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

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Abstract

The transcription factor Bcl6 is required for the development of the follicular helper T (T_{FH}) cells. Cytokines that activate Stat3 promote Bcl6 expression and T_{FH} cell differentiation. Previous studies with an acute virus infection model showed that T_{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_{FH} cells. In Peyer's patches (PPs), we found that compared to wild-type, Stat3-deficient T_{FH} cells developed at a 25% lower rate, and expressed increased IFNγ and IL-4. While PP germinal center B (GCB) cells developed at normal numbers with Stat3-deficient T_{FH} cells, IgG1 class switching was greatly increased. Following immunization with Sheep Red Blood Cells (SRBC), splenic Stat3-deficient T_{FH} cells developed at a slower rate than in control mice and splenic GCB cells were markedly decreased. Stat3-deficient T_{FH} cells developed poorly in a competitive bone marrow chimera environment. Under all conditions tested, Stat3-deficient T_{FH} cells over-expressed both IL-4 and Bcl6, a pattern specific for the T_{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_{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_{FH} cells, and that the specific immune environment determines the function of Stat3 in T_{FH} cells.

Introduction

In the course of an immune response, CD4 T helper cells differentiate into unique effector lineages that promote different immune responses via the secretion of specialized effector cytokines. Follicular T helper (T_{FH}) cells are a CD4 T cell lineage whose identified function is to promote formation of germinal centers (GCs) and select B cell clones that produce high-affinity antibodies (Abs) (reviewed in (1-5)). T_{FH} cells are typically identified as $CD4^+$ $CXCR5^+$ and PD-1^{high} T cells, and have an activated T cell phenotype but are $CD25^{neg}$. T_{FH} cells control the outcome of the GC B cell response, and are critical for memory B cell and

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plasma cell development. T_{FH} cells produce IL-21, a cytokine that potently promotes B cell activation and Ab secretion. While T_{FH} cells are required for the production of high affinity Abs, excessive numbers of T_{FH} cells can promote autoimmunity by helping B cells produce self-reactive Abs (6-8). The proper regulation of T_{FH} cell differentiation is therefore essential for strong antibody responses and preventing development of autoimmune disease.

The Bcl6 transcriptional repressor protein is up-regulated in T_{FH} cells and is considered a master regulator for the T_{FH} lineage (9-11). Signal transducer and activator of transcription (Stat) factors are upstream of Bcl6 in T_{FH} cell differentiation and receive cytokine signals to bind to the Bcl6 promoter and induce high levels of Bcl6 expression. Specifically, activated CD4 T cells exposed to the cytokines IL-6, IL-21 and IL-12 activate Stat3 and Stat4 to promote T_{FH} cell differentiation via up-regulation of Bcl6 (1, 2, 12-15). Stat1, activated by IL-6 or type I IFN can also promote Bcl6 transcription and T_{FH} cell differentiation (16, 17), although in certain contexts, type I IFN can inhibit T_{FH} cell differentiation (18).

Although Stat3 is not required for early T cell development (19, 20), Stat3 plays many important roles in T cell immune responses, particularly in the development of the pro-inflammatory T_H17 lineage (21-25) and formation of T cell memory (26, 27). Furthermore, Stat3 is required for T_H17-mediated colitis, as well as regulatory control of colitis (28, 29). Mutations in Stat3 can lead to disease termed Hyper IgE Syndrome (HIES), which is characterized by elevated IgE, repeated infections, chronic dermatitis and lack of Th17 cells (30). HIES has a complex pathology and aspects of the disease appear to be mediated by non-lymphoid and even non-hematopoietic cells (31).

Two previous studies on the role of Stat3 in T_{FH} cell differentiation following lymphocytic choriomeningitis virus (LCMV) infection revealed that T_{FH} cell development was not strictly dependent on Stat3 function (17, 18). Thus, LCMV induced a delayed (17) or weakened (18) T_{FH} cell response in CD4-cre $Stat3^{fl/fl}$ conditional KO mice, where Stat3 was deleted specifically in T cells. Ray *et al* (18) revealed that part of the defect in T_{FH} cell development in the LCMV system was due to the failure to properly up-regulate Bcl6 in the absence of Stat3, a defect that was partially 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 T_H1 phenotype (18), indicating that Stat3 has an important role in repressing T_H1 cell development. However, a major question unaddressed by these studies was whether the findings observed for Stat3 and T_{FH} cells in a LCMV infection model were broadly applicable to the role of Stat3 in T_{FH} cell responses in other immune contexts. We therefore used CD4-cre $Stat3^{fl/fl}$ conditional knockout mice to examine T_{FH} cell differentiation in the gut immune response, in Peyer's Patches, as well as with immunization with a strong inducer of the germinal center response, Sheep Red Blood Cells (SRBC). Our data contrasts with the Ray *et al* study on Stat3 in T_{FH} cells on the control of Bcl6 and IL-4 expression by Stat3 (18), and thus provides important new insights into the role of Stat3 in Bcl6 expression and T_{FH} cell differentiation.

