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An Inhibitory Role for the Transcription Factor Stat3 in Controlling IL-4 and Bcl6 Expression in Follicular Helper T Cells

Hao Wu,* Lin-Lin Xu,* Paulla Teuscher,* Hong Liu,* Mark H. Kaplan,*† and Alexander L. Dent*

The transcription factor Bcl6 is required for development of follicular helper T (T\textsubscript{FH}) cells. Cytokines that activate Stat3 promote Bcl6 expression and T\textsubscript{FH} cell differentiation. Previous studies with an acute virus infection model showed that T\textsubscript{FH} cell differentiation was decreased but not blocked in the absence of Stat3. In this study, we further analyzed the role of Stat3 in T\textsubscript{FH} cells. In Peyer’s patches, we found that compared with wild-type, Stat3-deficient T\textsubscript{FH} cells developed at a 25% lower rate and expressed increased IFN-\gamma and IL-4. Whereas Peyer’s patch germinal center B cells developed at normal numbers with Stat3-deficient T\textsubscript{FH} cells, IgG1 class switching was greatly increased. Following immunization with sheep RBCs, splenic Stat3-deficient T\textsubscript{FH} cells developed at a slower rate than in control mice, and splenic germinal center B cells were markedly decreased. Stat3-deficient T\textsubscript{FH} cells developed poorly in a competitive bone marrow chimera environment. Under all conditions tested, Stat3-deficient T\textsubscript{FH} cells overexpressed both IL-4 and Bcl6, a pattern specific for the T\textsubscript{FH} cell population. Finally, we found in vitro that repression of IL-4 expression in CD4 T cells by Bcl6 required Stat3 function. Our data indicate that Stat3 can repress the expression of Bcl6 and IL-4 in T\textsubscript{FH} cells, and that Stat3 regulates the ability of Bcl6 to repress target genes. Overall, we conclude that Stat3 is required to fine-tune the expression of multiple key genes in T\textsubscript{FH} cells, and that the specific immune environment determines the function of Stat3 in T\textsubscript{FH} cells. The Journal of Immunology, 2015, 195: 000–000.
due to heightened sensitivity to type I IFN signaling in Stat3-deficient CD4 T cells (18). Additionally, Stat3-deficient CD4 T cells responding to LCMV developed a strong Th1 phenotype (18), indicating that Stat3 has an important role in repressing Th1 cell development. However, a major question not addressed by these studies was whether the findings observed for Stat3 and T <sub>FH</sub> cells in an LCMV infection model were broadly applicable to the role of Stat3 in T <sub>FH</sub> cell responses in other immune contexts. We therefore used CD4<sup>-</sup>cre Stat3<sup>fl/fl</sup> conditional KO mice to examine T <sub>FH</sub> cell differentiation in the gut immune response, in Peyer’s patches (PPs), as well as with immunization with a strong inducer of the GC response, sheep RBCs (SRBCs). Our data contrast with the Ray et al. (18) study on Stat3 in T <sub>FH</sub> cells on the control of Bcl6 and IL-4 expression by Stat3, and thus provide important new insights into the role of Stat3 in B<sub>2</sub>2c expression and T <sub>FH</sub> cell differentiation.

Materials and Methods

Mice and immunizations

Stat3<sup>fl/fl</sup> mice (18) were backcrossed to CD4<sup>-</sup>cre transgenic mice and the C57BL/6 strain for at least six generations (32). TCR-transgenic OT-II mice and B6.JL-<i>Pr<sub>p</sub>rc</i> <i>Pep<sub>c</sub>Bo</i>Y<sub>j</sub> (BoyJ) mice were purchased from The Jackson Laboratory. Control mice for CD4<sup>-</sup>cre Stat3<sup>KO</sup> mice were littermate Stat3<sup>fl/fl</sup> (wild-type [WT]) mice. Mice were bred under specific pathogen-free conditions at the laboratory animal facility at the Indiana University School of Medicine and were handled according to protocols approved by the Indiana University School of Medicine Animal Use and Care Committee. For SRBC immunization, mice were i.p. injected with 1 <times> 10<sup>9</sup> SRBCs (Rockland Immunochemicals) and were sacrificed at the indicated day. For OVA (Sigma-Aldrich) immunization, 50 µg OVA was mixed with Injel Act (Pierce) and the mixture was injected i.p.

