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Stat4 Is Critical for the Balance between Th17 Cells and Regulatory T Cells in Colitis

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Abstract

Th17 play a central role in autoimmune inflammatory responses. Th1 are also necessary for autoimmune disease development. The interplay of Th1 signals and how they coordinate with Th17 during inflammatory disease pathogenesis are incompletely understood. In this study, by adding Stat4 deficiency to Stat6/T-bet double knockout, we further dissected the role of Stat4 in Th1 development and colitis induction. We showed that in the absence of the strong Th2 mediator Stat6, neither Stat4 nor T-bet is required for IFN-γ production and Th1 development. However, addition of Stat4 deficiency abolished colitis induced by Stat6/T-bet double-knockout cells, despite Th1 and Th17 responses. The failure of colitis induction by Stat4/Stat6/T-bet triple-knockout cells is largely due to elevated Foxp3$^+$ regulatory T cell (Treg) development. These results highlight the critical role of Stat4 Th1 signals in autoimmune responses in suppressing Foxp3$^+$ Treg responses and altering the balance between Th17 and Tregs to favor autoimmune disease.

Mice deficient in the Th1 transcription factors Stat4 or T-bet are severely impaired in their ability to produce IFN-γ and Th1 cells and are resistant to the development of experimental autoimmune encephalomyelitis or colitis (1–4). Thus IL-12, T-bet, and Th1 effector cells were initially suggested to be important in the molecular pathogenesis of inflammatory responses and autoimmune diseases (2, 5, 6). Later studies demonstrated that colitis is due more to the effects of IL-23 and Th17 cells than to Th1 cells and cytokines (7, 8). However, there are increased levels of IL-17 when T-bet is absent (9–11), which fail to induce colitis (2, 11), suggesting that Th1 responses may still be involved in the pathogenesis of autoimmunity. It is not clear how Stat4, T-bet, and Th1 signals coordinate with IL-17 and Th17 signals during autoimmune disease pathogenesis.

Regulation of IFN-γ production and Th1 development of CD4 T cells is mainly mediated by IFN-γ/Stat1/T-bet– and IL-12/Stat4–signaling pathways (12–17). We and other investigators

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showed that in addition to directly regulating IFN-γ and Th1 differentiation, T-bet is critical to prevent the IL-4/Stat6/GATA-3 Th2 signal cascade from suppressing IL-12Rβ2 expression and, thus, maintain the IL-12/Stat4–signaling pathway for IFN-γ regulation (11, 14). Restoration of IL-12/Stat4 signals by adding Stat6 deficiency to T-bet deficiency allows Th1 development and colitis induction (11), pointing to an important role for IL-12/Stat4 in Th1 and autoimmune pathogenesis. The direct role of Stat4 in Th1 development and autoimmunity requires further clarification.

$Foxp3^+$ regulatory T cells (Tregs) are central in immune regulation (18, 19), and TGF-β plays a critical role in inducing and maintaining Tregs and controlling autoimmune disease pathogenesis (20–25). Treg and Th17 developmental pathways are reciprocally regulated (26–29), so that TGF-β plus IL-6 drive Th17 pathways (9, 10, 26–28, 30). Tregs were suggested to be one of the main sources for TGF-β in Th17 differentiation (25), and the Th17 cytokines IL-21 and IL-23 suppress Foxp3 induction and Treg generation (31, 32). Th1 signals were shown to negatively regulate Th17 and Treg development. Mice defective in Th1 development have decreased Th1 but increased numbers of Th17 cells (9–11, 33). IL-12/Stat4 Th1 signals inhibit TGF-β from inducing Treg, through the indirect effects of IFN-γ/T-bet (34) and the direct effects of Stat4 binding to the Foxp3 locus (35). It has not been fully determined whether and how IL-12/Stat4 signals affect the balance of Th17 and Treg and autoimmune pathogenesis.

To dissect the precise role of IL-12/Stat4 in Th1, Th17, Treg development, and autoimmune colitis, we generated Stat4/Stat6/T-bet triple-knockout (TKO) mice. Our data revealed that neither Stat4 nor T-bet is necessary for IFN-γ production and Th1 development in an adoptive-transfer model of colitis. However, Stat4 is essential for CD4 T cells to be pathogenic. Without Stat4, Th17 development decreased, whereas Treg development increased. Our data indicated a critical role for Stat4 in skewing Th17 and Treg responses and autoimmune disease.

Materials and Methods

Mice and reagents

Stat4/Stat6/T-bet TKO mice were generated from Stat4-deficient (BALB/c background, The Jackson Laboratory, Bar Harbor, ME) and Stat6/T-bet double-knockout (DKO) (BALB/c background) (11) mice. C.B-17 SCID mice were purchased from Taconic Farms (Hudson, NY). Recombinant mouse IL-2, IL-4, IL-6, IL-12, IFN-γ, PE–anti–IL-17A, allophycocyanin–anti-CD11c, and PE–anti-CD103 mAbs were purchased from BD Pharmingen (San Diego, CA). rTGF-β was purchased from R&D Systems (Minneapolis, MN). Recombinant mouse IL-23, IL-27, anti-CD3ε, anti-CD28, anti–IFN-γ, anti–IL-12, allophycocyanin–anti-CD4, PE–anti-CD4, FITC–anti-CD45RB, PE–anti–IL-13, FITC–anti–IL-10, PE–anti–IL-4, PE–anti–IFN-γ, FITC–anti–IFN-γ, FITC–anti-Foxp3 mAbs, intracellular staining kit, monesin solution, and cell proliferation dye eFluor 670 were purchased from eBioscience (San Diego, CA). PMA and ionomycin were purchased from Sigma-Aldrich (St. Louis, MO).

T cell purification, activation, intracellular staining, and ELISA

$CD4^+CD25^-$ T cells were selected by flow cytometric sorting and seeded in 96-well round-bottom plates (5×10^4 cells/well). Cells were stimulated with plate-bound anti-CD3ε (5 μg/ml) and soluble anti-CD28 (1 μg/ml), along with various cytokines. After 3 d of culture, ELISA for IFN-γ was performed using the mouse IFN-γ ELISA Kit (eBioscience). Intracellular staining for Foxp3 expression was performed following the manufacturer's protocol (eBioscience). Intracellular cytokine staining was performed after restimulation

with PMA (100 ng/ml) and ionomycin (500 ng/ml) in the presence of monesin (1/1000 dilution) for 4 h. Analytical flow cytometry was performed with a FACSCanto (BD Biosciences).

Induction of colitis in SCID mice by adoptive cell transfer

CD4 T cells were enriched from spleens using a CD4 negative isolation kit (Invitrogen, Carlsbad, CA) and labeled with allophycocyanin–anti-CD4, PE–anti-CD25, and FITC–anti-CD45RB mAbs. $CD4^+CD25^-CD45RB^{high}$ cells were sorted by MoFlo (Dako Cytomation, Carpinteria, CA). A total of 4×10^5 cells in 200 μl PBS was injected i.p. into each recipient.

Histology and fluorescent immunohistochemistry

Colons were cut into small portions, fixed in 10% formalin in PBS (Fisher Scientific, Pittsburgh, PA), embedded in paraffin wax, and stained with H&E. Histologic sections were examined and scored using the following scale: grade 0, no evidence of inflammation; grade 1, low level of leukocyte infiltration, minimal structural changes; grade 2, moderate leukocyte infiltration, crypt elongation, bowel wall thickening but not extending beyond the mucosal layer; grade 3, high level of leukocyte infiltration, infiltration beyond the mucosal layer, and superficial ulcerations; and grade 4, marked degree of transmural leukocyte infiltration, elongated and distorted crypts, and extensive ulcerations. Frozen tissue sections of 7–8 μm thickness from colon, mesenteric lymph node (MLN), or thymus were collected onto glass slides in a cryostat. The slides were air dried, fixed in acetone at −20°C, blocked with 5% normal goat serum, and stained with primary Abs: rat anti-mouse CD4, Armenian hamster anti-mouse CD11c (BD Pharmingen), or rat anti-mouse Foxp3 (eBioscience), followed by fluorophore-conjugated secondary Abs (Cy3-goat anti-rat IgG, Cy5-goat anti-Armenian hamster IgG) (Jackson ImmunoResearch Laboratories, West Grove, PA) in the dark at room temperature. For additional direct immunofluorescence staining, the slides were further blocked with 20% normal rat serum and stained with FITC-rat anti-mouse CD68 (AbD Serotec, Raleigh, NC) or allophycocyanin-rat anti-mouse CD4 (eBioscience). The slides were mounted with Fluoro Gel II with DAPI (Electron Microscopy Sciences, Hatfield, PA), and coverslips were applied. Images were acquired by a Leica DMRA2 fluorescence microscope with a Hamamatsu digital charge-coupled device camera and analyzed by Volocity software.