Materials and Methods

Mice and immunizations

Stat3$^{fl/fl}$ mice (18) were backcrossed to CD4-cre transgenic mice and the C57BL/6 strain for at least six generations (32). TCR-transgenic OT-II mice and B6.SJL-Prprc^{a}Pepc^{b}/BoyJ (BoyJ) mice were purchased from The Jackson Laboratory (JAX). Control mice for CD4-cre Stat3$^{fl/fl}$ (STAT3KO) mice were litter-mate Stat3$^{fl/fl}$ (WT) mice. Mice were bred under specific pathogen-free conditions at the laboratory animal facility at IUSM and were handled according to protocols approved by the IUSM Animal Use and Care Committee. For sheep red blood cell (SRBC) immunization, mice were intraperitoneally (i.p.) injected with 1×10^9 SRBCs (Rockland Immunochemicals) and were sacrificed at the indicated day. For Ovalbumin (OVA; Sigma) immunization, 50 ug OVA was mixed with Imject Alum (Pierce), and the mixture was injected i.p.

Cell culture and retrovirus transduction

CD4+CD62L+ WT and STAT3KO OT-II T cells were isolated with the CD4+CD62L+ T cell isolation kit II (Miltenyi), then were activated with 1ug/ml OVA peptide (Anaspec) and spleen-derived APCs for 3 days (one round) or 5 additional days (two rounds) under T_H2 culture conditions: 40ng/ml IL-4 (Peprotech), 10ug/ml anti-IFNg and anti-IL-12 (BioXCell). After 3 or 8 days of culture, cells were collected for ICS as described below. For retroviral transduction, CD4+CD62L+ WT and STAT3KO T cells were activated with 1ug/ml anti-CD3, 2 ug/ml anti-CD28 and splenic APCs under Th2 condition. At 40h, cells were spin infected with control H2K^k, Bcl6-H2K^k-expressing retroviruses. After spin infection, supernatant were removed and fresh T_H2 condition medium with 10 U/ml rhIL-2 were added back to cell culture. 3 days later, cells were collected for ICS as described below. Cytokine levels of H2K^k+ cells were assessed using flow cytometry.

Flow cytometry reagents

Anti-CXCR5 (2G8), anti-CD95 (Jo2), GL7 (GL7), anti-active Caspase3 (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-13 (eBio13A), anti-IL-21 (mhalx21), anti-Gata3 (TWAJ), and anti-Foxp3 (FJK-16s) Abs were from eBioscience. Anti-CD4 (GK1.5), anti-B220 (RA3-6B2), anti-IgG1 (RMG1-1), anti-CD45.1 (A20), anti-PD-1 (29F.1A12), anti-IL-10 (JES5-16E3), anti-interferon-γ (XMG1.2) were from Biolegend.

Cell staining for flow cytometry

After red blood cell lysis, total spleen cells were incubated with anti-mouse CD16/CD32 (BioXcell) for 5 minutes at RT, followed by surface staining for the indicated markers. For intracellular transcription factor staining, after surface markers were stained, cells were fixed and stained with antibodies against transcription factors by following Foxp3 fixation kit (eBioscience) instructions. Cell events were collected on an LSR2 flow cytometer (Becton Dickonson).

Intracellular cytokine staining (ICS)

Cells were stimulated with PMA (75ng/mL) plus ionomycin (1ug/mL) for 5 hours 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.

Adoptive cell transfers and immunization

CD4+CD62L+ MACS-purified WT and STAT3KO OT-II T cells were labeled with 5 μM Cell Tracer Violet (Life Technologies). 5×10^5 cells were injected i.v. per BoyJ recipient mice. After 18 hours, 50 ug Ovalbumin (Sigma) mixed with Imject Alum (Pierce) was injected i.p. into recipient BoyJ mice. CD45.1− OT-II cells from spleen were analyzed by flow cyometry for CXCR5, PD1, Bcl6, Tbet and Gata3.

Peyer's patch isolation

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

Bone marrow chimeras

Recipient CD45.1+ BoyJ mice were lethally irradiated with 1,100 Rad and reconstituted with 2×10^6 a mixture of nucleated bone marrow (BM) cells from either CD45.2+ WT mice plus CD45.1+ BoyJ mice, or CD45.2+ STAT3KO mice plus CD45.1+ BoyJ mice by i.v. injection. Thus, WT chimeras were used as a control to compare the repopulation of Stat3+ CD45.2+ BM with the repopulation by STAT3KO CD45.2+ BM. This system has been described previously (9). Chimeric mice were immunized with SRBC approximately 12 weeks after reconstitution.

