Development of Colitis in Signal Transducers and Activators of Transcription 6-Deficient T-Cell Receptor α-Deficient Mice

A Potential Role of Signal Transducers and Activators of Transcription 6-Independent Interleukin-4 Signaling for the Generation of Th2-Biased Pathological CD4$^+$ββT Cells

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**Forbidden CD4$^+$ββ T cells, which produce interleukin (IL)-4 predominantly, are a pathological subset in the development of colitis in T-cell receptor α chain (TCRα)-deficient mice. Stimulation of naive CD4$^+$ T cells with IL-4 induces Th2 development via the activation of signal transducers and activators of transcription (STAT) 6. In the present study, we had found that IL-4 enhanced the expression of STAT6 in CD4$^+$ββ T cells isolated from TCRα$^{-/-}$ mice with colitis, suggesting that the IL-4 signal in the CD4$^+$ββ T cells is mediated by STAT6. To further investigate the role of STAT6 in the development of colitis induced by TCRα deficiency, we generated double-deficient mice by crossing TCRα$^{-/-}$ mice and STAT6$^{-/-}$ mice. Surprisingly, STAT6 deficiency did not result in decreased severity of colitis in TCRα$^{-/-}$ mice. STAT6-deficient CD4$^+$ββ T cells produced IL-4 and intraperitoneal injection of anti-IL-4 monoclonal antibody in the nondiseased TCRα$^{-/-}$ and STAT6 double-deficient mice prevented the colitis formation, thus indicating that the cells differentiated into the Th2 phenotype have the ability to mediate the development of the colitis in the absence of STAT6. *(Am J Pathol 2003, 162:263–271)*

Human inflammatory bowel disease (IBD) consists of two distinctive types, ulcerative colitis and Crohn's disease, on the basis of clinical and pathological features.[1–3] Distinct cytokine imbalance is considered important for the induction of these two types of disease.[4–6] Th1 cytokine [interferon-γ, interleukin (IL)-2, and tumor necrosis factor-α] seems to be predominant in Crohn's disease, whereas Th2 cytokine (IL-4, IL-5, and IL-6) tends to be associated with ulcerative colitis.[7–9]

Colitis spontaneously develops in various gene-manipulated murine models: T-cell receptor α chain-deficient (TCRα$^{-/-}$) mice, IL-2-deficient mice, IL-7-transgenic mice, IL-10-deficient mice, macrophage-specific signal transducers and activators of transcription 3 (STAT3)-deficient mice, Gα$_{i2}$-deficient mice, and severe combined immunodeficient mice restored with CD45RB^{hi}CD4$^+$ T cells.[3,10–16] Most of these rodent IBD models exhibit disruption of T-cell regulatory networks, with cytokine imbalance of pathogenic CD4$^+$ T cells predominantly shifted to the Th1 type. In contrast, the Th2 pathway is a major causative agent for the development of colitis in TCRα$^{-/-}$ mice,[17–20] hapten-induced murine colitis,[21] and oxazolone-induced colitis.[22]

TCRα$^{-/-}$ mice spontaneously develop chronic colitis under specific pathogen-free conditions at ~10 weeks of age.[18] We have previously shown that CD4$^+$ββ T cells,

Table 1. Histological Scoring System (Colon)

Histological changes	Score			
	0	1	2	3
Loss of goblet cells	None	A few	Numerous and focal	Numerous and diffuse
Crypt abscesses	0	1–4	>5	-
Epithelial erosion	None	Focal	Diffuse	-
Hyperemia	None	A few	Numerous and focal	Numerous and diffuse
Cellular infiltration in the lamina propria	None	A few	Numerous and focal	Numerous and diffuse
Thickness of colonic mucosa	<100%	101–140%	141–180%	>181%
Increase of the colonic gland	None	A few	Numerous and focal	Numerous and diffuse

which produce predominantly IL-4, are increased in the colons of TCR$\alpha^{-/-}$ mice with the disease.[17,18,23] Furthermore, administration of a monoclonal antibody (mAb) against IL-4 suppressed the onset of IBD in TCR$\alpha^{-/-}$ mice.[19] It has been reported also that TCR$\alpha^{-/-}$ × IL-4$^{-/-}$ double-mutant mice have a decreased propensity to develop IBD.[20] Thus, Th2-driven, IL-4-producing CD4$^+\beta\beta$ T cells seem to be a fundamental pathological element for the induction of colitis in TCR$\alpha^{-/-}$ mice.

STAT6 are members of the STAT family of proteins, which have been cloned and characterized as IL-4-activated transcription factors.[24,25] The binding of IL-4 to its receptor leads to the tyrosine phosphorylation of STAT6 by JAK1 and JAK3.[26] The phosphorylated STAT6 then forms homodimers, translocates to the nucleus, and binds to the promoter regions of IL-4-responsive genes to initiate the gene expression.[26] From analysis of mice lacking the *STAT6* gene it has been concluded that the signal transducer is essential for the biological functions of IL-4, including the development of Th2 cells from naive CD4$^+$ T cells, the class switching of B cells to the production of IgE and IgG1, induction of antigen-dependent airway hyperresponsiveness, and IL-4-mediated up-regulation of cell-surface molecules such as MHC class II and CD23.[24,25] These observations indicate that STAT6 is an essential signal transduction molecule for IL-4 signaling in T lymphocytes.[24,25]

To investigate the role of STAT6 in the development of IBD mediated by Th2-type pathological CD4$^+\beta\beta$ T cells, we crossed TCR$\alpha^{-/-}$ mice with STAT6$^{-/-}$ mice. Surprisingly, we found that the TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice spontaneously developed IBD, with characteristic clinical and histopathological abnormalities. STAT6-deficient CD4$^+\beta\beta$ T cells isolated from the diseased double-knockout mice retained the ability to produce IL-4, suggesting that the cells possessed the typical Th2-type phenotype. In addition, anti-IL-4 antibody (Ab) treatment in double-deficient mice prevented the development of colitis indicating that the STAT6-independent IL-4-signaling pathway is critical for the pathogenesis.