T cell proliferation and migration assay

CD4 T cells were enriched and labeled with PE–anti-CD4, FITC–anti-CD45RB, and cell-proliferation dye eFluor 670. eFluor 670-labeled $CD4^+CD45RB^{high}$ cells were sorted and injected i.p. into each SCID mouse. Proliferation analysis was performed on gated CD4 cells from MLN, spleen, and thymus by flow cytometry 5 or 15 d later. Frozen tissue sections were viewed under a fluorescence microscope using a Cy5 filter set for eFluor 670.

Quantitative real-time RT-PCR

Total RNA was isolated using an RNeasy Protect Mini Kit (Qiagen, Valencia, CA) and treated with DNAse I (Invitrogen), and cDNA was reverse transcribed using an Omniscript RT kit (Qiagen) with random primers. The primers for PCR were: *cyclophilin A*: 5′-AGG GTG GTG ACT TTA CAC GC-3′ and 5′-ATC CAG CCA TTC AGT CTT GG-3′; *c-Maf*: 5′-AGC AGT TGG TGA CCATGT CG-3′ and 5′-TGG AGATCT CCT GCT TGA GG-3′; *IL-10*: 5′-AAC TGC ACC CAC TTC CCA GTC-3′ and 5′-CAT TAA GGA GTC GGT TAG CAG-3′; *IL-4*: 5′-GAA GCC CTA CAG ACG AGC TCA-3′ and 5′-ACA GGA GAA GGG ACG CCAT-3′; *IFN-γ*: 5′-TGG CTC TGC AGG ATT TTC ATG-3′ and 5′-TCA AGT GGC ATA GAT GTG GAA GAA-3′; *IL-17A*: 5′-CTC CAG AAG GCC CTC AGA CTA-3′ and 5′-AGC TTT CCC TCC GCA TTG ACA-3′; and *Eomes*: 5′-TAC GGC CAG GGT TCT

CCG CTC TAC-3′ and 5′-GGG CCG GTT GCA CAG GTA GAC GTG-3′. Each reaction was performed with the LightCycler system (Roche, Indianapolis, IN) and the SYBR Green PCR kit (Qiagen). All experiments were performed at least three times. Amplification of the *cyclophilin A* gene was used as a housekeeping gene.

Coculture of CD4 T cells and CD11c^{high} subsets

Wild-type MLN dendritic cells (DCs) were first enriched using pan T (Thy1.2) and pan B (B220) Dynabeads (Invitrogen) to deplete Tand B cells. CD103^{-} or CD103^{+} CD11c^{high} DCs were then sorted by a MoFlo sorter gated on CD3^{-} cells. A total of 1×10^4 CD103^{-} or CD103^{+} MLN CD11c^{high} DCs and 5×10^4 CD4^{+}CD25^{-} T cells from Stat6/T-bet DKO or Stat4/Stat6/T-bet TKO mice were seeded in 96-well round-bottom plates and cultured in the presence of anti-CD3 mAb (1 μg/ml) and IL-2 (1 ng/ml). On day 3, intracellular staining for Foxp3, IL-17, and IFN-γ expression was performed and analyzed on gated CD4 cells. ELISA for IL-12 (p70) and IL-27 was performed using the mouse IL-12 p70 Platinum and IL-27 ELISA Kits (eBioscience).

Statistical analysis

Statistical significances between groups were determined by the Student t test; $p < 0.05$ was considered to indicate a significant difference.

Results

Stat4/Stat6/T-bet TKO model for dissecting the role of IL-12/Stat4 in Th cell development

Our recent studies showed that adding Stat6 deficiency to T-bet deficiency to limit interference from elevated levels of IL-4/Stat6/GATA-3 Th2-polarizing signals restored the IL-12/Stat4 Th1-development pathway (11). To further determine the precise role of IL-12 signals in IFN-γ production and Th1 development, we added Stat4 deficiency to Stat6/T-bet DKO and generated Stat4/Stat6/T-bet TKO mice. Stat4/Stat6/T-bet TKO mice are healthy, and the distribution of CD4 and CD8 T cells in thymus, spleen, and lymph nodes, as well as CD4 T cell surface markers CD62L, CD25, CD44, CD45RB, and CD69, were comparable to wild-type mice (data not shown). CD4^{+}CD25^{-} T cells from wild-type, Stat6/T-bet DKO, and Stat4/Stat6/T-bet TKO mice were purified and stimulated with plate-bound anti-CD3 plus soluble anti-CD28 mAbs, with or without IL-12 or IL-4. After 3 d of culture, intracellular staining was performed to measure IFN-γ (Th1) or IL-13 (Th2) production. As shown in Fig. 1*A*, addition of Stat4 deficiency abolished IL-12–mediated IFN-γ production. Similar results were confirmed by ELISA and quantitative RT-PCR (Fig. 1*B*). There was very limited IL-13 expression after IL-4 stimulation in DKO or TKO cells (Fig. 1*C*), indicating that even relieving Stat4 and T-bet Th1-inhibitory signals did not result in Th2 development, demonstrating the essential role of IL-4/Stat6 in Th2 development. We next examined whether Stat4 deficiency affected Th17 development. As shown in Fig. 1*D*, IL-6 alone induced similar IL-17 production in DKO and TKO cells. Similar IL-10 expression was observed in IL-6/TGF-β DKO or TKO treated cells, demonstrating that addition of Stat4 deficiency did not alter Th17 development or their capacity for IL-10 expression. These data indicated that Stat4/Stat6/T-bet TKO cells provided a viable model for dissecting the roles of IL-12/Stat4 in Th1, Th2, and Th17 effector development and inflammatory responses.

Stat4 is essential for colitis induction

We next examined the role of IL-12/Stat4 signals in autoimmune inflammatory disease pathogenesis in vivo. We chose the adoptive-transfer colitis model, in which CD4^{+}CD25^{-}CD45RB^{high} cells were transferred into SCID mice, thus focusing on naive

CD4 T cells and eliminating the potential confounding effects of Stat4, Stat6, or T-bet deficiency on APCs, and the pathological features of this model resemble those observed in human Crohn's disease. We first examined how Stat4 deficiency affected colitis development. Wild-type, DKO, and TKO $CD4^+CD25^-CD45RB^{high}$ T cells were purified and transferred into SCID mice, and body weights were monitored. As shown in Fig. 2*A*, recipients of wild-type and DKO CD4 T cells had progressive weight loss over 8 wk, whereas Stat4 deficiency in TKO T cells abolished wasting disease. Histologic analysis (Fig. 2*B*) showed that Stat4 deficiency resulted in diminished colonic inflammation. Immunohistochemistry for CD4, CD11c, and CD68 (Fig. 2*C*) showed decreased numbers of CD4 T cells, DCs, and macrophages in the recipients of TKO cells. No colitis was observed in mice receiving TKO cells for up to 12 wk (Fig. 2*A*–*C*), indicating that failure of colitis development was probably not due to a delayed response. These data indicated that Stat4 signals are critical for determining whether CD4 T cells are pathologically active.