Antibody titer analysis

Antibody titers of SRBC-specific in serum were measured by ELISA, as previously reported (33). Briefly, 96 well Nunc-Immuno plates (Sigma) 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), anti-mouse IgG or anti-mouse IgM Abs (Sigma) were used as secondary Ab.

Statistical Analysis

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

Results

T_{FH} cell development in the Peyer's patch can occur independent of Stat3, and Stat3 regulates the cytokine response of T_{FH} cells

In order to analyze the role of Stat3 in T_{FH} cell development, we obtained conditional knockout mice in which Stat3 is deleted from T cells via a CD4-cre transgene (CD4-cre Stat3$^{fl/fl}$ mice, termed STAT3KO mice)(32). Initially, we analyzed the T_{FH} cell and GCB cell responses that occur in the intestinal Peyer's patches (PPs), to determine if Stat3 in T cells was essential for normal homeostatic T_{FH} cell responses and the related GCB responses in the PP (Figure 1). The percentage and overall numbers of CD4 T cells in PP were decreased significantly in the STAT3KO mice (Fig. 1B, C). As shown in Figure 1D, the loss of Stat3 mildly inhibited the proportion of T_{FH} cells developing from the CD4 T cell compartment in the PP (Fig. 1D), and the absolute number of PP T_{FH} 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_{FH} cells did affect the rate of GCB cells in the PP switching to IgG1, as dramatically increased percentages of IgG1+ GCB cells were seen in the PP of STAT3KO mice (Fig. 1 I-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 PP, although there was a trend towards decreased levels of fecal IgA in STAT3KO mice (data not shown).

We next wondered what could account for the increased percentages of IgG1+ GCB cells in STAT3KO mice and decided to examine cytokine production by PP T_{FH} cells. As shown in Figure 2A-B, PD1^{hi} Bcl6^{hi} T cells (T_{FH} cells) from STAT3KO mice PP produce markedly higher percentages of IFNg- and IL-4-positive cells as detected by intracellular cytokine staining (ICS). Representative ICS cytokine flow plots are shown in Supplementary Figure 1. As expected for loss of Stat3 activity, IL-21 was significantly lower in STAT3KO PP T_{FH} cells (Fig. 2B). Non-T_{FH} cells produced much lower levels of cytokines (Fig. 2C), and STAT3KO PP non-T_{FH} cells expressed more IFNg, IL-10 and less IL-21. However IL-4 was not increased in the STAT3KO non-T_{FH} cells (Fig. 2C), indicating a unique regulation of IL-4 in T_{FH} cells. Notably, whereas WT PP T_{FH} cells had almost no double IFNg/IL-4-expressing cells or double IFNg/IL-10-expressing cells, the STAT3KO PP T_{FH} cells had far higher levels of these double cytokine-expressing cells (Fig. 2D, E). These results indicate that Stat3 regulates expression of cytokines by T_{FH} 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 T_{FH} cells by FACS, made RNA and cDNA directly from the purified cells and tested gene expression by QPCR. We found as expected, that in contrast to non-T_{FH} cells, T_{FH} cells constitutively express *Il4* (Figure 3A). We found 3-fold higher levels of Il4 and the master transcription factor for Th2 cells, Gata3, in the absence of Stat3 (Fig. 3 A,B). We also observed significant increases in the levels of *Bcl6* and *Prdm1* (Blimp1) in the STAT3KO PP T_{FH} cells (Fig. 3C, D). The gene encoding Tbet, *Tbx21*, was also elevated in T_{FH} cells and non-T_{FH} cells in the STAT3KO PP (Suppl. Fig. 2A).

Stat3 is required for acute T_{FH} cell development in the spleen and represses IL-4 expression by T_{FH} cells

In order to test if our results from analyzing Stat3 function in PP T_{FH} cells were applicable to other T_{FH} cell responses, we immunized mice with Sheep Red Blood Cells (SRBC), a potent inducer of GCB cell responses and T_{FH} cells. Like 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 Figure 4, Stat3 was required for development of a normal T_{FH} 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 days post-immunization, T_{FH} cells in STAT3KO spleens were 10-fold lower than WT (Fig. 4A-C), but by day 7, the response of the Stat3-deficient T_{FH} 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 T_{FH} cells, loss of Stat3 led to significantly higher Bcl6 expression specifically in T_{FH} cells (Fig. 4G, H). 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 (Fig. 5A-D). Consistent with a decreased GCB cell reaction, anti-SRBC IgG titers were several fold lower in the STAT3KO, while 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 to WT T_{FH} cells, found increased IL-4 and decreased IL-21 consistent with the PP T_{FH} cells. However in contrast to the PP T_{FH} cells, IFNg was decreased in STAT3KO T_{FH} cells (Fig. 6A). As in PP, the increase in IL-4 was specific to the STAT3KO T_{FH} cells, and STAT3KO non-T_{FH} cells showed an opposite pattern of IFNg expression (Fig. 6B). As in the PP T_{FH} cells, dual cytokine-expressing cells were significantly increased in the spleen T_{FH} cell population (Fig. 6 C, D).