Materials and Methods

Mice

TCR$\alpha^{-/-}$ mice, with a background of C57Bl/6, were obtained from the Jackson Laboratory (Bar Harbor, ME). TCRα and STAT6 double-knockout mice were generated by backcrossing TCR$\alpha^{-/-}$ mice into STAT6$^{-/-}$ mice with a background of C57Bl/6.[25] The animals were maintained in the Experimental Animal Facility at the Research Institute for Microbial Diseases, Osaka University, under specific pathogen-free conditions and given sterilized food and autoclaved distilled water *ad libitum*. To determine the genotype of the mice, polymerase chain reaction (PCR) analysis was performed by using tail DNA.[25,27]

Anti-IL-4 mAb Treatment

In this study, a standard protocol was used for mAb *in vivo* treatment.[18,19] TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice from the beginning of 4 weeks to 10 weeks of age were intraperitoneally injected with rat anti-mouse IL-4 mAb (1 mg/mouse) prepared from hybridoma (11B11; American Type Culture Collection, Manassas, VA) or rat IgG1 (R3-34, 1 mg/mouse; PharMingen, San Diego, CA) as mock Ab in 100 μl of phosphate-buffered saline (PBS) twice a week. These treatments did not induce any signs of serum sickness.

Histopathological Analysis

For the assessment of the severity of IBD, the colons of TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice and TCR$\alpha^{-/-}$ mice were examined histologically. Tissue samples obtained from the proximal, middle, and distal colon were fixed in 4% paraformaldehyde in PBS for 4 hours, embedded in paraffin, and sectioned at a thickness of 6 μm.[19] The tissue sections were stained with hematoxylin and eosin. The histopathological score shown in Table 1 was used for the assessment of the severity of the colitis. It is a modification of the scoring system described previously.[21,28] The scoring was performed blindly by two independent investigators (YO and YK). The thickness of the colonic mucosa was determined to compare with the mucosal layer of the equivalent colonic segment in normal C57Bl/6 mice using a micrometer. The total histological score represents the sum of each item score.

Preparation of Cell Suspensions

Mice anesthetized with ketamine (Sigma Chemical Co., St. Louis, MO) were sacrificed at 10 weeks age. The spleens and mesenteric lymph nodes (MLNs) were aseptically removed, and single-cell suspensions were pre-

pared by a standard mechanical procedure.[18,29,30] Mononuclear cells from the lamina propria (LP) of the colon were dissociated using type IV collagenase (Sigma) to obtain single-cell preparations as described.[18,29,30]

Flow Cytometric Analysis and Cell Sorting

For analysis of the distribution of CD4$^+$$\beta\beta$ T cells by flow cytometry, single-cell suspensions of the mononuclear cells (10^6/sample) prepared from various tissues were stained with optimal concentrations of phycoerythrin-conjugated anti-CD4 mAb (L3T4) and fluorescein isothiocyanate (FITC)-conjugated anti-TCR-β mAb (H57-597). The samples were then subjected to flow cytometric analysis by using a FACScan (Becton Dickinson, Mountain View, CA). Data were analyzed by using CellQuest software (Becton Dickinson). For the analysis of the cytokine mRNA expression by the CD4$^+$$\beta\beta$ T cells, the cells were purified by FACS Vantage (Becton Dickinson) as described previously.[19]

Quantitative Reverse-Transcriptase-PCR

To analyze the cytokine-specific mRNA expression by CD4$^+$$\beta\beta$ T cells isolated from colonic LP of the diseased TCRα⁻/⁻ × STAT6⁻/⁻ mice, a highly sensitive, quantitative RT-PCR was performed.[31–33] Total RNA was isolated from fluorescence-activated cell sorting-purified CD4$^+$$\beta\beta$ T cells by using TRIzol reagent (Invitrogen, Carlsbad, CA). The RNA was reverse-transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen), RNase inhibitor (Toyobo, Tokyo, Japan), oligo(dT)12-18 primer (Invitrogen), and dNTPs (Amersham Pharmacia Biotech, Arlington Heights, IL). The mixture was incubated at 42°C for 120 minutes and heated to 90°C for 5 minutes.[34] After treatment with RNase H (Toyobo), the synthesized cDNA was extracted by phenol/chloroform. Then, the cytokine-specific cDNA was quantified with LightCycler (Roche Diagnostics, Mannheim, Germany) technology by using LightCycler-DNA Master Hybridization Probes (Roche Diagnostics). For the amplification of cDNA, 20 μl of PCR mix was added to each tube to give a final concentration of 0.05 μmol/L 5′ primer, 0.05 μmol/L 3′ primer, 0.2 μmol/L FITC-labeled probe, 0.2 μmol/L LightCycler Red 640-labeled probe, 2 mmol/L MgCl$_2$, and 1× LightCycler-DNA master hybridization probes mix (Roche Diagnostics). The oligoprimers specific for the IL-4 (sense, 5′-ATGGGTCT-CAACCCCCAGCTAGT-3′; anti-sense, 5′-GCTCTTTAG-GCTTTCCAGGAAGTC-3′), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (sense, 5′-TTCACCACCATG-GAGAAGGC-3′; anti-sense, 5′-GGCATGGACTGTGGT-CATGA −3′) were used.[35,36] For detection of the target molecule, FITC-labeled hybrid probe and LightCycler Red 640 (LCR)-labeled hybrid probe to IL-4 (FITC, 5′-CGTTTG-GCACATCCATCTCCGT-3′; LCR, 5′-CATGGCGTCCCT-TCTCCTGTG −3′), and GAPDH (FITC, 5′-TGGGTGTGAAC-CACCAGAAATATGAC-3′; LCR, 5′-ACTCACTCAAGATTGT-CAGCAATGCA-3′) were prepared according to instructions provided by the manufacturer. After heating at 94°C for 2 minutes, cDNA were amplified for 40 cycles, each cycle