Neither Stat4 nor T-bet is required for Th1 development in vivo

To determine whether Stat4 deficiency affected CD4 T cell distribution, migration, or proliferation in vivo, which consequently resulted in their different pathogenic capacities, eFluor 670-labeled DKO or TKO $CD4^+CD45RB^{high}$ cells were sorted and transferred into SCID mice. Five or 15 d later, flow cytometry was performed on CD4 T cells from MLN, spleen, and thymus. As shown in Fig. 3*A*, there were no differences in CD4 expression or proliferation between mice receiving DKO and TKO cells. DKO and TKO cells were equally distributed in MLN, spleen, or thymus (Fig. 3*B*). These results showed that Stat4 deficiency did not affect migratory or proliferative properties of TKO CD4 T cells.

We next determined whether failure of colitis induction by TKO CD4 T cells was due to diminished IFN-γ production and Th1 development. MLN CD4 T cells were purified, and IFN-γ expression was examined by quantitative RT-PCR. Surprisingly, there was similar IFN-γ expression by recipients of TKO or DKO cells (Fig. 3*C*), indicating that abolishing IL-12/Stat4 signals did not affect IFN-γ production in vivo. We and other investigators showed that Eomes plays a complementary role for T-bet in IFN-γ regulation (11, 36–39). Eomes expression was examined in MLN CD4 T cells (Fig. 3*D*), and significantly higher levels were observed in recipients of DKO or TKO cells compared with wild-type cells, supporting a role for Eomes in IFN-γ production and Th1 development. Overall, the results showed equal IFN-γ and Th1 in DKO and TKO cells; therefore, failure to induce colitis was not due to changes in Th1 development.

Stat4 is required for optimal Th17 response in vivo

Although Fig. 1*D* showed similar IL-17 expression by DKO and TKO cells in vitro, we next determined how Stat4 deficiency affected Th17 development in vivo. Intracellular staining and quantitative RT-PCR of MLN CD4 T cells (Fig. 4*A*, 4*B*) showed significant IL-17 expression in wild-type cells. DKO cells expressed more IL-17, confirming that Stat6/T-bet double deficiency favored Th17 development (11). Stat4 deficiency did not abolish Th17 responses; however, there was 50% less IL-17 in TKO cells compared with DKO cells. This suggested that Stat4 played a positive regulatory role in IL-17 and Th17 responses for colitis induction. There was very little IL-4 expression (Fig. 4*B*) in wild-type, DKO, or TKO T cells, indicating that Stat4 deficiency did not alter IL-4/Th2 responses, and reduced IL-17 expression was not due to suppressive effects from IL-4 Th2 signals.

IL-10 was demonstrated to be critical for suppressing autoimmunity and inflammatory responses (40–43), and whether Th17 express IL-10 is crucial in determining their protective or pathological consequences (44, 45). We examined whether Stat4 affected IL-10 production in vivo. There was similar IL-10 expression in DKO and TKO CD4 T cells, and

no IL-17 cells produced IL-10, indicating that Stat4 deficiency did not alter total IL-10 or the composition of IL-17$^+$IL-10$^+$ nonpathogenic versus IL-17$^+$IL-10$^-$ colitogenic Th17 cells (Fig. 4*A*, 4*B*). c-Maf was recently shown to play an important regulatory role in IL-10 and IL-17 expression (46–48). There was no difference in c-Maf expression between DKO and TKO T cells, indicating that reduced IL-17 expression was not due to altered c-Maf expression (Fig. 4*B*).

IL-27 shares the common gp130 signal receptor chain with IL-6, and similar to IL-6, it activates Stat3 and Stat1. However, IL-27 suppresses Th17 development. We examined whether Stat4 affected the response to IL-27. In vitro stimulation showed that IL-27 suppressed Th17 while promoting IL-10 expression in wild-type, DKO, and TKO CD4$^+$CD25$^-$ T cells, and the responses of DKO and TKO cells were similar to each other (Fig. 4*C*). Thus, Stat4 deficiency did not alter the IL-17 or IL-10 responses to IL-27.

Stat4 deficiency results in higher levels of Foxp3$^+$ Tregs

Foxp3$^+$ Tregs play a critical role in controlling immune responses, and IL-12/Stat4 was implicated in promoting Th1 and suppressing Treg development (34, 35). We next determined whether Stat4 affected the Treg response. Immunohistochemistry was performed on colons and MLNs of mice receiving TKO or DKO cells. There were striking differences in the numbers of CD4$^+$Foxp3$^+$ Tregs in the two groups (Fig. 5*A*). Flow cytometry and quantitative RT-PCR further confirmed greater numbers of Foxp3$^+$ cells and levels of Foxp3 expression in TKO CD4 T cells than in DKO MLN CD4 T cells (Fig. 5*B*), indicating that Stat4 deficiency allowed a more robust Foxp3$^+$ Treg response. Very low levels of Foxp3 were observed in MLNs 2 wk after transfer (Fig. 5*B*), and IL-12Rβ2–deficient CD4$^+$CD25$^+$ Tregs had less proliferation capacity than wild-type Tregs (49), indicating that the high numbers of Foxp3$^+$ cells observed in TKO recipients were probably not due to contamination of Foxp3$^+$ cells during sorting, or TKO cells intrinsically favored Foxp3$^+$ Treg expansion.

IL-12/Stat4 signals alter the balance between Th17 and Treg development

The selective effects of Stat4 in the suppression of Tregs while promoting Th17 in vivo prompted us to examine how IL-12/Stat4 signals affected the balance of Th17 and Treg development. Because IL-12 also influences APC cytokine production, which, in turn, influences induction of Tregs and Th17, we used an APC-free culture system. Because resting CD4 T cells express little IL-12Rβ2, and IFN-γ is critical for IL-12Rβ2 induction (50), it was possible that differential IFN-γ production by various cells could lead to differential IL-12R expression and, thus, cloud the interpretation of direct effects of IL-12/Stat4. Therefore, we also added exogenous IFN-γ to the cultures. As shown in Fig. 6*A*, TGF-β induced very high numbers of Foxp3$^+$ cells in DKO and TKO cells compared with wild-type CD4 T cells. These results confirmed that Th1 and Th2 signals suppress Foxp3 expression and demonstrated that Stat4 did not regulate the intrinsic responses of CD4 T cells to TGF-β for Foxp3 expression. Exogenous IL-12 suppressed Foxp3 expression in wild-type (31% inhibition) or DKO (17% inhibition) cells but not in TKO (2% inhibition) cells. IL-6 profoundly suppressed Foxp3 expression (Fig. 6*A*) in wild-type (73%), DKO (65%), and TKO (66%) cells, indicating that Stat4 deficiency did not alter the response to IL-6. The combination of IL-12 plus IL-6 resulted in additive suppression of Foxp3 in wild-type (79%) and DKO (70%) cells but not in TKO (67%) cells (Fig. 6*A*), pointing to different mechanisms for Foxp3 suppression by IL-12/Stat4 signals and IL-6 signals. The combination of IL-23 plus IL-6 inhibited Foxp3 in all cells, with DKO and TKO having similar responses (Fig. 6*A*), also indicating that IL-12–mediated Foxp3 suppression was not due to secondary effects of IL-23. Intracellular staining for IL-17 on the same cells showed that IL-12 had little effect on IL-17 expression by IL-6 plus TGF-β or on the ability of IL-23

to promote Th17 development (Fig. 6*B*). Thus, changes in Foxp3 expression were not directly linked to changes in IL-17 expression. Together, these results demonstrated that IL-12/Stat4 Th1 signals specifically suppressed Foxp3.