Intrinsic effects of Stat3 on T_{FH} cell function

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

To further test T_{FH} cell intrinsic effects of Stat3, we utilized a mixed bone marrow (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 ($Stat3^{fl/fl}$) 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 days, and then were immunized with SRBC. Both WT and STAT3KO donor BM CD4 T cells re-populated 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 T_{FH} cells at a markedly lower rate than control cells in the PP, whereas splenic T_{FH} cells were less affected (Fig. 7C). These results suggest that in a competitive environment with wild-type T_{FH} cells, Stat3-deficient T_{FH} cells can develop to a significant degree, but in a chronic, ongoing response as in the PP, do not persist as well as wild-type T_{FH} cells. The proportion of GCB cells was not affected by the presence of Stat3-deficient T_{FH} cells, and likely the wild-type T_{FH} cells compensated for any defect of the Stat3-deficient T_{FH} cells (Fig. 7D). GCB cells showed a non-significant trend towards increased IgG1, which would fit with a model where increased IL-4 made by Stat3-deficient T_{FH} cells was diluted out by wild-type T_{FH} cells (Fig. 7E). In the chimeric PP, we examined cytokine production and consistent with our results in non-chimeric mice, we observed increased IFNg and IL-4, and decreased IL-21 in Stat3-deficient T_{FH} cells (Suppl. Fig. 2E-G). Thus, the altered cytokine expression in Stat3-deficient T_{FH} cells is cell intrinsic. In the chimeric spleen, we further examined the expression of Bcl6 and Gata3, and found these two key transcription factors were significantly increased in Stat3-deficient T_{FH} cells in both PP and SP (Fig. 7F, G). Thus, deregulated Bcl6 and Gata3 expression in Stat3-deficient T_{FH} cells is an intrinsic effect of loss of Stat3 in the T_{FH} cells, and is not a function of an abnormal immune environment present in STAT3KO mice.

To better understand how Stat3 was required for T_{FH} cell development, we wondered if Stat3-deficient CD4 T cells proliferated poorly or underwent greater apoptosis, thus leading to non-competitive T_{FH} cell responses. To test this, we used T cells from WT and STAT3KO OTII 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 the control cells (Suppl. Fig. 3A, B). We additionally analyzed the rate at which Stat3-deficient T_{FH} cells underwent apoptosis, using two different methods. We tested the *ex vivo* level of T_{FH} cells initiating apoptosis by staining T_{FH} cells for AnnexinV, both in spleen after SRBC immunization and in PP. We also tested whether the Stat3-deficient T_{FH} cells might undergo higher apoptosis if they were stimulated through the TCR. T_{FH} 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 T_{FH} cells had fewer or equal levels of apoptotic cells (Suppl. Fig. 3C-E).

Control of Bcl6 repressive activity by Stat3

To better dissect why Stat3-deficient T_{FH} cells expressed more IL-4, we used an Ova antigen plus APC *in vitro* culture system where OTII TCR control and OTII TCR Stat3-deficient CD4 T cells were activated under T_H2 conditions, and IFNg and IL-4 expression were monitored by ICS after 3 days (Fig. 8A). Consistent with what we observed for Stat3-deficient T_{FH} cells, Stat3-deficient CD4 T cells cultured under T_H2 conditions produced

about twice as much IL4, and like the Stat3-deficient T_{FH} 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 T_{FH} conditions, but the level of IL-4 single expressing cells was lower in the Stat3-deficient cells (Suppl. 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 the control Th2 cells (Fig. 8C). Bcl6 is known to 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 T_{FH} cells failed to control IL-4. We therefore tested whether forced expression of Bcl6 by retrovirus (RV) could suppress IL-4 in Stat3-deficient CD4 T cells cultured under T_H2 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 APC. Under these conditions, about 50% of control T cells expressed IL-4 and a higher level, about 70% of Stat3-deficient T cells expressed IL-4 (Fig. 8D). Whereas Bcl6 RV was able to suppress over 60% of the IL-4 in control T cells, it suppressed less than 30% of the IL-4 in Stat3-deficient T cells (Fig. 8D, G). 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, G) and Bcl6 showed only a slight defect in repressing IL-13 (Fig. 8F, G). 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 T_{FH} cells.