consisting of 95°C for 10 seconds, 55°C for 30 seconds, and 72°C for 30 seconds.[33] Once during the cycle in which the log-linear signal could be distinguished from background, it was possible to compare the target concentrations (external standard) in samples with an internal standard in the same samples.[33] After the PCR had been completed, the LightCycler software (Roche Diagnostics) automatically converted the raw data into copies of target molecules. In this study, the relative quantitative expression of cytokine-specific mRNA in each sample was expressed as the amount of cytokine mRNA divided by the amount of mRNA GAPDH.[37]

Cytokine Enzyme-Linked Immunosorbent Assay

Purified CD4$^+$ T cells were co-cultured with mAb anti-CD28 (2 μg/ml, 37.51; PharMingen) in anti-CD3 mAb (10 μg/ml, 145-2C11; PharMingen) precoated 96-well tissue culture plates (5 × 10^5 cells/well) for 60 hours.[38] Culture supernatants were then harvested for analysis of cytokine production by cytokine enzyme-linked immunosorbent assay kit (Amersham Pharmacia Biotech) for IL-4.

Western Blotting Analysis for the Expression of Phosphorylated STAT6

Purified CD4$^+$ T cells isolated from MLNs of wild-type C57Bl/6 mice and TCRα⁻/⁻ mice were stimulated *in vitro* with IL-4 (20 ng/ml) for 15 minutes, then lysed in cell lysis buffer (5% sodium dodecyl sulfate, 0.5 mol/L Tris-HCl, pH 6.8, 0.5 mol/L ethylenediaminetetraacetic acid, 1 mmol/L dithiothreitol, 1 mmol/L phenylmethyl sulfonyl fluoride, and 10 μg leupeptin) for 30 minutes at 4°C, and centrifuged at 10,000 rpm for 30 minutes. The supernatants were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and probed with anti-phospho STAT6Ab (Phospho-STAT Antibody Sampler; Cell Signaling, Beverly, MA) and anti-STAT6Ab (Santa Cruz Biotechnology, Santa Cruz, CA). The membrane was finally developed by use of an enhanced chemiluminescence kit (ECL plus; Amersham Pharmacia Biotech).

Statistical Analysis

Data were statistically analyzed by Student's two-tailed *t*-test or Pearson's correlation coefficient test with $P <$ 0.05 considered statistically significant.

Results

STAT6 Mediated the IL-4 Signaling in CD4$^+$$\beta\beta$T Cells

It has been shown that IL-4-mediated differentiation of naive CD4$^+$ T cells into Th2 cells is dependent on STAT6.[24,25] Thus, it was important to examine whether the STAT6-mediated signaling pathway was involved in the activation of IL-4-producing pathological CD4$^+$$\beta\beta$ T
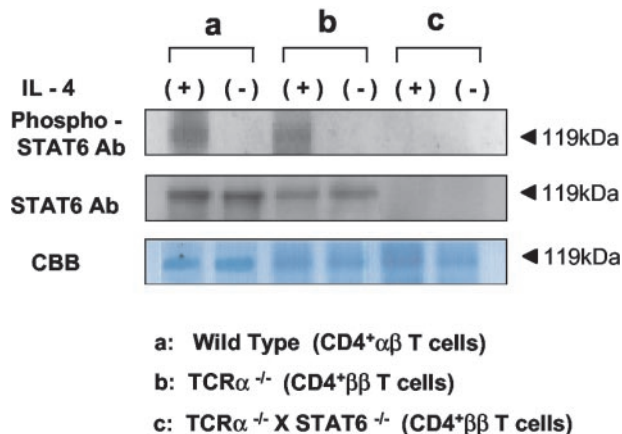
Figure 1. Expression of phosphorylated STAT6 in CD4$^+$$\beta\beta$ T cells from TCR$\alpha^{-/-}$ mice and CD4$^+$$\alpha\beta$ T cells from wild-type C57Bl/6 mice. MACS-purified CD4$^+$ T cells from MLNs were stimulated *in vitro* with recombinant IL-4 (20 ng/ml) for 15 minutes, and then phosphorylated STAT6 protein was examined by Western blotting analysis. **Top**, Anti-phospho STAT6 Ab; **middle**, anti-STAT6 Ab; **bottom**, Coomassie brilliant blue staining for the confirmation of that equivalent amounts of cellular protein were present. The data are representative from one of three independent experiments.

Figure 2. Decreased body weight in TCR$\alpha^{-/-}$ $\times$ STAT6$^{-/-}$ mice and TCR$\alpha^{-/-}$ mice but not in STAT6$^{-/-}$ mice or wild-type C57Bl/6 mice. Body weight of the mice was measured weekly for 14 weeks. All of the mice used in this study have the same inbred C57Bl/6 background. Statistical analysis was performed using Student's two-tailed *t*-tests to compare TCR$\alpha^{-/-}$ $\times$ STAT6$^{-/-}$ mice, TCR$\alpha^{-/-}$ mice, and STAT6$^{-/-}$ mice with C57Bl/6 mice. The results are expressed as mean $\pm$ SEM of three independent experiments (10 mice/group). *, $P < 0.05$.

cells clonally expanded in the diseased TCR$\alpha^{-/-}$ mice. According to the method described previously,[18,19,23] the CD4$^+$$\beta\beta$ T cell-enriched fraction was prepared by MACS separation. Western blotting analysis revealed that phosphorylated STAT6 was induced in MLN-derived $\beta\beta$ T cell-enriched CD4$^+$ T cells after stimulation with IL-4, thus indicating that STAT6 was involved in the IL-4 signaling event in the cells (Figure 1).

