 min at 4°C. Cells were stained using pSTAT4 Ab (BD Pharmingen) for 30 min at room temperature and analyzed by flow cytometry.

**Results**

*Th1 cells expressing STAT4β secrete significantly more TNF-α upon TCR stimulation than STAT4α expressing Th1 cells*

Although previous studies demonstrated that T cells expressing either STAT4α or STAT4β could differentiate into Th1 cells, STAT4α was more efficient than STAT4β in the induction of IFN-γ following IL-12 stimulation. To extend these findings, we examined supernatants from naive CD4^+^ T cells undergoing Th1 differentiation in the presence of IL-12 for IFN-γ production (Fig. 1A). Consistent with previous literature, there was significantly less IFN-γ present in the supernatant throughout the differentiation period in STAT4β-expressing and STAT4-deficient cultures. Despite these differences, upon anti-CD3 stimulation of differentiated Th1 cells, there were no significant differences in IFN-γ production between the isoforms (Fig. 1B). These results suggest that the differences in endogenous IFN-γ production stimulated by the STAT4 isoforms during the differentiation period did not affect the process of differentiation.
Although IFN-γ levels were not different between STAT4α- and STAT4β-expressing Th1 cells, we wanted to examine the levels of other cytokines. The dependence of TNF-α production on STAT4 either in vitro or in vivo during the development of disease is not clear (10, 13). To examine STAT4-dependent TNF-α production, wild-type and Stat4−/− naive CD4+ T cells were cultured in Th1 priming conditions for 5 days. At the end of the 5-day culture, the cells were stimulated with IL-12, IL-12 plus IL-18, anti-CD3, or PMA plus ionomycin and analyzed for TNF-α and IFN-γ production. Maximal TNF-α production, as assessed by intracellular cytokine staining and mRNA levels, was dependent upon STAT4 (Fig. 1, C and D). Although the percentage of TNF-α positive CD4+ T cells did not differ drastically between wild-type and Stat4−/− cells, the mean fluorescence intensity at 4 h and the secretion of TNF-α over a 24-h time period showed TNF-α production significantly reduced in the absence of Stat4 (Fig. 1, C and E). In contrast, TNF-α production was not detected following stimulation with IL-12, in the presence or absence of IL-18 (data not shown).

Having demonstrated the STAT4-dependence in TNF-α production, we wanted to examine the ability of the STAT4 isoforms to prime Th1 cells to secrete TNF-α. Naive CD4+ T cells expressing either STAT4α or STAT4β were cultured under Th1 culture conditions for 5 days and stimulated with anti-CD3 before examining the levels of TNF-α and IL-12 using ELISA. The Th1 cells expressing STAT4β consistently secreted significantly more TNF-α compared with the CD4+ T cells expressing STAT4α while IL-2 levels between cells expressing the STAT4 isoforms were similar (Fig. 1E). Similar to the data for Stat4−/− cells, decreased TNF-α production from STAT4α-expressing Th1 cells was due to decreased TNF-α per cell compared with STAT4β cultures, with only minor differences in the percentage of TNF-α− cells, as assessed by intracellular cytokine staining (data not shown). These results suggest that IL-12 stimulation of STAT4β differentially programs the developing Th1 cells to secrete more TNF-α and that this programming is specific and independent of the concentration of IFN-γ throughout the culture period. Thus, these data suggest that STAT4 isoforms can dictate differential cytokine expression in Th1 cells.

To determine whether differential activation of STAT4 contributed to the production of distinct Th cytokines, we stained developing Th1 cultures for phospho-STAT4 (pSTAT4) levels over the first three days of culture. Wild-type and STAT4β-expressing cells showed similar percentages of pSTAT4+ cells at all of the time points examined (Fig. 2A). In contrast, there was less pSTAT4 in STAT4α transgenic cells than in wild-type cells or STAT4β transgenic cells at all of the time points (Fig. 2A). During this time period, there were modest changes in the expression of total STAT4 in each of the cell types (Fig. 2B). After 5 days of differentiation, IL-12 stimulation resulted in greater induction of pSTAT4 in wild-type and STAT4β-expressing cells than in STAT4α-expressing cells, despite similar levels of total STAT4 expression (Fig. 2, B and C). Moreover, STAT4 expression did not change over the course of the stimulation (data not shown). Consistent with our previous report (26), STAT4α phosphorylation decreased over time while STAT4β phosphorylation stayed constant over the 48-h assay period (Fig. 2C). Despite lower levels of pSTAT4α during Th1 differentiation and following IL-12 restimulation, STAT4α was still more potent than STAT4β in the acute production of IFN-γ (Fig. 2D). These data suggest that the differential activation of the isoforms in response to IL-12 can contribute to differential gene expression but that the amount of activated STAT4 does not directly correlate with IFN-γ gene transcription.