Discussion

T_{FH} cells control germinal center reaction and the production of high affinity antibodies to antigen, and the pathways for how T_{FH} 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 T_{FH} cell differentiation and if other pathways besides Stat3 could induce Bcl6 expression in T_{FH} cells. Recent studies have revealed that T_{FH} cells can develop at a low level in the absence of Stat3 (18), and that Stat4 and Stat1 can participate in inducing Bcl6 during T_{FH} cell differentiation (14, 16, 17). The work of Ray et al (18) established that Stat3 is critical factor for not only activating Bcl6 in T_{FH} cells but also for repressing a Type I IFN pathway that induces a T_H1-like effector program in T_{FH} cells. However, since 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 T_{FH} 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 $Stat3^{fl/fl}$; STAT3KO) for other types of T_{FH} cell responses. In the ongoing chronic T_{FH} cell/GCB cell response of the PP, we found that loss of Stat3 did not affect the proportion of the T_{FH} cells within the PP, nor did loss of Stat3 in T_{FH} cells affect the level of PP GCB cells. Furthermore, there was a shift towards the expression of both T_H1 (IFNg) and T_H2 (IL-4) cytokines in Stat3-deficient PP T_{FH} cells. We observed that Bcl6 expression was notably higher in Stat3-deficient PP T_{FH} cells. These data indicate that Stat3 function in T_{FH} cells can vary depending on the type of immune response, and that Stat3 appears to insulate T_{FH} cells from differentiating into different effector pathways depending on the cellular micro-environment. Most dramatic, though was our finding that

PP T_{FH} cells and GCB cell responses were present at normal levels in the STAT3KO mice, with increased Bcl6 expression by T_{FH} cells. The function of Stat3 in regulating Bcl6 expression in T_{FH} cells is therefore heavily influenced by the local immune environment. In a virus infection with high levels of Type I IFN, Stat3 is required for up-regulating Bcl6 in T_{FH} cells, but in the immune response against commensal organisms in the PP, Stat3 actually restrains the up-regulation of Bcl6 in T_{FH} cells. In the anti-virus 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 antigens where Type I IFN is not produced, such as Ova-Alum, Stat5 is likely not activated in T_{FH} cells. We propose that lack of Stat3 in this inactive Stat5 T_{FH} 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 IL4 and IFNg are up-regulated in Stat3-deficient T_{FH} cells. We observed increased IL-4 in all four types of T_{FH} cell responses we tested (PP, SRBC immunization, OVA-Alum immunization and *in vitro* activation under T_H2 conditions), and thus it seems to be a general effect in our hands. IFNg was increased in Stat3-deficient PP T_{FH} cells and the *in vitro* cultures. Thus, the immune environment is key for the exact type of cytokine response that Stat3 regulates in T_{FH} 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 hyper-activated 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 co-factor 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, though 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 T_H2 cell differentiation (32). A possible explanation for this discrepancy is that T_{FH} cells regulate IL-4 gene expression by different regulatory elements than the regulatory elements that T_H2 cells use for IL-4 expression (37, 38), and overall it is clear that T_{FH} cells are a separate lineage from T_H2 cells. At the same time, we observed increased IL-4 expression by Stat3-deficient T cells cultured under T_H2 conditions (Fig. 8). Critically, in the *in vitro* T_H2 cultures, we analyzed IL-4 and other cytokines in these Stat3-deficient T_H2 cells at an early time point: 3 days after initial activation. When we examined Stat3-deficient T_H2 cells cultured for longer periods and after re-stimulation (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 hours 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 T_H2 cell stability and/or survival.

Curiously, we found that Stat3-deficient T_{FH} cells survive about the same or slightly better than wild-type T_{FH} cells (Suppl. Fig. 3C-E), which contradicts the standard view that Stat3 is a pro-tumor and pro-survival transcription factor (15, 20, 40). One explanation is that the increased survival of Stat3-deficient T_{FH} cells is due to increased Bcl6 expression, since we have previously shown a survival effect of Bcl6 in T_{FH} cells (33). Whether this effect is unique to T_{FH} cells and holds up tp further scrutiny will require investigation.