Cell culture and retrovirus transduction

CD4<sup>+</sup>CD62L<sup>+</sup> WT and STAT3KO OT-II T cells were isolated with the CD4<sup>+</sup>CD62L<sup>+</sup> T cell isolation kit II (Miltenyi Biotec) and then were activated with 1 µg/ml OVA peptide (Anaspec) and spleen-derived APCs for 3 d (one round) or 5 additional days (two rounds) under Th2 culture conditions: 40 ng/ml IL-4 (PeproTech), 10 µg/ml anti-IFN-γ and anti-IL-12 (Bio X Cell). After 3 or 8 d of culture, cells were collected for intracellular cytokine staining (ICS) as described below. For retroviral transduction, CD4<sup>+</sup>CD62L<sup>+</sup> WT and STAT3KO T cells were activated with 1 µg/ml anti-CD3, 2 µg/ml anti-CD28, and splenic APCs under the Th2 condition. At 40 h, cells were spin infected with control H2K<sup>k</sup>, Bcl6–H2K<sup>k</sup>– expressing retroviruses (RVs). After spin infection, supernatants were removed and fresh Th2 condition medium with 10 U/ml recombinant human IL-2 was added back to cell culture. Three days later, cells were collected for ICS as described below. Cytokine levels of H2K<sup>k</sup><sup>+</sup> cells were assessed using flow cytometry.

Flow cytometry reagents

Anti-TCR<sub>α</sub>CR5 (2G8), anti-CD95 (Jo2), GL7 (GL7), anti-active caspase-3 (C92-605), anti-IL-4 (11B11), and anti-Bcl6 (K112-91) Abs were from BD Biosciences. Fixable viability dye, annexinV detection kit, and anti-CD38, anti-IL-10 (eBioscience), anti-IL-21 (mAb21), anti-IgG1 (RMG1-1), anti-CD45.1 (A20), anti–PD-1 (C92-605), anti–IL-4 (11B11), and anti-Bcl6 (K112-91) Abs were from BioLegend.

Cell staining for flow cytometry

After RBC lysis, total spleen cells were incubated with anti-mouse CD16/CD32 (Bio X Cell) for 5 min at room temperature, followed by surface staining for the indicated markers. For intracellular transcription factor staining, after surface markers were stained, cells were fixed and stained with Abs against transcription factors by following Foxp3 fixation kit (eBioscience) instructions. Cell events were collected on an LSR II flow cytometer (Becton Dickinson).

Intracellular cytokine staining

Cells were stimulated with PMA (75 ng/ml) plus ionomycin (1 µg/ml) for 5 h in DMEM cell culture medium, then fixed and stained for indicated cytokines as described (33). GolgiStop and GolgiPlug (BD Biosciences) were used during the stimulation to inhibit cytokine secretion.

Adaptive cell transfers and immunization

CD4<sup>+</sup>CD62L<sup>+</sup> MACS-purified WT and STAT3KO OT-II T cells were labeled with 5 µM CellTrace Violet (Life Technologies). Cells (5 <times> 10<sup>5</sup>) were injected i.v. per BoyJ recipient mice. After 18 h, 50 µg OVA (Sigma-Aldrich) mixed with Injel Act (Pierce) was injected i.p. into recipient BoyJ mice. CD45<sup>+</sup> OT-II cells from spleen were analyzed by flow cytometry for CXCRC5, PD-1, Bcl6, Tbet, and Gata3.

PP isolation

PPs were cut from the small intestine using scissors and then incubated for 10 min at 37°C in PBS containing 1% FBS, 4 mM EDTA, and 15 mM HEPES (PH 7.2). PPs were washed twice with vigorous vortexing before spinning, in PBS. Soluble cell debris in supernatants was removed after centrifugation. Isolated PPs were broken apart between two frosted glass microscope slides to generate a single-cell suspension for flow cytometry staining or ICS described above.

Bone marrow chimeras

Recipient CD45<sup>+</sup> BoyJ mice were lethally irradiated with 1100 rad and reconstituted with 2 <times> 10<sup>6</sup> cells from a mixture of nucleated bone marrow (BM) cells from either CD45<sup>+</sup> WT mice plus CD45<sup>+</sup> BoyJ mice or from CD45<sup>+</sup> STAT3KO mice plus CD45<sup>+</sup> BoyJ mice by i.v. injection. Thus, WT chimeras were used as a control to compare the repopulation of Stat3<sup>−/−</sup> BM with the repopulation by STAT3KO CD45<sup>+</sup> BM. This system has been described previously (9). Chimeric mice were immunized with SRBCs ∼12 wk after reconstitution.

Ab titer analysis

Ab titers (SRBC specific) in serum were measured by ELISA, as previously reported (33). Briefly, 96-well Nunc-Immu plates (Sigma-Aldrich) were coated with SRBC membrane protein overnight at 4°C. Wells were blocked with 10% FCS and diluted serum was added and incubated at room temperature for 2 h. A peroxidase-conjugated anti-mouse IgG1 (BD Biosciences), anti-mouse IgG, or anti-mouse IgM Abs (Sigma-Aldrich) were used as secondary Ab.