TCR$\alpha^{-/-}$ $\times$ STAT6$^{-/-}$ Mice Developed Colitis

To assess the role of STAT6 in the development of colitis in the TCRα-deficient mice, we generated TCRα- and STAT6-double-mutant (TCR$\alpha^{-/-}$ $\times$ STAT6$^{-/-}$) mice. The expression of STAT6 protein was not detected in CD4$^+$$\beta\beta$ T cells isolated from MLNs of TCR$\alpha^{-/-}$ $\times$ STAT6$^{-/-}$ mice (Figure 1). Unexpectedly, the TCR$\alpha^{-/-}$ $\times$ STAT6$^{-/-}$ mice, like TCR$\alpha^{-/-}$ mice, lost significant body weight from 11 weeks of age when compared with C57Bl/6 wild-type mice and STAT6$^{-/-}$ mice (Figure 2). The average body weight at the end of observation was: wild-type mice, 26.42 $\pm$ 0.62 g; STAT6$^{-/-}$ mice, 26.19 $\pm$ 0.62 g; TCR$\alpha^{-/-}$ mice, 21.17 $\pm$ 0.93 g; and TCR$\alpha^{-/-}$ $\times$ STAT6$^{-/-}$ mice, 20.63 $\pm$ 0.73 g. TCR$\alpha^{-/-}$ $\times$ STAT6$^{-/-}$ mice also displayed the physical changes typical of those in TCRα-deficient mice with colitis, ie, hunched posture, anorectal prolapse, and diarrhea. Some TCR$\alpha^{-/-}$ $\times$ STAT6$^{-/-}$ mice had died after 8 to 10 weeks of age, and all were dead by 15 weeks. Histologically, colons of the TCR$\alpha^{-/-}$ $\times$ STAT6$^{-/-}$ mice at age 10 weeks were thickened, eroded, and infiltrated with inflammatory mononuclear cells, just as in TCR$\alpha^{-/-}$ mice with IBD (Figure 3A). In contrast, the colons of STAT6$^{-/-}$ mice and wild-type mice had no evident inflammatory changes. We further compared the degree of histopathological changes present in the colonic tissues of TCR$\alpha^{-/-}$ $\times$ STAT6$^{-/-}$ mice with those in other mice by
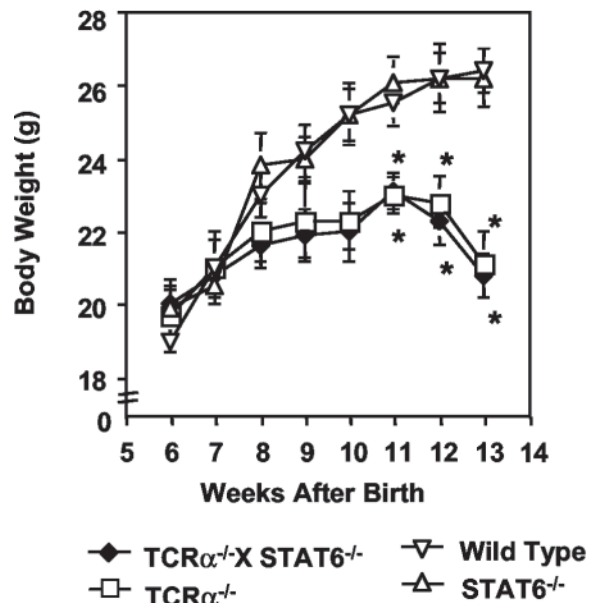
using the histological scoring system as described (Table 1).[21,28] The scores in TCR$\alpha^{-/-}$ $\times$ STAT6$^{-/-}$ mice (8.7 $\pm$ 1.1) were comparable to those in TCR$\alpha^{-/-}$ mice with colitis (9.4 $\pm$ 0.5) (Figure 3B), whereas the scores in C57Bl/6 wild-type and STAT6$^{-/-}$ mice were both 0.

STAT6-Deficient CD4$^+$$\beta\beta$T Cells Were Present in TCR$\alpha^{-/-}$ $\times$ STAT6$^{-/-}$ Mice with Colitis

Because CD4$^+$$\beta\beta$ T cells reportedly are crucial for the induction of IBD in TCR$\alpha^{-/-}$ mice,[18,23,39] we next assessed the presence of this unique subset of T cells in TCR$\alpha^{-/-}$ $\times$ STAT6$^{-/-}$ mice with IBD. When mononuclear cells isolated from the colonic LP and MLNs of the TCR$\alpha^{-/-}$ $\times$ STAT6$^{-/-}$ mice with IBD were examined for the subset of CD4$^+$$\beta\beta$ T cells, increased numbers of the cells were present (MLN, 1.89 $\pm$ 0.41%; colonic LP, 19.24 $\pm$ 3.90%). The degree of increase was similar to those in TCR$\alpha^{-/-}$ mice with IBD [MLN, 1.67 $\pm$ 0.44%; colonic lamina propria lymphocytes (LPLs), 19.86 $\pm$ 2.22%] (Figure 4, A and B).

It has been reported also that the severity of IBD in TCR$\alpha^{-/-}$ mice correlates with the increase of colonic CD4$^+$$\beta\beta$ T cells.[18] We observed a similar and significant correlation in the TCR$\alpha^{-/-}$ $\times$ STAT6$^{-/-}$ mice with IBD when the correlation coefficient between histopathological changes and increase of CD4$^+$$\beta\beta$ T cells was calculated ($r = 0.85$, $P < 0.05$) (Figure 4C).