In summary, we have revealed a much more complete picture of how Stat3 regulates T_{FH} cell development and function. Stat3 function in T_{FH} cells appears to be strongly dependent on the immune environment, and Stat3 plays very different regulatory roles in T_{FH} cells that develop during virus infection than in T_{FH} cells that develop in response to commensal organisms in the gut, as well to model antigens 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 T_{FH} cells to either repress Bcl6 or to activate Bcl6, depending on the activation of other Stat factors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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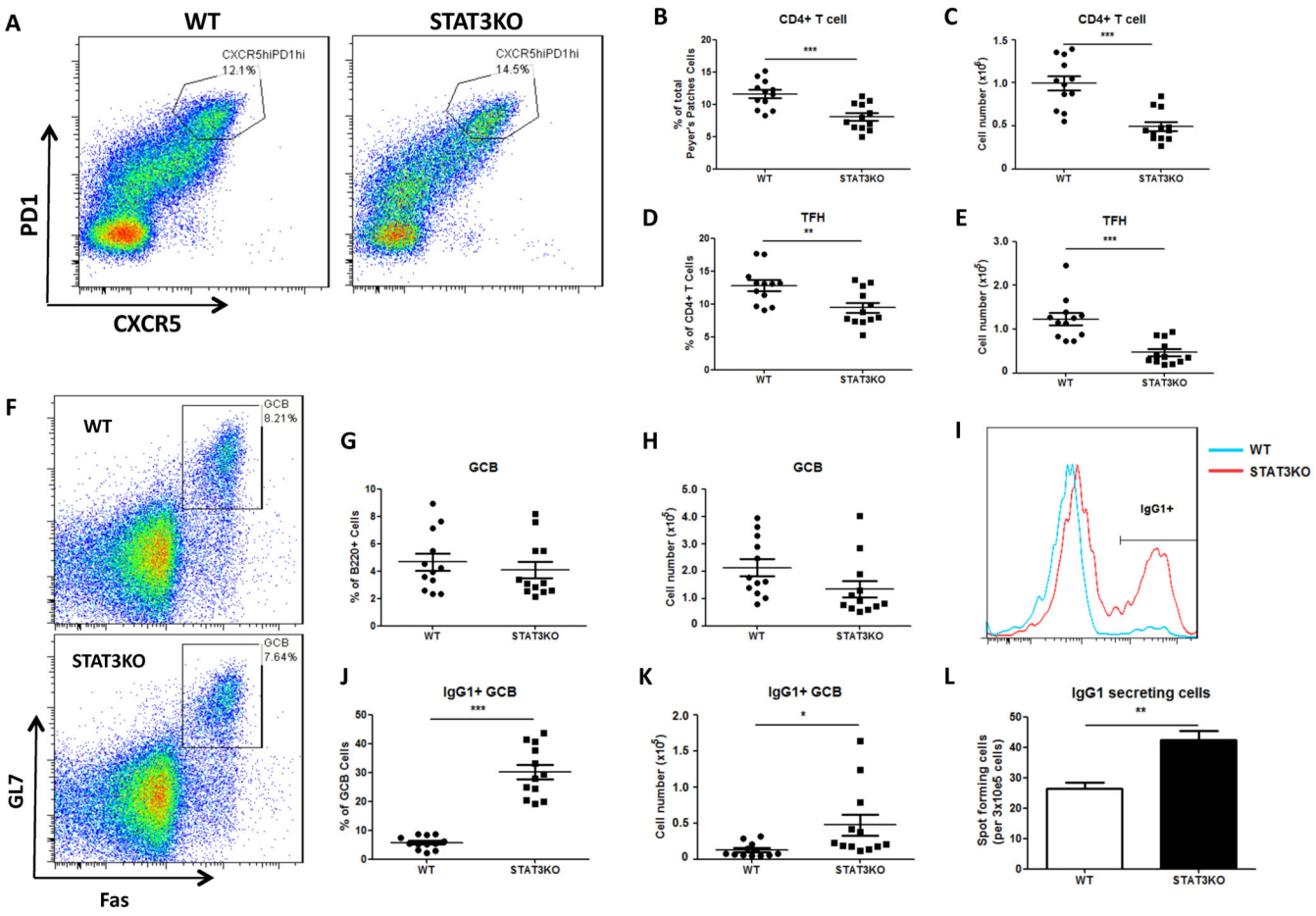

Figure 1. Effect of the loss of Stat3 in T cell on T_{FH} cell numbers, germinal center B (GCB) cell numbers, and IgG1 class switching in the Peyer's Patch (PP)