Statistical analysis

All data analysis was done using GraphPad Prism software. Unless otherwise stated, a Student t test or ANOVA with a Tukey post hoc analysis were used. Only significant differences (p < 0.05) are indicated in figures.

Results

T<sub>FH</sub> cell development in the PP can occur independent of Stat3, and Stat3 regulates the cytokine response of T<sub>FH</sub> cells

To analyze the role of Stat3 in T<sub>FH</sub> cell development, we obtained conditional KO mice in which Stat3 is deleted from T cells via a CD4<sup>-</sup>cre transgene (CD4<sup>-</sup>cre Stat3<sup>fl/fl</sup> mice, termed STAT3KO mice) (32). Initially, we analyzed the T<sub>FH</sub> cell and GCB cell responses that occur in the intestinal PPs to determine whether Stat3 in T cells was essential for normal homeostatic T<sub>FH</sub> cell responses and the related GCB cell responses in the PP (Fig. 1). The percentage and overall numbers of CD4<sup>+</sup> T cells in PPs were decreased significantly in the STAT3KO mice (Fig. 1B, 1C). As shown in Fig. 1D, the loss of Stat3 mildly inhibited the proportion of T<sub>FH</sub> cells developing from the CD4<sup>+</sup> T cell compartment in the PP (Fig. 1D), and the absolute number of PP T<sub>FH</sub> cells in STAT3KO mice was about one third of the number in WT PP (Fig. 1E). However, the loss of Stat3 in T cells did not significantly affect the development of PP GCB cells (Fig. 1G). Loss of Stat3 in T<sub>FH</sub> cells did affect the rate of GCB cells in the PP switching to IgG1, as dramatically increased percentages of IgG1<sup>+</sup> GCB cells were seen in the PP of STAT3KO mice (Fig. 1I–K). This result was confirmed by ELISPOT assays showing significantly increased numbers of IgG1-producing cells in the PP of STAT3KO mice (Fig. 1L). IgA-producing plasma cells were not altered in the STAT3KO PPs, although there was a trend toward decreased levels of fecal IgA in STAT3KO mice (data not shown).

We next wondered what could account for the increased percentages of IgG1<sup>+</sup> GCB cells in STAT3KO mice and decided to...
examine cytokine production by PP T_{FH} cells. As shown in Fig. 2A and 2B, PD-1^hiBcl6^hi T cells (TFH cells) from STAT3KO mice PPs produce markedly higher percentages of IFN-γ^+ and IL-4^+ cells as detected by ICS. Representative ICS cytokine flow plots are shown in Supplemental Fig. 1. As expected for loss of Stat3 activity, IL-21 was significantly lower in STAT3KO PP TFH cells (Fig. 2B). Non–TFH cells produced much lower levels of cytokines (Fig. 2C), and STAT3KO PP non–TFH cells expressed more IFN-γ and IL-10 and less IL-21. However, IL-4 was not increased in the STAT3KO non–TFH cells (Fig. 2C), indicating a unique regulation of IL-4 in TFH cells. Notably, whereas WT PP TFH cells had almost no double IFN-γ^+/IL-4^+ cells or double IFN-γ^+/IL-10-expressing cells, the STAT3KO PP TFH cells had far higher levels of these double cytokine-expressing cells (Fig. 2D, 2E). These results indicate that Stat3 regulates expression of cytokines by TFH cells and inhibits dual expression of cytokines. To further confirm these results and gain insight into the mechanism for increased IL-4 expression, we obtained WT and STAT3KO PP TFH cells by FACS, made RNA and cDNA directly from the purified cells, and tested gene expression by quantitative PCR. We found as expected, that in contrast to non–TFH cells, TFH cells constitutively express Il4 (Fig. 3A, 3B). We also observed significant increases in the levels of Bcl6 and Prdm1 (Blimp1) in the STAT3KO PP TFH cells (Fig. 3C, D). The gene encoding Tbet, Tbx21, was also elevated in TFH cells and non–TFH cells in the STAT3KO PPs (Supplemental Fig. 2A).