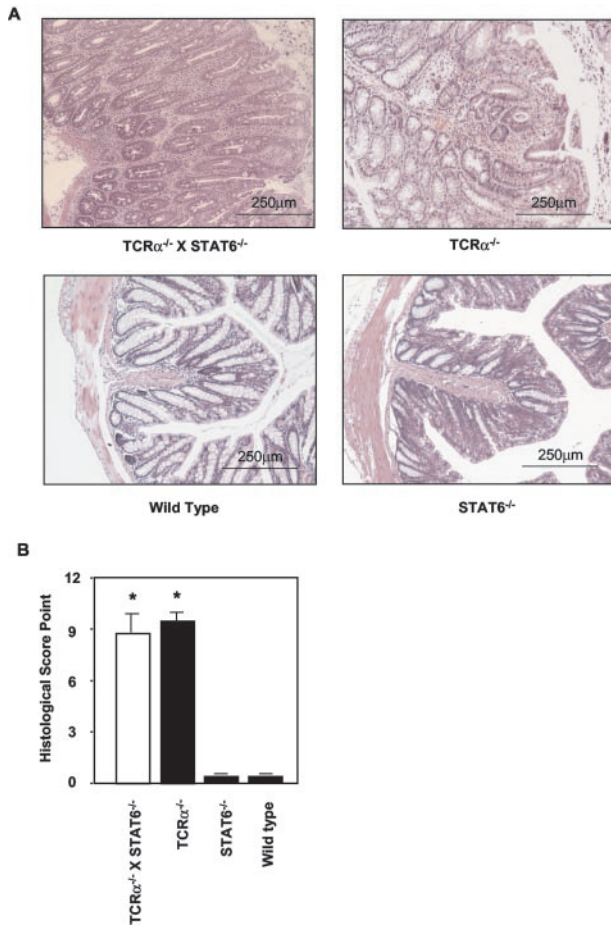**Figure 3.** Histological analysis of colons isolated from TCRα$^{-/-}$ × STAT6$^{-/-}$ mice, TCRα$^{-/-}$ mice, STAT6$^{-/-}$ mice, and wild-type C57Bl/6 mice. All of the mice were sacrificed at 10 weeks of age. **A:** The tissue sections prepared from paraffin-embedded fixed colon were stained with H&E. **B:** The severity of the colitis was also examined by using the histological disease scoring system (Table 1) as described previously.[21,28] The histological score shown in each strain of mice ($n = 7$) was determined according to the diagnosis and grading of the colitis and was expressed as mean ± SD. Statistical analysis was performed for the comparison of TCRα$^{-/-}$ × STAT6$^{-/-}$ mice, TCRα$^{-/-}$ mice, and STAT6$^{-/-}$ mice. *, $P < 0.05$.

STAT6-Deficient Intestinal CD4$^+$ββT Cells Produced IL-4 in the Double-Mutant Mice with Colitis

Th2-type pathological CD4$^+$ββ T cells from TCRα$^{-/-}$ mice produce predominantly IL-4,[17,18] and the administration of mAb against IL-4 reportedly suppressed the onset of IBD in TCRα$^{-/-}$ mice.[19] Because CD4$^+$ββ T cells were increased in the colons of our TCRα$^{-/-}$ × STAT6$^{-/-}$ colitic mice, we next investigated cytokine production by CD4$^+$ββ T cells isolated from the mice. Total RNA isolated from purified pathogenic CD4$^+$ββ T cells of colonic LP and MLNs was subjected to cytokine-specific quantitative RT-PCR. An equivalent or slightly increased amount of IL-4-specific mRNA was found in the cells of these diseased animals as compared with amounts expressed in conventional CD4$^+$ββ T cells isolated from TCRα$^{-/-}$ mice with IBD (Figure 5A). We also examined, by enzyme-linked immunosorbent assay, the levels of the cytokine in the culture supernatant of purified CD4$^+$ T
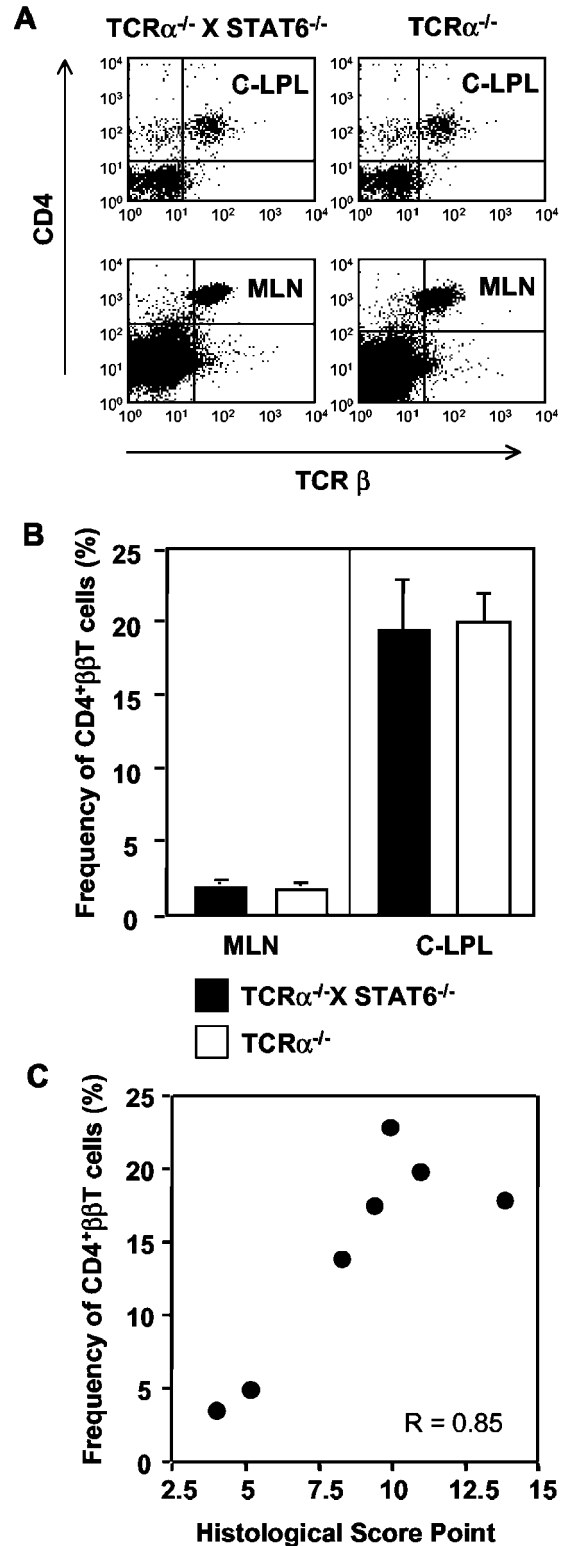