A) T_{FH} cells are gated on Foxp3^{-}CXCR5^{hi}PD1^{hi} cells within the CD4+ population, examples from PP of Stat3$^{fl/fl}$ (WT) and CD4-cre Stat3$^{fl/fl}$ (STAT3KO) mice are shown. Percentages within the PP (B) and absolute numbers (C) of total PP CD4 cells. D) Average PP T_{FH} cell percentages within the CD4 T cell population in WT and STAT3KO mice. E) Absolute numbers of PP T_{FH} cells in WT and STAT3KO mice. F) GCB cells are gated on Fas+GL7+ cells within the B220+ population, examples from PP of WT and STAT3KO mice are shown. Average PP GCB cell populations by percentage (G) and absolute number (H). (I) Histogram showing IgG1+ GCB cells from WT and STAT3KO, gated on the GCB population. Average IgG1+ GCB cell populations in terms of percentage (J) and absolute number (K). L) IgG1 secreting cells in 3×10^5 WT and STAT3KO total PP cells, assayed by ELISPOT. (n=3, mean ± SEM). Each symbol represents one mouse (n=12, mean ± SEM). Data are combined from four independent experiments, except for ELISPOT, which was repeated once. **P* <0.05, ***P* <0.01, ****P* <0.001 by *t*-test.

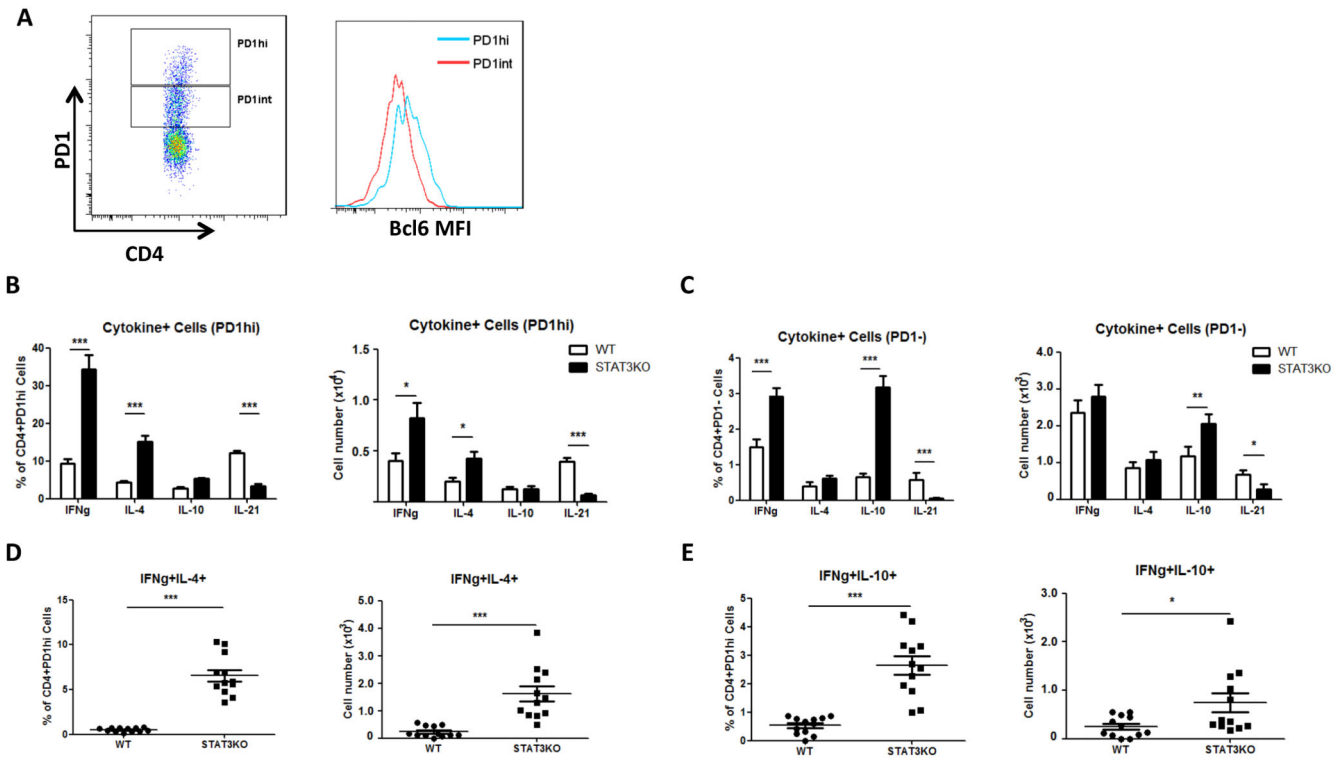

Figure 2. Deregulated cytokine production in Stat3-deficient T_{FH} cells in the PP

(A) $PD1^{hi}$ and $PD1^{int}$ $CD4^+$ 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 $CD4^+PD1^{hi}$. Average percentage and absolute number of cytokine-producing (IFNg+ (IFNγ+), IL-4+, IL-10+ and IL-21+) in $CD4^+PD1^{hi}$ T_{FH} (B) and $CD4^+PD1^{neg}$ (C) cells from WT and STAT3KO PP measured by intracellular cytokine staining (ICS) (n=12, mean ± SEM). Average percentage and absolute number of double cytokine-producing (D) $IFNg^+IL\text{-}4^+$ and (E) $IFNg^+IL\text{-}10^+$ populations in $PD1^{hi}CD4^+$ T cells. 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-C) or *t*-test (D-E).