**STAT4 isoforms are equally efficient in promoting Th17 differentiation**

IL-23 also activates STAT4 and induces Th17 cells to secrete IL-17 (4). Because we observed a differential induction of Th1 cells to secrete TNF-α by the STAT4 isoforms, we examined the ability of Th17 cells expressing STAT4 isoforms to secrete IL-17...
We differentiated naive T cells with TGF-β1, IL-6, and IL-23 for 5 days and stimulated cells with anti-CD3 or PMA plus ionomycin (Fig. 3A). There were no significant differences between the percentage of TNF-α positive cells in Th17 cells expressing either isoform although the percentage of TNF-α positive cells was considerably higher following PMA plus ionomycin stimulation, compared with anti-CD3 (Fig. 3A). The Th17 cells expressing either isoform had similar capabilities to produce IL-17. As we have previously shown that generation of Th17 cells by TGF-β plus IL-6 is independent of STAT4 (31), we also examined the effects of culture with IL-23 on IL-17 production from STAT4 isoform-expressing T cells. After a week of culture in IL-23 cells were restimulated with anti-CD3 and IL-17 production was analyzed using ELISA. There was also no defect in IL-17 production from T cells expressing either STAT4 isoform, and production was increased compared with wild-type cells (Fig. 3B). To assess the responsiveness of the STAT4 isoforms to IL-23-induced cytokine production, we examined IL-17 levels by ELISA after 24 h of stimulating the cells with IL-23 and IL-18 (Fig. 3C). T cells expressing the STAT4α isoform secreted similar amounts to wild-type cells and significantly more IL-17 than cells expressing the STAT4β isoform. Thus, although either STAT4 isoform is sufficient for the generation of Th17 cells, activation of STAT4α by IL-23 can more efficiently induce IL-17 than the STAT4β isoform.

STAT4β promotes more severe colitic inflammation than STAT4α

Because we observed some differences in the ability of T cells expressing STAT4α or STAT4β to secrete inflammatory cytokines, we wanted to test the ability of the T cells expressing each isoform to mediate inflammation. Therefore, we reconstituted SCID mice with CD4+CD45RBhigh or CD4+CD45RBlow T cells that expressed either STAT4α or STAT4β and examined the weight loss kinetics of the mice. There was no significant difference in the kinetics of weight loss or the end point weight loss...
between the SCID mice reconstituted with either isoform or wild-type mice (Fig. 4A). However, there was a significant difference between the weight loss of mice reconstituted with the CD4\(^+\) CD45RB\(^{high}\) cells compared with the mice reconstituted with CD4\(^+\) CD45RB\(^{low}\) cells, indicating that the CD4\(^+\)CD45RB\(^{high}\) T cells expressing either isoform were sufficient to induce colitis (Fig. 4A). As wild-type mice had the same overall disease course as STAT4 isoform-expressing cells, we focused further analysis on the comparison between cells expressing the transgenic STAT4 isoforms. To determine whether the differences in T cell proliferation between the STAT4 isoforms seen previously in vitro (26) resulted in differences in cell reconstitution in vivo, we determined the absolute CD4\(^+\) cell numbers in MLN cells and the percentage of CD4\(^+\) T cells in the splenocytes and observed no significant difference between the repopulation efficiency of the CD4\(^+\) T cells expressing either isoform (Fig. 4B and data not shown). Similar to protein levels seen in Fig. 2B, STAT4 mRNA expression was slightly higher in STAT4\(^{\beta}\)-expressing cells than STAT4\(^{\alpha}\)-expressing cells in vivo (Fig. 4B).