Figure 4. Flow cytometric analysis of CD4$^+$ββ T cells in TCRα$^{-/-}$ × STAT6$^{-/-}$ mice with IBD. **A:** Lymphocytes were isolated from the colonic LP (C-LPLs) and MLNs of the double-mutant and TCRα$^{-/-}$ mice with IBD and co-stained with appropriate fluorescence-conjugated anti-CD4 (L3T4) and anti-TCR-β (H57-597) mAb for fluorescence-activated cell sorting analysis. **B:** The frequency of CD4$^+$ββ T cells in the C-LPLs and MLNs of TCRα$^{-/-}$ × STAT6$^{-/-}$ mice (■) and TCRα$^{-/-}$ mice (□) was calculated. **C:** The increase of CD4$^+$ββ T cells and the disease score in each TCRα$^{-/-}$ × STAT6$^{-/-}$ mouse was correlated ($P = 0.016$, $r = 0.85$, $n = 7$). The data are representative of three independent experiments.
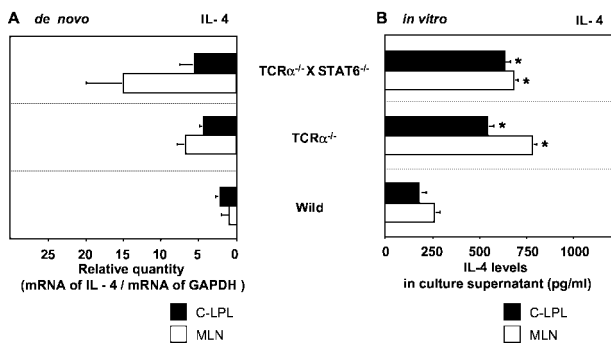
Figure 5. IL-4 production by CD4$^+\beta\beta$ T cells isolated from TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice with colitis. **A:** Relative quantity of cytokine-specific mRNA expression by CD4$^+\beta\beta$ T cells in the mucosal compartment of TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice and TCR$\alpha^{-/-}$ mice *de novo.* CD4$^+\beta\beta$ T cells in the C-LPLs (■) and MLNs (□) were purified by flow cytometry, and cytokine-specific mRNA expression was analyzed by quantitative RT-PCR. Cytokine-specific mRNA production was expressed as the amounts of relative quantity against GAPDH. (C-LPL: TCR$\alpha^{-/-}$ × STAT6$^{-/-}$, 5.39 ± 2.42; TCR$\alpha^{-/-}$, 4.16 ± 0.81; wild-type, 3.01 ± 1.08; MLN: TCR$\alpha^{-/-}$ × STAT6$^{-/-}$, 14.94 ± 5.72; TCR$\alpha^{-/-}$, 6.58 ± 1.53; wild-type, 0.75 ± 0.13). **B:** Cytokine production by MACS-purified CD4$^+$ T cells isolated from TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice and TCR$\alpha^{-/-}$ mice with IBD. The T lymphocytes from the C-LPLs (■) and MLNs (□) were cultured *in vitro* with precoated anti-CD3 mAb in the presence of anti-CD28 mAb for 60 hours. Then culture supernatants were collected and cytokine production was analyzed by cytokine enzyme-linked immunosorbent assay. (C-LPL: TCR$\alpha^{-/-}$ × STAT6$^{-/-}$, 634.2 ± 47.6 pg/ml; TCR$\alpha^{-/-}$, 542.2 ± 51.4 pg/ml; wild-type, 219.5 ± 28.2 pg/ml; MLN: TCR$\alpha^{-/-}$ × STAT6$^{-/-}$, 682.6 ± 35.8 pg/ml; TCR$\alpha^{-/-}$, 784.0 ± 36.3 pg/ml; wild-type, 249.9 ± 16.5 pg/ml). The results are expressed as mean ± SEM of three independent experiments. *, $P < 0.05$.

cells from TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice (Figure 5B), and found levels similar to those produced by cells from TCR$\alpha^{-/-}$ mice. Thus, even in the absence of STAT6, CD4$^+\beta\beta$ T cells in the TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice with colitis produced much IL-4.