Stat4 differentially affects Treg and Th17 development by mesenteric DCs

It was demonstrated that two functionally distinct $CD11c^{high}$ DC subsets reside in the MLNs: $CD103^{+}CD11c^{high}$ DCs drive Treg development, whereas $CD103^{-}CD11c^{high}$ DCs promote the inflammatory response (51–53). To further investigate how IL-12/ Stat4 signals affect Treg and Th17 responses, $CD103^{+}$ or $CD103^{-}$ MLN $CD3^{-}B220^{-}NK1.1^{-}CD11c^{high}$ DCs were isolated from wild-type BALB/c mice and cultured with DKO or TKO $CD4^{+}$ $CD25^{-}$ T cells in the presence of anti-CD3 mAb plus IL-2, and intracellular staining for Foxp3, IL-17, and IFN-γ was performed after 5 d. As shown in Fig. 7*A*, $CD103^{+}CD11c^{high}$ DCs induced two to three times more $Foxp3^{+}$ cells than did $CD103^{-}CD11c^{high}$ DCs, confirming that $CD103^{+}CD11c^{high}$ DCs favor Treg development (51–53). In contrast to previous observations with wild-type CD4 T cells (51–53), we also observed that $CD103^{-}CD11c^{high}$ DCs induced some Foxp3 in DKO and TKO cells, supporting observations that limiting Th1 and Th2 signals favored Treg development. Importantly, Stat4 deficiency resulted in higher levels of Foxp3 expression (54% increase) by $CD103^{-}$, but not $CD103^{+}$, $CD11c^{high}$ DCs, suggesting that Stat4 activation by $CD103^{-}CD11c^{high}$ DCs negatively regulates $Foxp3^{+}$ Treg development. $CD103^{-}$, but not $CD103^{+}$, $CD11c^{high}$ DCs induced significant IFN-γ and IL-17 expression in DKO cells, and Stat4 deficiency resulted in reduced levels of IL-17 but not IFN-γ expression (Fig. 7*A*). Thus, Stat4 was not required for IFN-γ and Th1 development, but it played a role in optimal Th17 development driven by inflammatory $CD11c^{high}$ DCs. Measurements of IL-27 showed that differential IL-17 production was not due to differential IL-27 production (Fig. 7*B*). Together, these data demonstrated that Stat4 activation altered the balance between Th17 and Tregs driven by mesenteric $CD11c^{high}$ DCs.

Discussion

IL-12/Stat4 signals have long been associated with Th1 development, inflammatory responses, and autoimmune disease pathogenesis, although their precise roles remain incompletely understood. By generating and studying Stat4/Stat6/T-bet TKO cells, we further dissected IL-12/Stat4 in Th1, Th2, Th17, and Treg responses. We showed that IL-12/ Stat4 did not necessarily play a direct role in Th1/Th2 polarization; rather, it selectively suppressed Tregs, skewing the balance between Th17 and Treg responses and consequently favoring inflammatory colitis development.

IL-12/Stat4–driven IFN-γ participates in the IFN-γ/Stat1/T-bet Th1-developmental signal cascade, whereas T-bet is required for maintaining IL-12Rβ2 expression and IL-12/Stat4 signal transduction (11, 14). This reciprocal interaction complicates the ability to examine the independent roles of these pathways. Our finding that, after eliminating the inhibitory effects from Th2-development signals by Stat6 deficiency, IFN-γ production was observed in cells deficient in T-bet (Stat6/T-bet DKO) or Stat4 and T-bet (Stat4/Stat6/T-bet TKO), indicated that neither Stat4 nor T-bet is necessary for directly regulating IFN-γ expression and Th1 development. Their functions in Th1 development are mainly for amplifying existing IFN-γ expression and for preventing inhibition by Stat6/GATA-3 Th2 signals. IFN-γ production observed in vivo (Fig. 3*C*) but not in vitro (Fig. 1*A*, 1*B*) implied that, in addition to IL-12, other cytokine(s) may regulate IFN-γ expression. Eomes was shown to be involved in IL-21–mediated CD4 IFN-γ regulation (39), and the observation that IFN-γ production corresponded with elevated Eomes expression (Fig. 3*C*), suggests a possible role for IL-21/Eomes in IFN-γ production and Th1 development (11, 39). Because it is too difficult to add Eomes deficiency to Stat4/Stat6/T-bet deficiency, our data do not exclude

the possibility that additional signal pathways may be involved in IFN-γ and Th1 development in vivo. In contrast to Th1 responses, relieving T-bet and Stat4 Th1 signals did not result in significantly higher levels of Th2 development when Stat6 was absent (Figs. 1*C*, 4*B*), indicating a fundamental difference between Th1 and Th2 differentiation; although Th1 responses are driven by multiple pathways, Th2 responses are driven almost exclusively through Stat6.

Stat4 is required for IL-17 production driven by IL-23 plus IL-18 (54), and decreased IL-17 production is observed following *Bacteroides fragilis* infection (55) and during allergic airway inflammation (56) in Stat4-deficient mice. We found that Stat4 deficiency also resulted in reduced IL-17 production in an inflammatory colitis model. Because similar IFN-γ Th1 and IL-4 Th2 responses were observed in DKO and TKO cells (Figs. 3*C*, 4*B*), the reduced IL-17 expression observed in TKO cells was unlikely to be due to secondary effects from Th1 or Th2 signals. Similar to IL-6, IL-27 activates Stat3 and Stat1 (57, 58); however, IL-27 does not induce IL-17 but rather inhibits Th17 development (44, 59, 60). Because IL-27 is produced mostly by APCs, and our model used adoptive transfer of CD4 T cells into the same SCID mice, it is unlikely that reduced IL-17 expression was due to differential IL-27 production by APCs. In addition, similar IL-27 inhibition of IL-17 responses was observed in DKO and TKO cells (Fig. 4*C*), excluding the possibility of differential responses of DKO and TKO to IL-27. IL-12/Stat4 signals were shown to negatively regulate Th17 development (11, 61, 62). However, we showed that when exogenous IFN-γ was provided and T-bet was absent, IL-12 did not inhibit IL-17 production (Fig. 6*B*). These data revealed that, in contrast to IFN-γ or T-bet Th1 signals, IL-12/Stat4 have a limited role in directly suppressing IL-17 expression. Overall, IL-12/Stat4 signals do not directly affect IL-17 expression and Th17 development.

IL-10 plays a critical role in suppressing autoimmunity and inflammatory responses, and its production by Th17 cells restrains the pathologic effects of Th17 (44, 45). Stat4 has been implicated in IL-10 regulation by the Notch pathway (63). Similar IL-10 production was observed in mice receiving Stat6/T-bet DKO and Stat4/Stat6/T-bet TKO cells (Fig. 4*A*, 4*B*), suggesting that Stat4 does not play a significant role in IL-10 regulation during colitis induction. Our results also showed that neither Th17 IL-10 nor IL-27–induced IL-10 played a role in the failure of colitis induction by Stat4 deficiency.

By examining Stat6/T-bet DKO and Stat4/Stat6/T-bet TKO cells, we were able to show direct inhibitory effects of IL-12/Stat4 on Foxp3 expression (Fig. 6*A*). IL-23 activates Stat3 and Stat4 and was shown to skew the balance between Th17 and Treg response (32). Because IL-23 suppressed Foxp3 expression equally efficiently in DKO and TKO cells (Fig. 6*A*), this indicated that IL-23 inhibition was not through Stat4 activation, and the higher levels of Foxp3-expressing cells observed in TKO compared with DKO recipients and cells (Figs. 5, 7) were due to impaired IL-12/Stat4 signals rather than IL-23/Stat4 signals. Therefore, our data showed that, in addition to IL-23/Stat3 signals, IL-12/Stat4 signals skew the balance between Th17 and Tregs. Together, the opposing effects of Th17 and Tregs in autoimmune colitis demonstrated that the balance between Th17 and Tregs is critical in determining the outcome of disease pathogenesis. Because we used cells deficient in multiple transcription factors (Stat4, Stat6, and T-bet), these multiple genetic-deficient cells may have limitations with regard to reflecting or predicting human disease development. Nevertheless, our data showed an important function for IL-12/Stat4 Th1 signals in skewing the balance between Th17 and Tregs by suppressing Tregs, demonstrating the necessity to suppress IL-12/Stat4 Th1 signals for immunotherapy designed to engage the protective functions of Tregs in inflammatory responses.