Although weight loss was not significantly different between the SCID mice reconstituted with either STAT4 isoform, gross examination of the colon and scoring of the slides showed that the SCID mice reconstituted with the CD4\(^+\) CD45RB\(^{high}\) cells expressing the STAT4\(^{\alpha}\) isoform had more significant mucosal inflammation than the SCID mice reconstituted with the STAT4\(^{\alpha}\) as assessed by area and severity of the lesion (Fig. 4, C–E). There was no difference in mucosal hyperplasia between the mice reconstituted with STAT4\(^{\alpha}\) or STAT4\(^{\beta}\) expressing T cells. Importantly, SCID mice reconstituted with the CD4\(^+\) CD45RB\(^{low}\) cells had essentially no inflammatory infiltrates into the tissues (Fig. 4E).

**FIGURE 5.** Cytokine production from STAT4\(^{\alpha}\)- and STAT4\(^{\beta}\)-expressing T cells ex vivo. A and B, Cells were isolated and stimulated as described in Materials and Methods and concentration of cytokines were determined by ELISA and are displayed as mean ± SEM (STAT4\(^{\alpha}\) n = 9; STAT4\(^{\beta}\) n = 10). *, p < 0.05; **, p < 0.02 using unpaired Student’s t test. C, Cells were isolated and stimulated as described in Materials and Methods. The concentration of cytokines were determined by ELISA and are displayed as mean ± SD of pooled MLNs from the SCID mice reconstituted with the CD45RB\(^{high}\) subset of the indicated STAT4 isoform. *, p < 0.05 using unpaired Student’s t test.

Because previous data (26) and data in Figs. 2 and 3 show that STAT4\(^{\alpha}\) is more efficient than STAT4\(^{\beta}\) in cytokine stimulated production of IFN-\(\gamma\) and IL-17, we next examined the MLN cells from colitic mice for their ability to produce these cytokines following treatment with IL-12 and IL-18 or IL-23 and IL-18 for 72 h. Although the IL-23- and IL-18-stimulated cells from the SCID mice reconstituted with STAT4\(^{\alpha}\) secreted more IL-17, similar to results from in vitro differentiated cells, there was no significant difference in the amount of IFN-\(\gamma\) secreted from the cells isolated from the SCID mice reconstituted with either isoform (Fig. 5C). Overall, these data indicate that the increased inflammatory disease caused by STAT4\(^{\beta}\)-expressing T cells correlates with increased inflammatory cytokine production.

Previous literature suggested that TNF-\(\alpha\) and GM-CSF are important in neutrophil chemotaxis to inflamed tissues (19, 32). To examine whether the increased TNF-\(\alpha\) secretion from STAT4\(^{\beta}\)-expressing T cells correlated with increased neutrophils in the lamina propria, we analyzed microscopic sections of the colon for PMN infiltration. Consistent with the increased TNF-\(\alpha\) seen in the
SCID mice reconstituted with the STAT4β isoform, there were also increased neutrophils present in the lamina propria compared with the SCID mice reconstituted with STAT4α (Fig. 6A). Because anti-TNF therapies have been shown to inhibit GM-CSF production, we next wanted to look at GM-CSF levels in the mice with colitis (19). We examined supernatants from stimulated MLN cell cultures to assess GM-CSF production (Fig. 6B). Consistent with the increased neutrophil infiltration, GM-CSF was significantly increased from STAT4β-expressing T cells, further supporting the ability of T cells expressing the STAT4β isoform to mediate potent inflammatory responses.

Because there was increased GM-CSF production from STAT4β-expressing cells ex vivo, we wanted to define whether this reflected an increased propensity for STAT4β-expressing T cells to produce GM-CSF or whether it was a result of the in vivo inflammatory environment. To test this, we isolated naive T cells expressing either isofrom and differentiated them in Th1 or Th17 conditions for five days and stimulated them with anti-CD3 to examine their ability to secrete GM-CSF. Production of GM-CSF in Th1 cultures was dependent upon STAT4 (Fig. 7A). Consistent with what we observed in the ex vivo stimulated cells, the STAT4β-expressing Th1 cells secreted significantly more GM-CSF than STAT4α expressing Th1 cells. In contrast, there was no STAT4-dependence for GM-CSF production from Th17 cells and no significant difference in the amount of GM-CSF produced by Th17 cells expressing either STAT4 isoform (Fig. 7B). No detectable GM-CSF was secreted upon acute stimulation with IL-12 or IL-23 with or without IL-18, suggesting that STAT4 does not directly induce transcription of GM-CSF (data not shown). Together, these data demonstrate the increased inflammatory propensity of T cells expressing STAT4β and suggest that the increased inflammatory cytokine production by STAT4β-expressing T cells results in greater inflammatory disease in vivo.