Anti-IL-4 mAb Administration Suppressed the Onset of Colitis in TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ Mice

To clarify that IL-4 plays an essential role for the development of IBD in TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice, the double-mutant mice were treated intraperitoneally with anti-IL-4 mAb from the beginning of 4 weeks to 10 weeks of age. The mice treated with mock IgG developed IBD, including anorectal prolapse, diarrhea, hemorrhagic stool, and the weight loss. In contrast, the mice treated with anti-IL-4 mAb showed no sign for the development of IBD. Histological examination of the colon demonstrated anti-IL-4 mAb treatment prevented the formation of colonic inflammation (Figure 6A). We further compared the degree of histopathological changes present in the colonic tissues of TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice with and without anti-IL-4 mAb by using the histological scoring system (Table 1). The score in anti-IL-4 mAb treated mice (0.6 ± 0.4) was significantly less than that in mock IgG-treated mice (8.3 ± 1.2) (Figure 6B). We next assessed the alteration of CD4$^+\beta\beta$ T cells in TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice with and without the anti-IL-4 mAb treatment. A significant difference was not observed between anti-IL-4-treated (0.91 ± 0.20%) and untreated (or mock IgG treated, 1.78 ± 0.38%) groups in the frequency of CD4$^+\beta\beta$ T cells in MLNs ($P = 0.26$). However, the frequency of the patho-
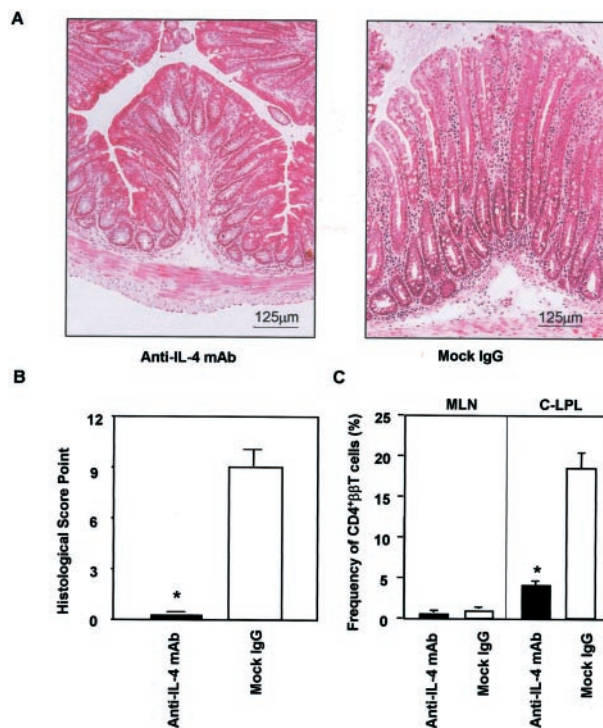

Figure 6. The suppressive effect of anti-IL-4 mAb treatment for the development of colitis in TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice. **A:** Histological analysis of the colon in TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice treated intraperitoneally with anti-IL-4 mAb or mock Ab (three mice/group). The treatment was initiated at 4 weeks of age and all of the mice were then sacrificed at 10 weeks of age. The tissue sections were prepared from colon and stained with H&E. **B:** The severity of the colitis was also determined in each group of mice ($n = 3$) by using the histological disease scoring system (Table 1) and was expressed as mean ± SD. The administration of anti-IL-4 Ab ameliorated the severity of the colitis. **C:** Flow cytometric analysis of CD4$^+\beta\beta$ T cells in TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice with and without anti-IL-4 mAb. Lymphocytes were isolated from the C-LPLs and MLNs, then co-stained with anti-CD4 (L3T4) and anti-TCRβ (H57-597) mAbs. The results are expressed as mean ± SEM, *, P < 0.05.

genic CD4$^+\beta\beta$ T cells was significantly decreased in the colon of double-knockout mice treated with anti-IL-4 mAb (anti-IL-4 mAb, 4.60 ± 0.87%; mock IgG, 18.59 ± 3.76%) (Figure 6C). These findings indicated that IL-4 plays a critical role for the onset of IBD in TCR$\alpha^{-/-}$ × STAT6$^{-/-}$ mice despite in the absence of STAT6.

Discussion

IL-4 produced by CD4$^+\beta\beta$ T cells is critical for the development of IBD in TCR$\alpha^{-/-}$ mice.[18–20] Previously we reported that the administration of anti-IL-4 antibody changed the pattern of cytokine production in CD4$^+\beta\beta$ T cells from dominant Th2 to Th1 and resulted in reduced ability to induce IBD in TCR$\alpha^{-/-}$ mice.[19] Others have reported that TCR$\alpha^{-/-}$ and IL-4$^{-/-}$ double-knockout mice, but not TCR$\alpha^{-/-}$ and interferon-$\gamma^{-/-}$ double-knockout mice, have a decreased frequency of IBD when compared to TCR$\alpha^{-/-}$ mice.[20] STAT6 is a transcriptional molecule that is a constitutive ingredient for IL-4 functions;[24,25,40] in the absence of STAT6, IL-4 failed to activate naive T cells to differentiate into Th2 cells or to enhance the proliferation of differentiated

Th2 cells.[24,25,40,41] Further, STAT6 was shown to be an essential transcriptional molecule for IL-4-driven Th2 differentiation and cell expansion in CD4$^+$ T cells.[42] Also, in a murine model of allergy, the loss of STAT6 abrogated antigen-induced respiratory hyperresponsiveness.[43,44] Thus, it was logical to postulate that the removal of the *STAT6*-specific gene from the pathological CD4$^+$$\beta\beta$ T cells would result in diminished development of colitis in TCRα⁻/⁻ mice.