**Stat3 is required for acute TFH cell development in the spleen and represses IL-4 expression by TFH Cells**

To test whether our results from analyzing Stat3 function in PP TFH cells were applicable to other TFH cell responses, we immunized mice with SRBCs, a potent inducer of GCB cell responses and TFH cells. Similar to CD4 T cells in the PP, the total number of CD4 T cells was also decreased in the spleen of STAT3KO mice (data not shown). As shown in Fig. 4, Stat3 was required for development of a normal TFH cell response in the spleen, particularly in the early stage of the reaction, analogous to what was previously reported in LCMV infection (17). Thus, at 3 d post-immunization, TFH cells in STAT3KO spleens were 10-fold lower than in the WT (Fig. 4A–C), but by day 7, the response of the Stat3-deficient TFH cell response increased such that it trended lower but was not significantly different (Fig. 4D–F). We also analyzed Bcl6 expression by intracellular staining and found that as in the PP TFH cells, loss of Stat3 led to significantly higher Bcl6 expression.
specifically in T_{FH} cells (Fig. 4G, 4H). However, in contrast to the PP, the loss of Stat3 strongly impacted GCB cell and GCB IgG1+ cell development in the spleen: both responses were impaired at the early time point and never developed above the WT level in the STAT3KO mice (Fig. 5A–D). Consistent with a decreased GCB cell reaction, anti-SRBC IgG titers were several fold lower in the STAT3KO mice, whereas anti-SRBC IgM levels were not affected (Fig. 5E–G). We next examined cytokine production by splenic STAT3KO T_{FH} cells after SRBC immunization, and, compared with WT T_{FH} cells, we found increased IL-4 and decreased IL-21, consistent with the PP T_{FH} cells. However, in contrast to the PP T_{FH} cells, IFN-γ was decreased in STAT3KO T_{FH} cells (Fig. 6A). As in PPs, the increase in IL-4 was specific to the STAT3KO T_{FH} cells, and STAT3KO non–T_{FH} cells showed an

**FIGURE 2.** Deregulated cytokine production in Stat3-deficient T_{FH} cells in the PP. (A) PD-1_{hi} and PD-1_{int} CD4+ cell gates and increased Bcl6 mean fluorescent intensity (MFI) on the PD1_{hi} population. Fixation for ICS degrades CXCR5 staining, and thus for ICS, T_{FH} cells are defined as CD4^{+}PD-1_{hi}. Average percentage and absolute number of cytokine-producing (IFN-γ^{+}, IL-4^{+}, IL-10^{+}, and IL-21^{+}) in CD4^{+}PD-1_{hi} T_{FH} (B) and CD4^{+}PD-1_{int} (C) cells from WT and STAT3KO PPs measured by ICS are shown (n = 12, mean ± SEM). Average percentage and absolute number of double cytokine-producing (D) IFN-γ^{+}IL-4^{+} and (E) IFN-γ^{+}IL-10^{+} populations in PD-1^{+}CD4^{+} T cells are shown. Each symbol represents one mouse (n = 12, mean ± SEM). Data are combined from four independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001 by ANOVA (B and C) or t test (D and E).

**FIGURE 3.** Altered gene expression in Stat3-deficient T_{FH} cells in the PP. Relative mRNA expression was determined by quantitative RT-PCR. (A) Il4 gene expression from freshly isolated PP T_{FH} and non–T_{FH} cells (CXCR5^{−}PD-1^{−}) from WT and STAT3KO mice (n = 4, mean ± SEM). *p < 0.05 (ANOVA). (B–D) Bcl6, Prdm1, and Gata3 gene expression in freshly isolated PP T_{FH} cells. T_{FH} cells were gated for flow cytometry as in Fig. 1. Each symbol represents one mouse (n = 4, mean ± SEM). *p < 0.05, **p < 0.01, ***p < 0.001 (t test).
opposite pattern of IFN-γ expression (Fig. 6B). As in the PP TFH cells, dual cytokine-expressing cells were significantly increased in the spleen TFH cell population (Fig. 6C, 6D).

Intrinsic effects of Stat3 on TFH cell function

To confirm and extend our results and test whether the alterations in cytokine expression were an intrinsic effect of Stat3 deficiency on CD4 T cells, we used the OT-II TCR transgenic system, where we generated both OT-II TCR+ Stat3fl/fl mice and OT-II TCR+ CD4-cre Stat3fl/fl mice. These OVA-specific CD4 T cells were transferred to congenic recipients, immunized with OVA-Alum, and donor TFH cell cytokine production was assayed by flow cytometry (Fig. 6E, 6F). In this system, Stat3-deficient T cells produced TFH cells, but at a significantly lower percentage than control T cells (Fig. 7A). Notably, and consistent with our earlier results, the Stat3-deficient TFH cells produced significantly more IL-4 and also had an increase in double cytokine-expressing cells. The OVA-specific Stat3-deficient TFH cells also expressed higher levels of Gata3 and Bcl6 as well as Tbet (Supplemental Fig. 2B–D), consistent with the Stat3-deficient TFH cells in the PP. Thus, Stat3 plays a critical role in controlling IL-4 expression in TFH cells and is intrinsically required for the de novo generation of TFH cells in an acute Ag challenge environment.