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Abbreviations used in this article

DC	dendritic cell
DKO	double knockout
MLN	mesenteric lymph node
TKO	triple knockout
Treg	regulatory T cell

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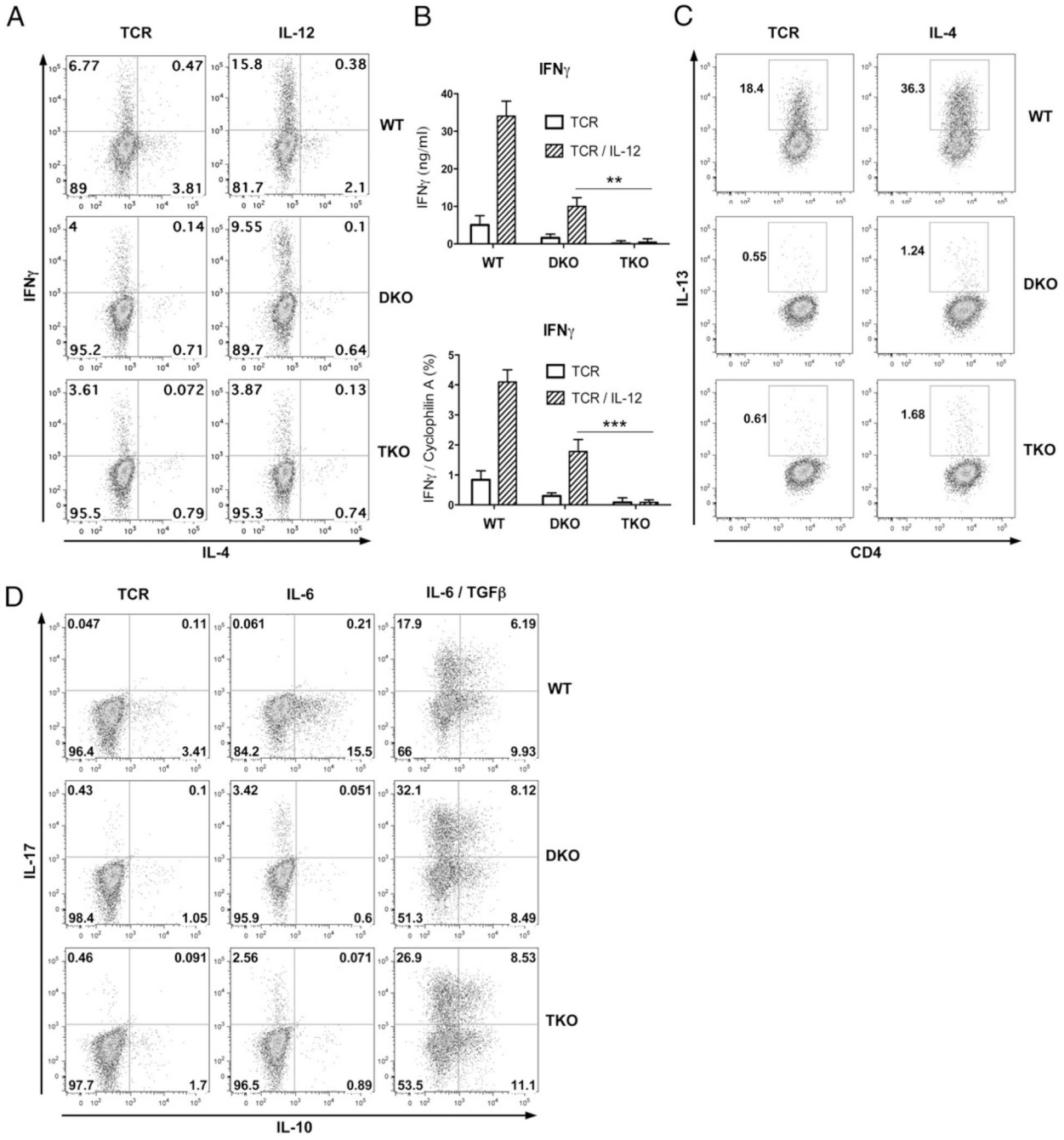

FIGURE 1.

Stat4/Stat6/T-bet TKO have Th17 but not Th1 or Th2 responses. *A* and *B*, Stat4 deficiency abolished IL-12–mediated IFN-γ production. Wild-type, Stat6/T-bet DKO, and Stat4/Stat6/T-bet TKO $CD4^+CD25^-$ T cells were stimulated with plate-bound anti-CD3 (5 μg/ml) and soluble anti-CD28 (1 μg/ml), with or without IL-12 (10 ng/ml). Quantitative RT-PCR for IFN-γ expression was performed on day 2; ELISA for IFN-γ and intracellular staining for IFN-γ and IL-4 were performed on day 3. $^{**}p < 0.01$, $p < 0.001$. *C*, Stat4 deficiency did not alter Th2 differentiation. Intracellular staining for IL-13 in $CD4^+CD25^-$ T cells after anti-CD3 plus anti-CD28 stimulation, with or without IL-4 (20 ng/ml) for 3 d. *D*, Stat4 deficiency did not alter composition of IL-10 expression in Th17 cells. Intracellular IL-10

and IL-17 staining in $CD4^+CD25^-$ T cells after 3 d of stimulation with anti-CD3 and anti-CD28 combined with IL-6 (10 ng/ml) or IL-6 plus TGF-β (5 ng/ml).

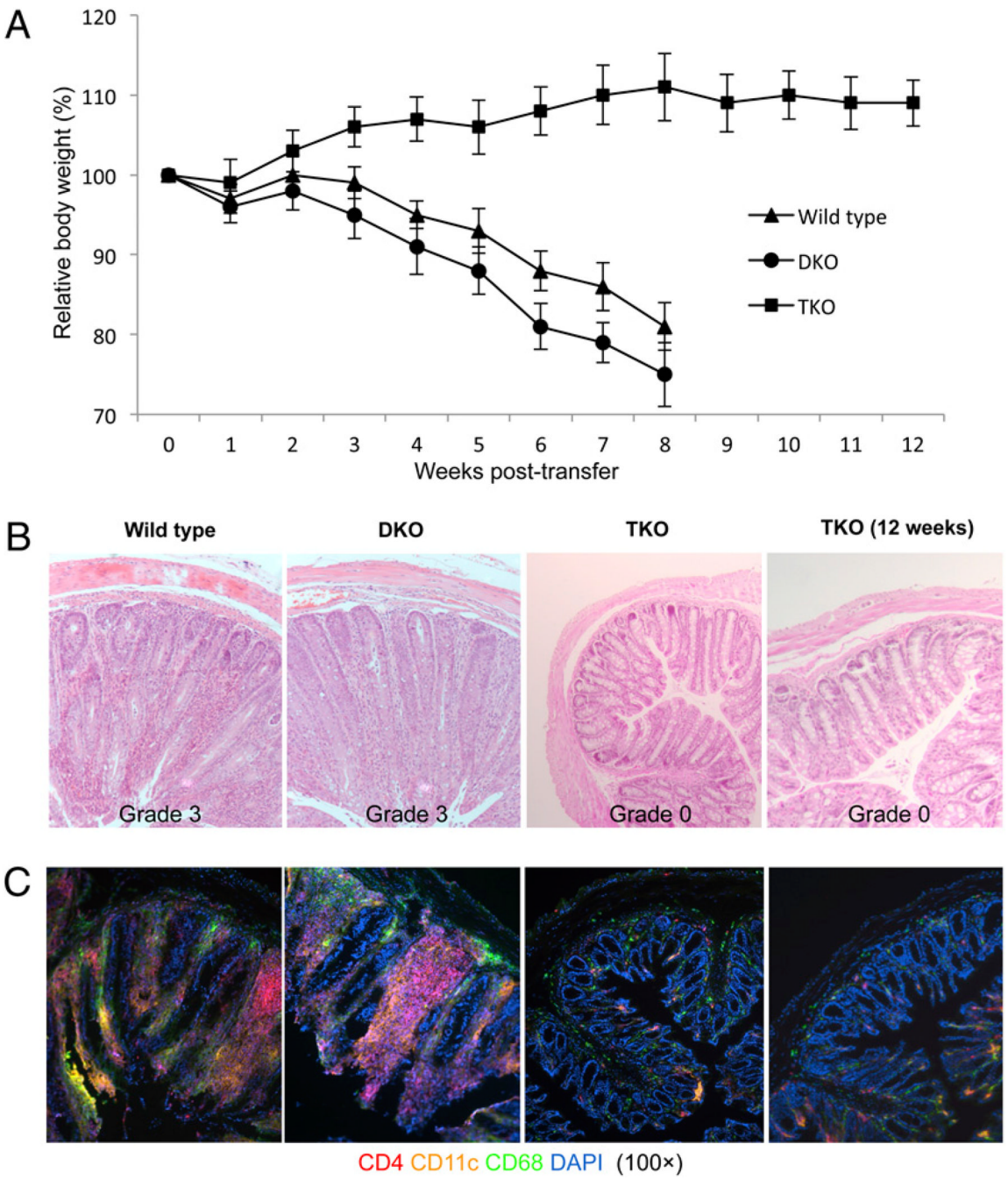

FIGURE 2.