Contrary to this postulate, we found that TCRα⁻/⁻ × STAT6⁻/⁻ mice developed IBD similar in severity to that seen in TCRα⁻/⁻ mice (Figures 2 and 3). Because anti-IL-4 mAb treatment suppressed the induction of IBD in TCRα⁻/⁻ × STAT6⁻/⁻ mice, a STAT6-independent signaling pathway responsible for the induction of IL-4-producing CD4$^+$$\beta\beta$ T cells in murine colitis was implied. Such a possibility seems reasonable especially because several additional molecules reportedly are critical for IL-4 signaling in Th2-type cells.[26,45] Ouyang and colleagues[46] reported that Th2 development is dependent on GATA-3 expression independent of IL-4 and STAT6, and activation of GATA-3 is a central player in Th2 differentiation.[46,47] GATA-3 has been shown to express naive T cells,[48] followed by a substantial increase during Th2 development with down-regulation of Th1 development.[49] Thus, GATA-3 can inhibit Th1 development by repressing IL-12Rβ expression.[49,50] Further, GATA-3 provides an instructive signal for the development of Th2 type cells,[47] and GATA-3 generates stability of Th2 commitment via the chromatin remodeling of Th2-specific cytokine loci.[51,52] Our preliminary results indicated that the levels of expression of GATA-3 were enhanced in CD4$^+$$\beta\beta$ T cells purified from the diseased TCRα⁻/⁻ × STAT6⁻/⁻ mice, implying that GATA-3 is at least one alternative component responsible for Th2 development of CD4$^+$$\beta\beta$ T cells in the double-knockout mice with colitis (data not shown). This point is now carefully addressed in a separate study.

In addition to GATA-3, STAT6-independent IL-4 signaling reportedly is mediated via several other molecules, such as phosphotyrosine-binding domain proteins;[26] insulin receptor substrate;[53] src homology 2 domain-containing α2 collagen-related protein (Shc);[54] IL-4 receptor-interacting protein FRIP;[55] and Th2-related transcription factors such as GATA-3[48,56] c-Maf,[57] NF-AT,[58] and BCL-6. A proto-oncogene, BCL-6, binds to the same DNA-binding motifs of STAT transcription factors as a transcriptional repressor.[59,60] The removal of the *BCL-6* gene resulted in the overproduction of Th2 cells, leading to severe inflammation of the heart and lung[59,61] and creation of double-mutant mice in both BCL-6 and either STAT6 or IL-4 unexpectedly resulted in the development of lethal Th2-type inflammation.[62] These findings further support our present result in which pathological Th2 type CD4$^+$$\beta\beta$ T cells developed in the absence of STAT6. Thus, there might be multiple redundant pathways for IL-4 production by CD4$^+$$\beta\beta$ T cell, exhibiting equivalent effects on the differentiation of the unique CD4$^+$$\beta\beta$ T cells into the Th2-biased phenotype.

STAT6 deficiency did not affect the ability of CD4$^+$$\beta\beta$ T cells to secrete IL-4 and proliferate. These results imply

that STAT6 is not an absolute requirement for Th2 differentiation of CD4$^+$$\beta\beta$ T cells, although it has been reported that STAT6 is necessary and sufficient to mediate both IL-4-driven Th2 differentiation and cell expansion in naive CD4$^+$ T cells in normal mice.[42] It should be noted that the concept of a primary role of IL-4 and STAT6 signaling in the induction of Th2-type cells has recently been put in doubt: in mice genetically deficient in IL-4, IL-4R, and STAT6, Th2-type cells and those associated immune responses emerged in the absence of the IL-4/IL-4R and STAT6 signaling cascade,[63–66] and classical IL-4- and IL-5-producing Th2-type cells developed in helminth-infected STAT6-deficient mice.[67] Further, repetitive anti-CD3 stimulation of memory type CD4$^+$CD62L^{high} T cells from STAT6-deficient mice resulted in the generation of IL-4-producing Th2-type cells.[68] Moreover, the number of CD4$^+$$\beta\beta$ T cells was not different in TCRα⁻/⁻ × IL-4⁻/⁻ and TCRα⁻/⁻ mice with IBD.[20] Taken together, the previous and present findings strongly suggest that the STAT6 signaling pathway is not essential for the development of pathological Th2-type CD4$^+$$\beta\beta$ T cells.

Our previous data also showed that anti-IL-4 treatment did not influence the number of CD4$^+$$\beta\beta$ T cells in TCRα⁻/⁻ mice.[19] Attempts to culture CD4$^+$$\beta\beta$ T cells isolated from TCRα⁻/⁻ mice failed to replicate these aberrant T cells under *in vitro* Th2-skewing-conditions (data not shown). Because IL-4 did not act as a trophic factor for CD4$^+$$\beta\beta$ T cells it probably does not directly affect the development of CD4$^+$$\beta\beta$ T cells. This implies that a unique environment exists *in vivo* that is capable of driving Th2 responses independent of IL-4-STAT6 signaling. Interestingly, CD4$^+$$\beta\beta$ T cells were not detected in TCRα⁻/⁻ × MHC class II⁻/⁻ mice.[30] Therefore, CD4$^+$$\beta\beta$ T cells may develop and expand in response to unidentified MHC class II-restricted antigens. TCRα⁻/⁻ mice with IBD showed humoral immune responses to food, self antigens,[18] and intestinal bacteria, such as *Bacteroides vulgatus*.[38] Perhaps then CD4$^+$$\beta\beta$ T cells exhibit Ag specificities against innocuous luminal antigens for the production of Th2-type cytokines, eventually leading to the development of IBD.

The results of the present study illustrate the complexity of mechanisms involved in the pathogenesis of colitis in TCRα-deficient condition. Further investigation of signaling molecules other than STAT6 involved in IL-4 signaling may clarify the molecular mechanism behind Th2-type colitis. Moreover, the TCRα⁻/⁻ × STAT6⁻/⁻ mouse strain is a useful animal model for investigating the mechanism of STAT6-independent Th2 differentiation of the unique pathogenic CD4$^+$$\beta\beta$ T cells.

Acknowledgment

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