To further test TFH cell–intrinsic effects of Stat3, we used a mixed BM chimera system where BM from CD45.2+ STAT3KO mice was mixed with CD45.1+ WT BoyJ BM and transferred into lethally irradiated WT BoyJ mice. Mice where BM from CD45.2+ WT (Stat3fl/fl) mice was mixed with CD45.1+ WT BoyJ BM and similarly transferred into lethally irradiated CD45.1 WT BoyJ mice were used as the control for the STAT3KO chimeras. The mice were allowed to repopulate their lymphoid systems for at least 90 d and then were immunized with SRBCs. Both WT and STAT3KO donor BM CD4 T cells repopulated the spleen normally in the absence of Stat3 (Fig. 7B). However, in contrast to our results with whole conditional mutant animals, Stat3-deficient CD4 T cells in chimeric mice formed TFH cells at a markedly lower rate than did control cells in the PP, whereas splenic TFH cells were less affected (Fig. 7C). These results suggest that in a competitive environment with WT TFH cells, Stat3-deficient TFH cells can develop to a significant degree, but in a chronic, ongoing response as in the PP, they do not persist as well as WT TFH cells. The proportion of GCB cells was not affected by the presence of Stat3-deficient TFH cells, and likely the WT TFH cells compensated for any defect of the Stat3-deficient TFH cells (Fig. 7D). GCB cells showed a nonsignificant trend toward increased IgG1, which would fit with a model where increased IL-4 made by Stat3-deficient TFH cells was diluted out by WT TFH cells (Fig. 7E). In the chimeric PP, we examined cytokine production and, consistent with our results in nonchimeric mice, we observed increased expression of IL-4.

**FIGURE 4.** Delayed TFH cell responses in the spleen of Stat3-deficient conditional KO mice after immunization. (**A**) TFH cells are gated on CD4^+^Foxp3^−^CXCR5^hi^PD-1^hi^ cells within the splenocyte population. Average TFH cell percentages (**B**) and absolute numbers (**C**) in WT and STAT3KO mice (n = 4, mean ± SEM) 3 d postimmunization (dpi) with SRBCs are shown. (**D**) Day 7 TFH cells are gated as in (**A**). Average TFH cell percentages (**E**) and absolute numbers (**F**) in WT and STAT3KO mice (n = 4, mean ± SEM) 7 dpi with SRBCs are shown. (**G**) Bcl6 mean fluorescence intensity (MFI) of TFH cells at 7 dpi (n = 3, mean ± SEM). (**H**) Bcl6 MFI of non–TFH cells at 7 dpi (n = 3, mean ± SEM). **p < 0.01, ***p < 0.001 (t test). Each symbol in graphs represents one mouse. Data are representative of two independent experiments with similar results.
IFN-γ and IL-4 and decreased IL-21 in Stat3-deficient TFH cells (Supplemental Fig. 2E–G). Thus, the altered cytokine expression in Stat3-deficient TFH cells is cell intrinsic. In the chimeric spleen, we further examined the expression of Bcl6 and Gata3 and found that these two key transcription factors were significantly increased in Stat3-deficient TFH cells in both PPs and spleen (Fig. 7F, G). Thus, deregulated Bcl6 and Gata3 expression in Stat3-deficient TFH cells is an intrinsic effect of loss of Stat3 in the TFH cells, and it is not a function of an abnormal immune environment present in STAT3KO mice.

To better understand how Stat3 was required for TFH cell development, we wondered whether Stat3-deficient CD4 T cells proliferated poorly or underwent greater apoptosis, thus leading to noncompetitive TFH cell responses. To test this, we used T cells from WT and STAT3KO OT-II TCR mice, where the cells were labeled with a cell tracking dye prior to transfer. Six days after OVA-Alum immunization, we found that the Stat3-deficient T cells demonstrated slightly greater proliferation than did the control cells (Supplemental Fig. 3A, 3B). We additionally analyzed the rate at which Stat3-deficient TFH cells underwent apoptosis, using two different methods. We tested the ex vivo level of TFH cells initiating apoptosis by staining TFH cells for annexin V, both in spleen after SRBC immunization and in PPs. We also tested whether the Stat3-deficient TFH cells might undergo higher apoptosis if they were stimulated through the TCR. TFH cells were isolated by FACS from spleens of SRBC-immunized mice, stimulated overnight with anti-CD3 Ab, and then measured for levels of active caspase-3. Contrary to expectations from the lack of in vivo competitiveness in chimera mice, the Stat3-deficient TFH cells had fewer or equal levels of apoptotic cells (Supplemental Fig. 3C–E).