Discussion

Although STAT4 is well known as an important regulator of inflammatory responses, the abilities of the STAT4 isoforms, STAT4α and STAT4β, to mediate inflammatory disease has not been well characterized. In this report, we use a model of inflammatory bowel disease to demonstrate differing abilities of STAT4 isoforms to promote tissue inflammation. We demonstrate that TNF-α and GM-CSF production are STAT4-dependent in Th1 cells and that STAT4β more effectively programs the secretion of these cytokines following subsequent Ag receptor stimulation. Thus, STAT4 isoforms may have differing roles in the development of inflammation.

IBD consists of two chronic, inflammatory diseases of the gastrointestinal tract, Crohn’s disease and ulcerative colitis, where CD4+ T cells play an important role in the dysregulated immune response. Crohn’s disease is typically associated with a Th1 and Th17 mediated response while ulcerative colitis is associated with Th2 response (33). In genetically susceptible individuals, it is thought that CD4+ T cells activated by environmental Ags and enteric bacteria secrete proinflammatory cytokines and stimulate macrophages within the lamina propria to release a variety of soluble proinflammatory mediators. These mediators recruit leukocytes and stimulate the release of cytokines that damage the epithelial cells and the mucosal tissues, creating the ulcerations and edema that characterize these disorders. To date, the most effective therapy has been aminosalicylates, sulfasalazine, corticosteroids, and anti-TNF-α therapy, all of which either limit the production or activity of proinflammatory cytokines secreted by the leukocytes (34). As STAT4 has been implicated as a pathogenic factor in Th1- and Th17-mediated autoimmune diseases, including IBD (17), we chose an IBD model system where colitis is induced in SCID mice upon reconstitution with CD4+ CD62Lhigh T cells to test the roles of STAT4 isoforms in disease. This model system has the advantage of being able to truly test the ability of T cells expressing the STAT4 isoforms to mediate pathogenesis with minimal manipulation after reconstitution.

Many cytokines seem to play an important role in the development of colitis in this model of IBD. That TNF-α production plays an essential, nonredundant role in the pathogenesis of colitis in this

FIGURE 6. Increased lamina propria neutrophil infiltration correlates with increased GM-CSF levels seen in the SCID mice reconstituted with the STAT4β isoform. A. PMN scores were determined as described in Materials and Methods. Data are presented as mean ± SEM. *, p < 0.05 using Mann-Whitney U test. B. Single cell suspensions from MLNs were pooled from the indicated mice, stimulated with anti-CD3 for 72 h and cell-free supernatants were analyzed by ELISA for GM-CSF. Data are presented as mean ± SEM. *, p < 0.05 using unpaired Student’s t test.

FIGURE 7. STAT4β Th1 cells are programmed to secrete more GM-CSF than STAT4α Th1 cells. A. CD4+ CD62L+ T cells were primed for Th1 differentiation using the same conditions as in Fig. 1. After 5 days, cells were stimulated for 24 h and cell-free supernatants were analyzed by ELISA for GM-CSF. Results are represented as mean ± SD and are representative of three independent experiments. *, p < 0.05 using unpaired Student’s t test. B. Cells cultured under Th17 conditions as in Fig. 3A for 5 days were stimulated for 24 h and analyzed by ELISA for GM-CSF production. Results are presented as mean ± SD and are representative of two independent experiments.
disease model (35) is important as that is one of the cytokines that we observe to be differentially regulated by STAT4 isoforms. IFN-γ was also produced from STAT4β cells at higher levels ex vivo and has a role in disease development. Although IL-17 is also critical for disease development, it was produced equally by STAT4α- and STAT4β-expressing T cells, suggesting that levels were sufficient for the establishment of disease. Similarly, we did not observe any difference in the potential of STAT4α- or STAT4β-expressing T cells to differentiate into adaptive T regulatory cells in vitro (data not shown). The differential effect of STAT4 isoforms on TNF-α and GM-CSF is interesting as there are some studies highlighting the cross-regulation of these two cytokines (36, 37). Importantly, infliximab treatment causes a concomitant decrease in both TNF-α and GM-CSF which is thought to contribute to apoptosis of PMNs and lead to decreased inflammation (19). It is possible that the differential expression of the STAT4 isoforms promote Th1 heterogeneity independent of IFN-γ production, due to the ability of STAT4β to enhance the secretion of TNF-α and GM-CSF.