Addition of Stat4 deficiency prevents colitis by Stat6/T-bet DKO cells. *A*, Relative body weight of SCID mice after transfer of 4×10^5 wildtype, DKO, or TKO $CD4^+CD25^-CD45RB^{high}$ cells. H&E staining (*B*) and fluorescent immunohistochemistry (*C*) for CD4, CD11c, and CD68 in the distal colons of SCID mice 8 or 12 wk after T cell reconstitution (original magnification ×100). Representative sections are shown from 10 mice in each group. Histological scoring of colitis was described in *Materials and Methods*.

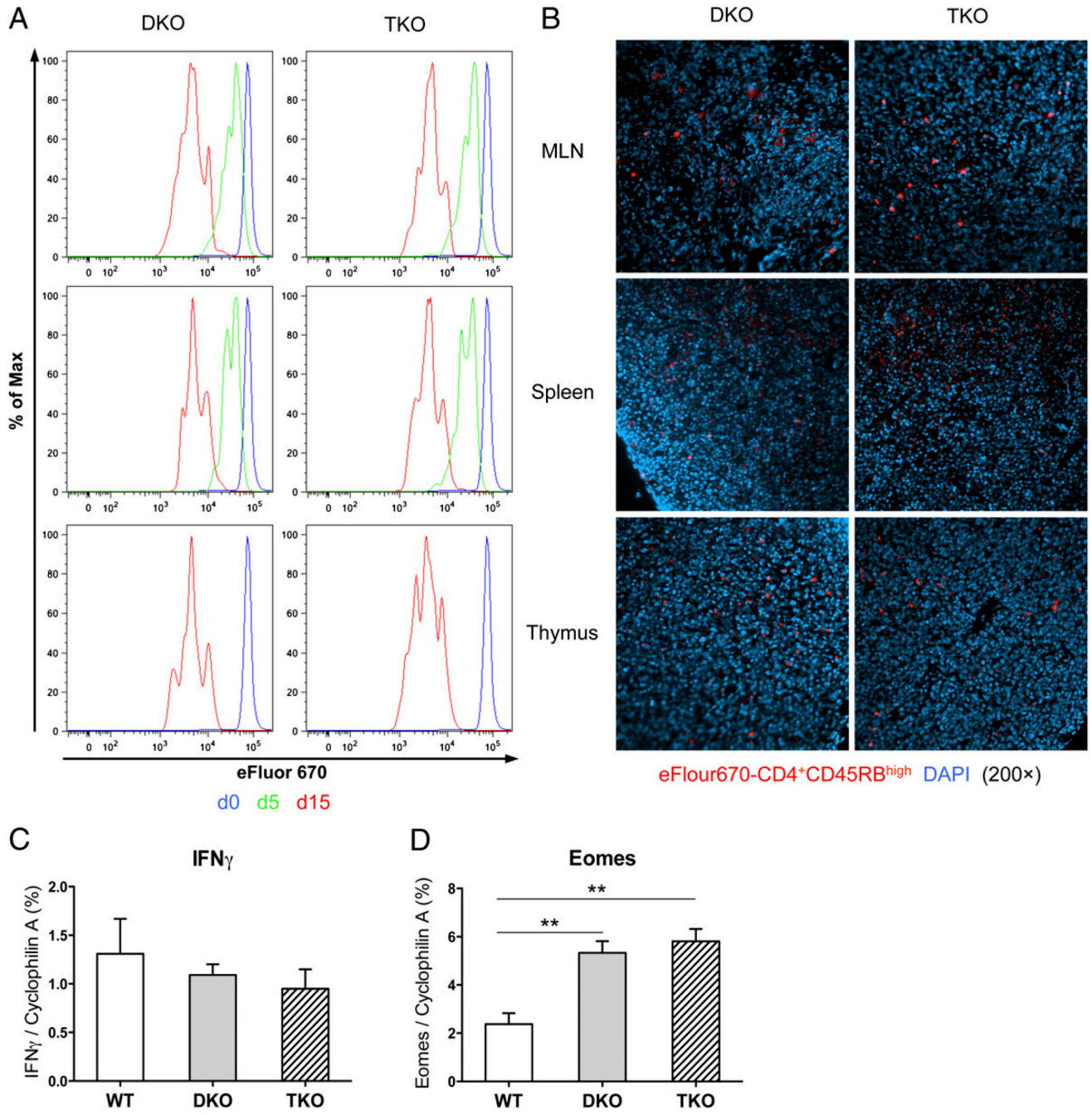

FIGURE 3.

Neither Stat4 nor T-bet is required for Th1 development. *A*, Cell distribution, migration, and proliferation after adoptive transfer. eFluor 670-labeled $CD4^{+}CD45RB^{high}$ cells from DKO or TKO mice were injected into SCID mice and harvested 5 or 15 d later, and proliferation analysis was performed on gated CD4 cells from MLN, spleen, and thymus. *B*, Distribution of eFluor 670-labeled $CD4^{+}CD45RB^{high}$ cells in MLN, spleen and thymus 5 d after T cell reconstitution (original magnification ×200). Representative sections from five mice in each group are shown. *C* and *D*, Quantitative RT-PCR for IFN-γ and Eomes expression in MLN CD4 T cells purified 8 wk after adoptive transfer. $^{**}p < 0.01$.