Control of Bcl6 repressive activity by Stat3
To better dissect why Stat3-deficient TFH cells expressed more IL-4, we used an OVA Ag plus APCs in vitro culture system where OT-II TCR control and OT-II TCR Stat3-deficient CD4 T cells were activated under Th2 conditions, and expression of IFN-γ and IL-4 was monitored by ICS after 3 d (Fig. 8A). Consistent with what we observed for Stat3-deficient TFH cells, Stat3-deficient CD4 T cells cultured under Th2 conditions produced about twice as much IL-4, and similar to the Stat3-deficient TFH cells, double cytokine-expressing cells were also increased (Fig. 8B). Similar increases in double cytokine-expressing cells were also observed in Stat3-deficient CD4 T cells cultured under TFH conditions, but the level of IL-4 single-expressing cells was lower in the Stat3-deficient cells (Supplemental Fig. 4). Notably, the increased IL-4 production by STAT3KO Th2 cells was transient, as STAT3KO T cells cultured longer under Th2 conditions produced significantly less IL-4 than did the control Th2 cells (Fig. 8C). Bcl6 is known to

FIGURE 5. Weak GCB cell responses and Ab responses following SRBC immunization when Stat3 is absent in T cells. Mice were immunized with SRBCs and spleen analyzed 3 or 7 d postimmunization (dpi). (A and C) GCB cells and (B and D) IgG1+ GCB cells were gated as in Fig. 1. Average GCB cell populations in terms of percentage of B220+ cells and absolute number at 3 dpi (A and B) and 7 dpi (C and D). Each symbol represents one mouse (n = 3–4, mean ± SEM). *p < 0.05, **p < 0.01 (t test). Data are representative of two independent experiments with similar results. (E–G) Anti-SRBC Ab responses measured by ELISA at 7 dpi. (E) Anti-SRBC IgM, (F) anti-SRBC IgG, and (G) anti-SRBC IgG1 titers (n = 4, mean ± SEM). *p < 0.05, **p < 0.01, ***p < 0.001.

NOVEL INSIGHTS INTO Stat3 FUNCTION IN TFH CELLS

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suppress IL-4 expression in CD4 T cells in part by suppressing Gata3 transcriptional activity (34, 35), so we wondered why the increased Bcl6 expression in Stat3-deficient TFH cells failed to control IL-4. We therefore tested whether forced expression of Bcl6 by RV could suppress IL-4 in Stat3-deficient CD4 T cells cultured under TH2 conditions. For this experiment, CD4 T cells from control and STAT3KO mice were activated under Th2 conditions using anti-CD3 and anti-CD28 Abs plus APCs. Under these conditions, ∼50% of control T cells expressed IL-4 and a higher level, ∼70% of Stat3-deficient T cells, expressed IL-4 (Fig. 8D). Whereas Bcl6 RV was able to suppress 60% of the IL-4 in control T cells, it suppressed 30% of the IL-4 in Stat3-deficient T cells (Fig. 8D, 8G). This strong loss of Bcl6 repressive activity was unique to IL-4, as Bcl6 repressed IL-10 slightly better in Stat3-deficient T cells (Fig. 8E, 8G) and Bcl6 showed only a slight defect in repressing IL-13 (Fig. 8F, 8G). These data show that Bcl6 is defective in repressing IL-4 expression in the absence of Stat3, and this can explain the abnormal expression of IL-4 by Stat3-deficient TFH cells.

Discussion
TFH cells control GC reaction and the production of high-affinity Abs to Ag, and the pathways for how TFH cells develop and regulate B cell responses are a major part of the adaptive immune response. When we initiated this study, we were interested in elucidating how critical Stat3 was for TFH cell differentiation and whether other pathways besides Stat3 could induce Bcl6 expression in TFH cells. Recent studies have revealed that TFH cells can develop at a low level in the absence of Stat3 (18), and that Stat4 and Stat1 can participate in inducing Bcl6 during TFH cell differentiation (14, 16, 17). The work of Ray et al. (18) established that Stat3 is critical factor for not only activating Bcl6 in TFH cells but also for repressing a type I IFN pathway that induces a TH1-like effector program in TFH cells. However, because this study was done solely in the context of acute LCMV infection where a great deal of type I IFN is produced, whether this pathway represented the dominant Stat3-mediated control pathway in TFH cells, or only was operative in the case of virus infection, was unclear. Thus, we analyzed T cell–specific Stat3 conditional KO mice (CD4-cre Stat3fl/fl; STAT3KO) for other types of TFH cell responses. In the ongoing chronic TFH cell/GCB cell response of the PP, we found that loss of Stat3 did not affect the proportion of the TFH cells within the PP, nor did loss of Stat3 in TFH cells affect the level of PP GCB cells. Furthermore, there was a shift toward the expression of both Th1 (IFN-γ) and Th2 (IL-4) cytokines in Stat3-deficient PP TFH cells. We observed that Bcl6 expression was notably higher in Stat3-deficient PP TFH cells. These data indicate that Stat3 function in TFH cells can vary depending on the type of immune response, and that Stat3 appears to insulate TFH cells from differentiating into different effector pathways depending on the cellular microenvironment. Most dramatic, however, was our finding that PP TFH cells and GCB cell responses were present at normal levels in the STAT3KO mice, with increased Bcl6 expression by TFH cells. The function of Stat3 in regulating Bcl6 expression in TFH cells is therefore heavily influenced by the local immune environment. In a virus infection with high levels of type I IFN, Stat3 is