The in vitro and in vivo data were divergent with regard to IFN-γ production upon anti-CD3 stimulation. Although the IFN-γ levels were similar in the in vitro differentiated Th1 cells, there was significantly more IFN-γ in the ex vivo stimulated cells of the SCID mice reconstituted with the STAT4β isoform. This was not due to more T cells being present in the splenocytes or MLNs as the percentage of CD4+ cells in those organs was not significantly different between the SCID mice reconstituted with either isoform (Fig. 3B and data not shown). It is possible that, given the ability of STAT4β to promote IL-12-stimulated proliferation to a greater extent than STAT4α (26) that there might be more differentiated Th1 cells expressing STAT4β in vivo. This would also explain the results with IL-12 and IL-18 stimulation where STAT4β-expressing cells were less responsive following in vitro stimulation, but produced similar levels of IFN-γ to STAT4α-expressing cells when stimulated with cytokine ex vivo. More Th1 cells, even if they produced less cytokine per cell, would generate the result observed. It is also possible that the increased IFN-γ is produced by accessory cells in the ex vivo cultures that might be differentially stimulated by interactions with STAT4α- or STAT4β-expressing cells.

As mentioned above, TNF-α may be involved in the regulation of GM-CSF (36, 37). This raised the issue of whether TNF-α might be regulating other aspects of the differentiation of STAT4α or STAT4β-expressing T cells. However, we did not find evidence of significant TNF-α effects on Th1 differentiation. First, expression of Tnfrsf1a and Tnfrsf1b was not different among WT, STAT4α-expressing, STAT4β-expressing, or STAT4-deficient cells (data not shown). Addition of TNF-α to differentiating cultures of WT, STAT4α-, or STAT4β-expressing T cells did not affect growth and led to a modest decrease in IFN-γ production from all cultures (data not shown). Expression of Ifngr1 and Ifngr2 was similar in WT, STAT4α-expressing and STAT4β-expressing T cells, although Ifngr2 was increased in Stat4α−/− cells, likely due to a lack of ligand-induced down-regulation (data not shown) (38). Il12rb2 was similar in WT and STAT4α-expressing cells, though ~50% decreased in STAT4β-expressing cells (data not shown) and also decreased in Stat4α−/− cells as previously noted (39). Importantly, the diminished Il12rb2 expression in STAT4β-expressing cells was still sufficient for IL-12-induced STAT4 phosphorylation (Fig. 2C). Culture with TNF-α did not significantly effect expression of Ifngr1, had minor effects on Ifngr2, and only had significant effects on Il12rb2 expression in STAT4β-expressing cells where expression was induced to WT levels (data not shown).

Overall, these data suggest that TNF-α does not have a major effect on Th1 development in this culture system.

There is potential clinical relevance to this study. The understanding of STAT4 isoform regulation in vitro or in vivo is unknown. For other human STAT isoforms, including STAT3 and STAT5, differentiation signals like G-CSF can induce the β isoform in myeloid cells (40, 41). In human T cell lines and PBMC, both STAT4α and STAT4β are detectable (26), though it is not known whether there are signals that might regulate splicing between the isoforms. It is also unclear whether an individual T cell expresses both isoforms or whether one isoform is preferentially expressed. It will be important to ascertain the relative amounts of STAT4 isoforms in humans and whether an increase in the STAT4β isoform has any relevance to severity of disease or susceptibility to disease in human patients. It is plausible that there could be an association with an increase in the Stat4β isoform and Th1-mediated autoinflammatory diseases like colitis.

The importance of STAT4 in human disease has been demonstrated both by the requirement for STAT4 in human IL-12 signaling (42) and the association of STAT4 single nucleotide polymorphisms with autoimmune diseases (43–46). A further understanding of the signals that regulate STAT4 mRNA splicing and the mechanisms through which STAT4 isoforms result in distinct effector phenotypes will be important in characterizing progression in human disease and may provide additional targets for the treatment of disease.

Disclosures

The authors have no financial conflict of interest.

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