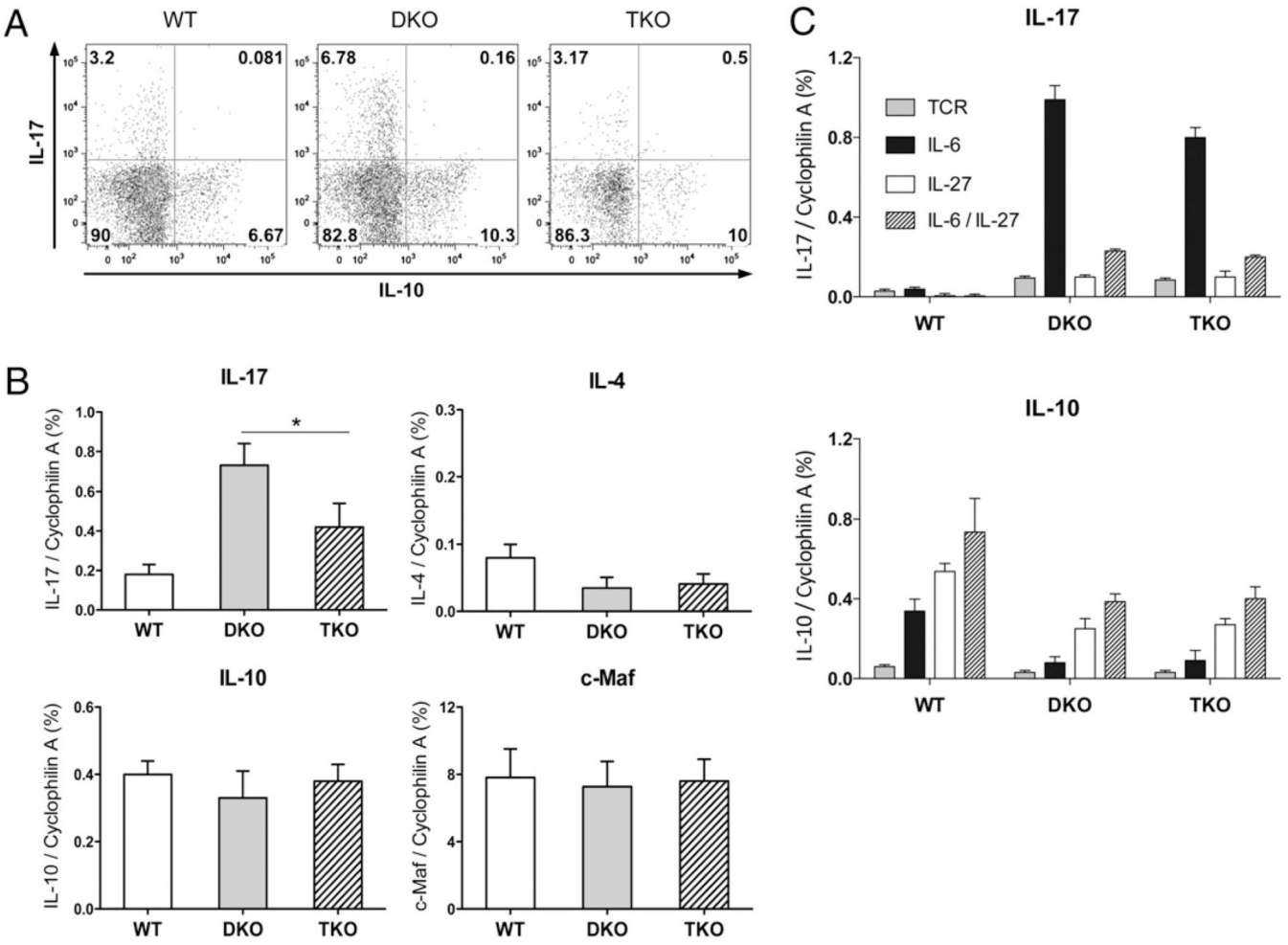

FIGURE 4.

Stat4 is required for optimal IL-17 but not IL-4 or IL-10 expression. *A*, Eight weeks after cell transfer, T cells were isolated from MLN and restimulated with PMA and ionomycin in the presence of monesin for 4 h, and IL-17 and IL-10 expression on gated CD4 cells was measured by intracellular staining. *B*, Real-time RT-PCR for IL-17, IL-4, IL-10, and c-Maf expression in MLN CD4 T cells. *$p < 0.05$. *C*, Real-time RT-PCR for IL-17 and IL-10 expression in wild-type, DKO, and TKO $CD4^+CD25^-$ T cells after 2 d of culture with anti-CD3 and anti-CD28, combined with IL-6, IL-27, or IL-6 plus IL-27.

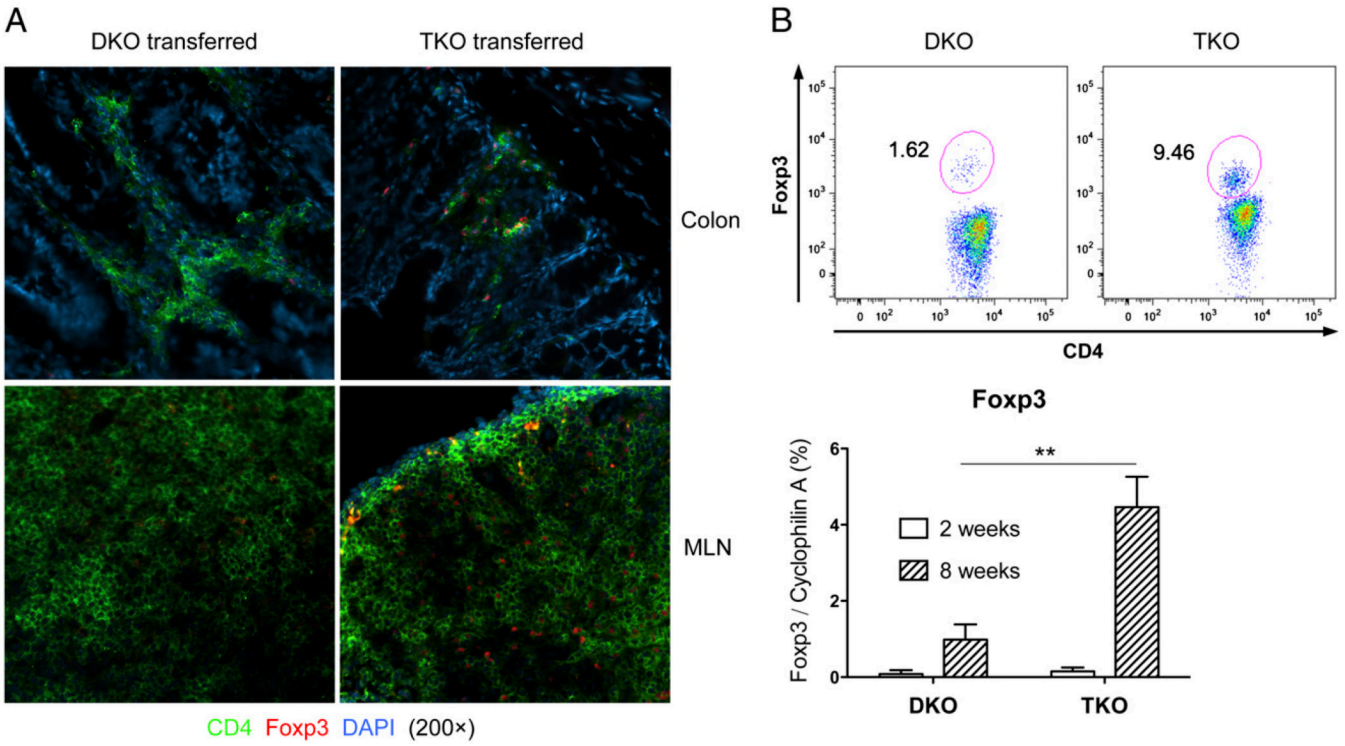

FIGURE 5.

Stat4 deficiency results in higher levels of Foxp3$^+$ Tregs. *A*, Immunohistochemistry for CD4 and Foxp3 in the distal colons and MLN in SCID mice 8 wk after T cell reconstitution (original magnification ×200). Representative sections are shown from 10 mice in each group. *B*, Intracellular staining for Foxp3 on gated MLN CD4 cells 8 wk after adoptive transfer (*upper panels*) and real-time RT-PCR for Foxp3 expression on MLN CD4 T cells 2 or 8 wk after adoptive transfer (*lower panel*). $^{**}p < 0.01$.