![Figure 6](http://www.jimmunol.org/Downloadedfrom/fig6.jpg)
required for upregulating Bcl6 in T<sub>FH</sub> cells, but in the immune response against commensal organisms in the PP, Stat3 actually restrains the upregulation of Bcl6 in T<sub>FH</sub> cells. In the antivirus response, type I IFN suppresses Bcl6 expression, and it was proposed that type I IFN leads to IL-2 gene transcription and downstream Stat5 activation, where Stat5 then directly binds to and represses Bcl6 (18). In contrast, for the gut-PP immune environment and for Ags where type I IFN is not produced, such as OVA-Alum, Stat5 is likely not activated in T<sub>FH</sub> cells. We propose that lack of Stat3 in this inactive Stat5 T<sub>FH</sub> context allows other Stats, such as Stat1 and Stat4, to bind to the Bcl6 promoter and more strongly activate transcription than when Stat5 is active.

A related question is why IL-4 and IFN-γ are upregulated in Stat3-deficient T<sub>FH</sub> cells. We observed increased IL-4 in all four types of T<sub>FH</sub> cell responses we tested (PP, SRBC immunization, OVA-Alum immunization, and in vitro activation under Th2 conditions), and thus it seems to be a general effect in our hands. IFN-γ was increased in Stat3-deficient PP T<sub>FH</sub> cells and the in vitro cultures. Thus, the immune environment is key for the exact type of cytokine response that Stat3 regulates in T<sub>FH</sub> cells. Our data with Bcl6 RV show that Bcl6 is unable to suppress IL-4 in the absence of Stat3, but the mechanism for this effect is not clear. One possibility is the increased Gata3 we observe in CD4 T cells in the absence of Stat3. Gata3 may put the IL-4 gene chromatin in a hyperactivated state that is resistant to repression by Bcl6. A related possibility is that there is differential regulation of IL-4 by Stat factors in the absence of Stat3. This model is analogous to the model for regulation of Bcl6 transcription in the absence of Stat3 described above. Thus, this model would propose that IL-4 gene expression is controlled by loss of Stat3 differently in the virus infection system versus immune responses where type I IFN is not produced, such as the responses we analyzed in this study. In the absence of type I IFN and activation of Stat1, other Stat factors may bind to IL-4 or Gata3 regulatory regions and induce high-level expression of these genes. Yet another possibility is that Stat3 regulates the transcription of a cofactor required for repression of IL-4 by Bcl6. The exact mechanism for how Bcl6 represses IL-4 expression in CD4 T cells is not known, although the current model is that...
Bcl6 represses Gata3 transcriptional activity, and thus indirectly suppresses IL-4 gene expression (35, 36).

Our data showing increased IL-4 by T cells in the absence of Stat3 contrasts with published work showing that Stat3 is required for proper full TI2 cell differentiation (32). A possible explanation for this discrepancy is that TI2 cells regulate IL-4 gene expression by different regulatory elements than the regulatory elements that TI2 cells use for IL-4 expression (37, 38), and overall it is clear that TI2 cells are a separate lineage from TI2 cells. At the same time, we observed increased IL-4 expression by Stat3-deficient T cells cultured under TI2 conditions (Fig. 8). Critically, in the in vitro TI2 cultures, we analyzed IL-4 and other cytokines in these Stat3-deficient TI2 cells at an early time point, that is, 3 d after initial activation. When we examined Stat3-deficient TI2 cells cultured for longer periods and after restimulation (Fig. 8C), the increased IL-4 expression was lost. Indeed, data in the previous report also showed that IL-4 production within the first 24–48 h was not deficient in the absence of Stat3 (39). Thus, we can reconcile these findings then by proposing that Stat3 indeed suppresses expression of IL-4 shortly after activation, but that Stat3 is actually important for long-term TI2 cell stability and/or survival.

Curiously, we found that Stat3-deficient TI2 cells survive about the same or slightly better than do WT TI2 cells (Supplemental Fig. 3C–E), which contradicts the standard view that Stat3 is a protumoral and prosurvival transcription factor (15, 20, 40). One explanation is that the increased survival of Stat3-deficient TI2 cells is due to increased Bcl6 expression, because we have previously shown a survival effect of Bcl6 in TI2 cells (33). Whether this effect is unique to TI2 cells and holds up to further scrutiny will require investigation.

In summary, we have revealed a much more complete picture of how Stat3 regulates TI2 cell development and function. Stat3 function in TI2 cells appears to be strongly dependent on the immune environment, and Stat3 plays very different regulatory roles in TI2 cells that develop during virus infection than in TI2 cells that develop in response to commensal organisms in the gut, as well to model Ags that do not provoke a strong type I IFN response. In the larger picture, our data indicate a critical role for Stat3 antagonizing the activity of other Stat factors that are activated in CD4 T cells during the immune responses. Notably, Stat3 can act in TI2 cells to either repress Bcl6 or to activate Bcl6, depending on the activation of other Stat factors.

Disclosures
The authors have no financial conflicts of interest.

References
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Supplemental Figure 1. Examples of cytokine staining and gates showing staining controls. Spleen cells were stimulated with PMA plus ionomycin and stained intracellularly for the indicated cytokines, 7 days after SRBC immunization as in Figure 6. Plots are gated on CD4+ cells. Gated regions show cytokine-positive cells. Isotype controls for each Ab were used to set gates; for isotype controls, 0-1% of the cells were in the gated region for all cytokines analyzed.
Supplementary Figure 2. Tfh cell intrinsic regulation of transcription factors and cytokines by Stat3. (A) \(T_{\text{FH}}\) cells in the Peyer’s patches (PPs) of WT and STAT3KO mice were sorted by FACS and RNA isolated post-sort. Tbx21 (Tbet) mRNA levels were assayed by QPCR. (B-D) WT and STAT3KO OTII TCR transgenic CD45.2+ CD4+ T cells were transferred to CD45.1+ BoyJ mice, and the recipient BoyJ mice were immunized with OVA-Alum. \(T_{\text{FH}}\) cells were analyzed at 7 days after immunization. Graphs show Tbet, Bcl6 and Gata3 MFIs of CD45.1-TFH cells, where each symbol represents one mouse (n=3, mean ± SEM). (E-G) Cytokines in \(T_{\text{FH}}\) cell populations in the PPs of bone marrow chimeras were measured by intracellular cytokine staining (ICS) following PMA plus ionomycin staining in vitro.
Supplemental Figure 3. Increased proliferation and decreased death of Stat3-deficient CD4 T cells. A-B) WT and STAT3KO OTII TCR transgenic CD45.2+ CD4+ T cells were transferred to CD45.1+ BoyJ mice and immunized with OVA-Alum as in Figure 6. A) Histogram plot of Cell Tracker Violet (CTV)-labeled WT and STAT3KO OTII CD4+ T cells at 6 dpi with OVA-Alum. B) Graphs of average MFI of CTV-labeled WT and STAT3KO OTII CD4+ T cells. Each symbol represents one mouse (n=3, mean ± SEM). **P <0.01 (t-test).  C) Annexin V+ percentage of T<sub>FH</sub> cells assayed ex vivo from spleen 7 dpi with SRBC in WT and STAT3KO mice. Each symbol represents one mouse (n=4-5, mean ± SEM). *P <0.05 (t-test). D) Annexin V+ percentage of T<sub>FH</sub> cells assayed ex vivo from PP (n=4-5, mean ± SEM). **P <0.01 (t-test). E) 7 dpi with SRBC, average percentage of activated Caspase3+ (aCaspase3+) cells in FACS-isolated spleen T<sub>FH</sub> cells and non-T<sub>FH</sub> cell populations, stimulated with anti-CD3 Ab overnight in vitro. (n=3, mean ± SEM). ***P <0.001 (ANOVA).
Supplemental Figure 4. Defective Suppression of IL-4 by Bcl6 in Stat3-deficient T cells. WT and STAT3KO OTII TCR transgenic CD4+ T cells were cultured under Th1 conditions with APC and OVA peptide in vitro for 3 days. (A) Cells were stimulated with PMA plus ionomycin and analyzed for IFNγ and IL-4 ICS and flow cytometry after just 3 days of culture. (B) Part of the primary culture was put into secondary culture under Th2 conditions with APC and OVA peptide in vitro for 5 days, before being stimulated with PMA plus ionomycin and analyzed for IFNγ and IL-4 ICS and flow cytometry. Graphs show average percentages of IFNγ+IL-4-, IFNγ+IL-4+ and IFNγ-IL-4+ cell CD4+ T cells (n=3, mean ± SEM). ***p <0.001 (ANOVA).