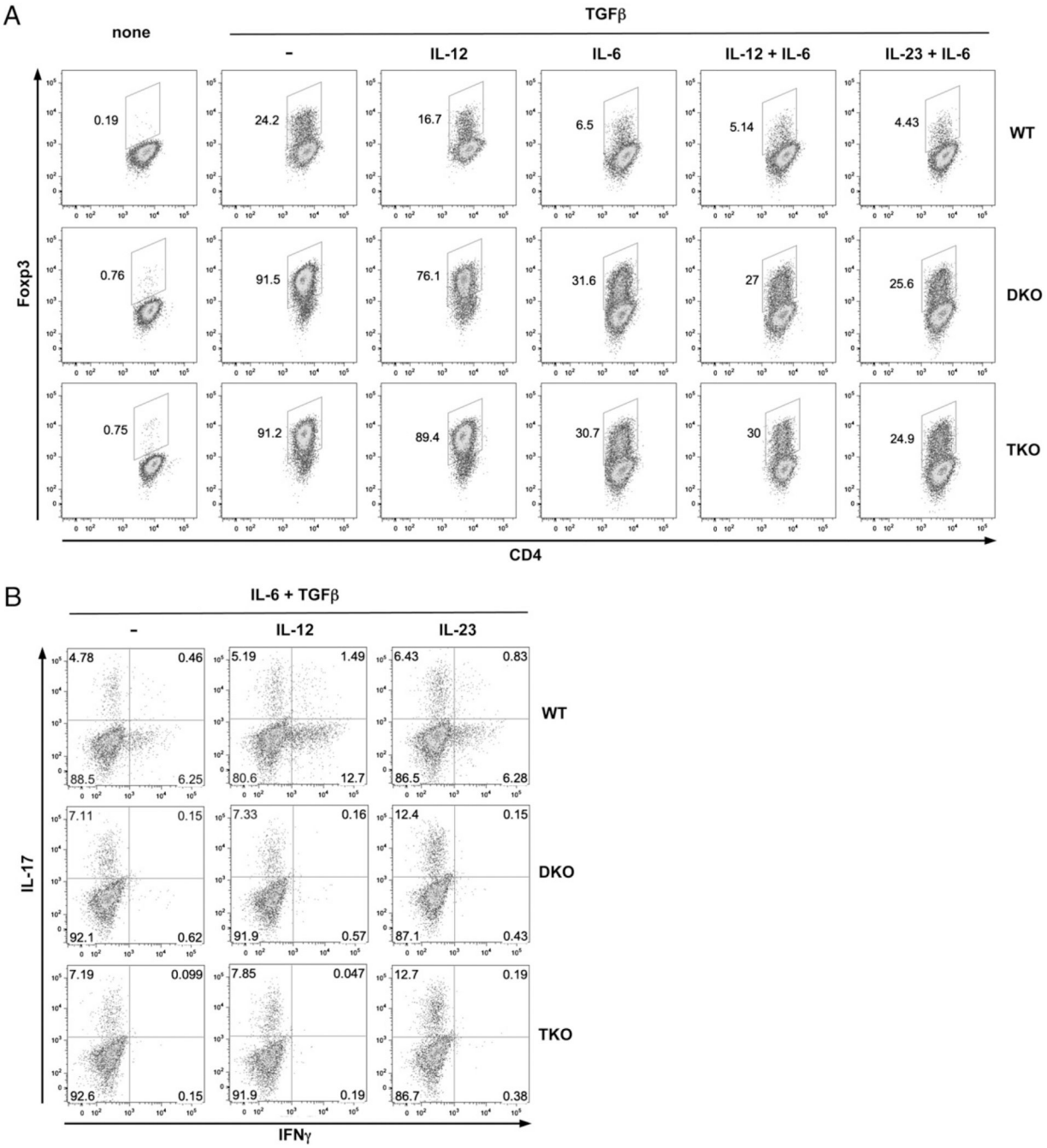

FIGURE 6.

IL-12/Stat4 signals alter the balance between Th17 and Tregs. *A*, IL-12/Stat4 suppresses Foxp3 expression. Wild-type, DKO, and TKO $CD4^{+}CD25^{-}$ T cells were stimulated with anti-CD3 and anti-CD28 in the presence of low-dose IFN-γ (1 ng/ml) for 3 d. Intracellular staining for Foxp3 was performed under Treg conditions (TGF-β), with or without IL-12, IL-6, IL-12 plus IL-6, or IL-23 plus IL-6. *B*, IL-12 does not affect Th17 development. Intracellular IL-17 and IFN-γ expression were measured under Th17 conditions (IL-6 + TGF-β), with or without IL-12 or IL-23. Data are representative of three independent experiments with similar results.

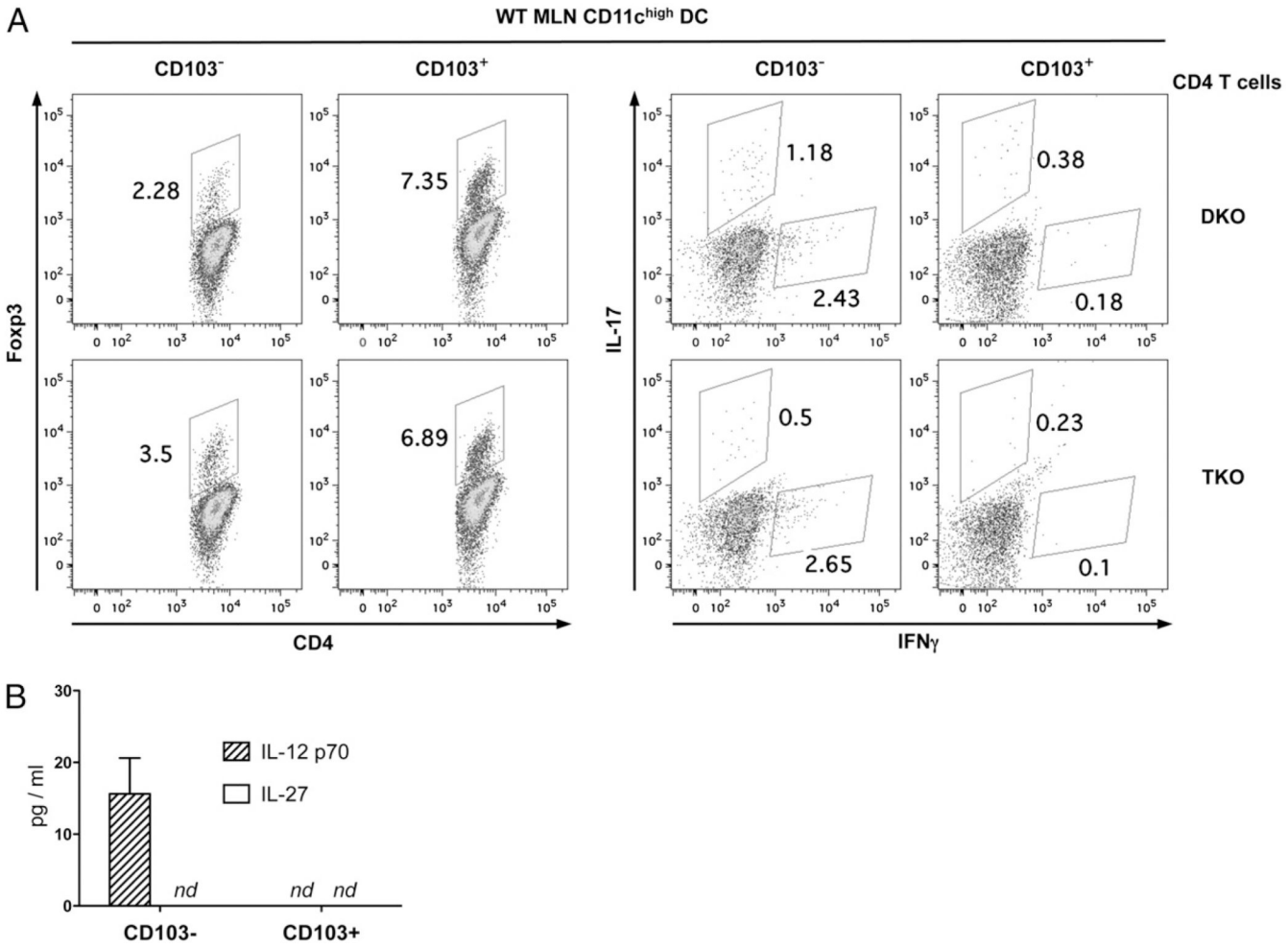

FIGURE 7.

Stat4 inhibits Tregs while promoting Th17 development driven by CD103^{-} CD11c^{high} MLN DCs. CD4^{+}CD25^{-} T cells from DKO and TKO mice were cultured with wild-type CD103^{-} or CD103^{+} MLN CD11c^{high} DCs in the presence of anti-CD3 (1 μg/ml) and IL-2 (1 ng/ml) for 3 d. *A*, Intracellular staining for Foxp3, IL-17, and IFN-γ expression was performed and analyzed on gated CD4 cells. *B*, The amounts of IL-12 p70 and IL-27 in the culture supernatants were measured by ELISA. Data are representative of three independent experiments with similar results. nd, not detected.