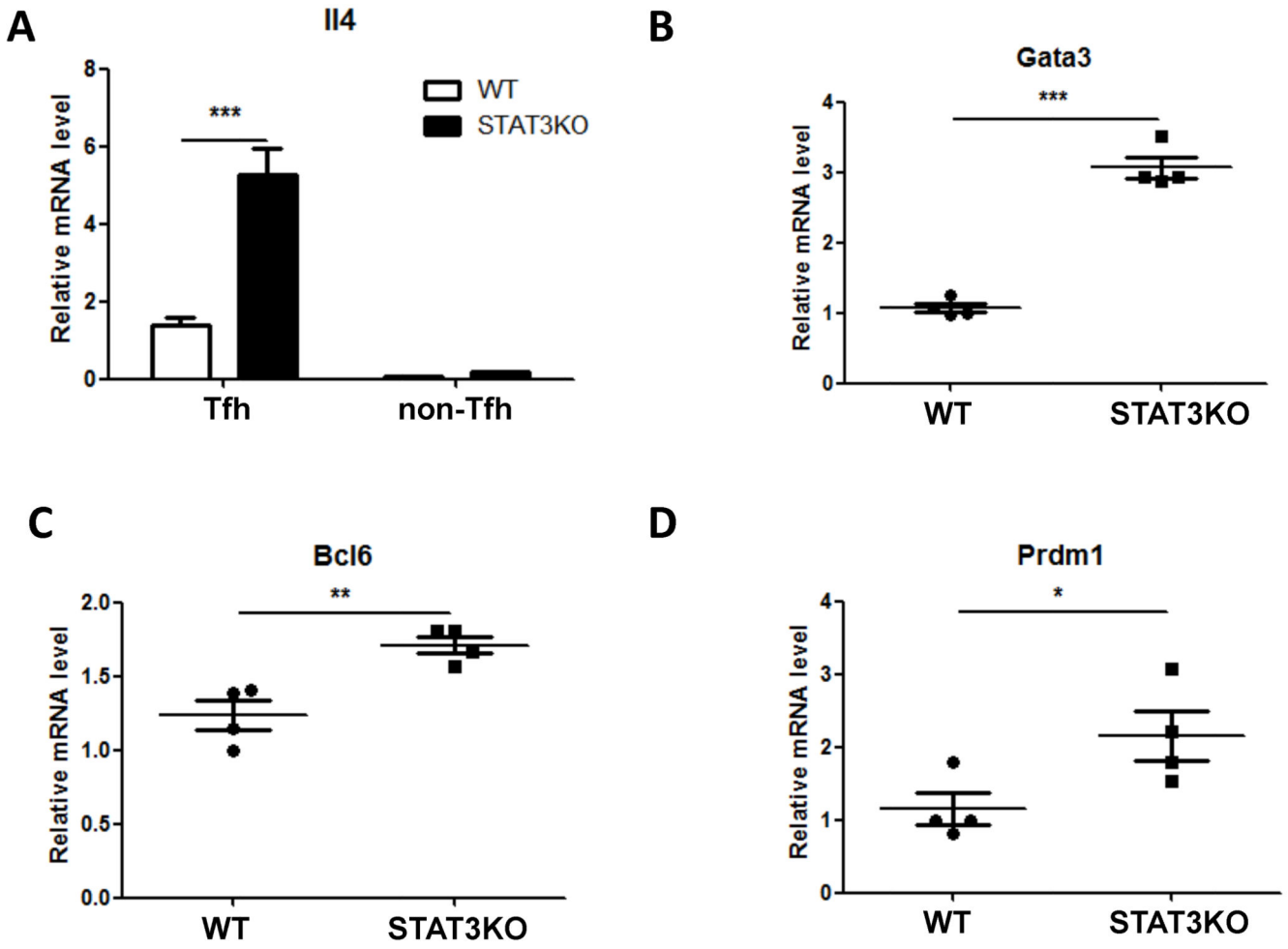

Figure 3. Altered gene expression in Stat3-deficient T_{FH} cells in the PP

Relative mRNA expression was determined by Quantitative RT-PCR (Q-RT-PCR). A) Il4 gene expression from freshly-isolated PP T_{FH} and non-T_{FH} cells (CXCR5-PD1-) 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 Figure 1. Each symbol represents one mouse (n=4, mean ± SEM). **P* <0.05, ***P* <0.01, ****P* <0.001 (*t*-test).

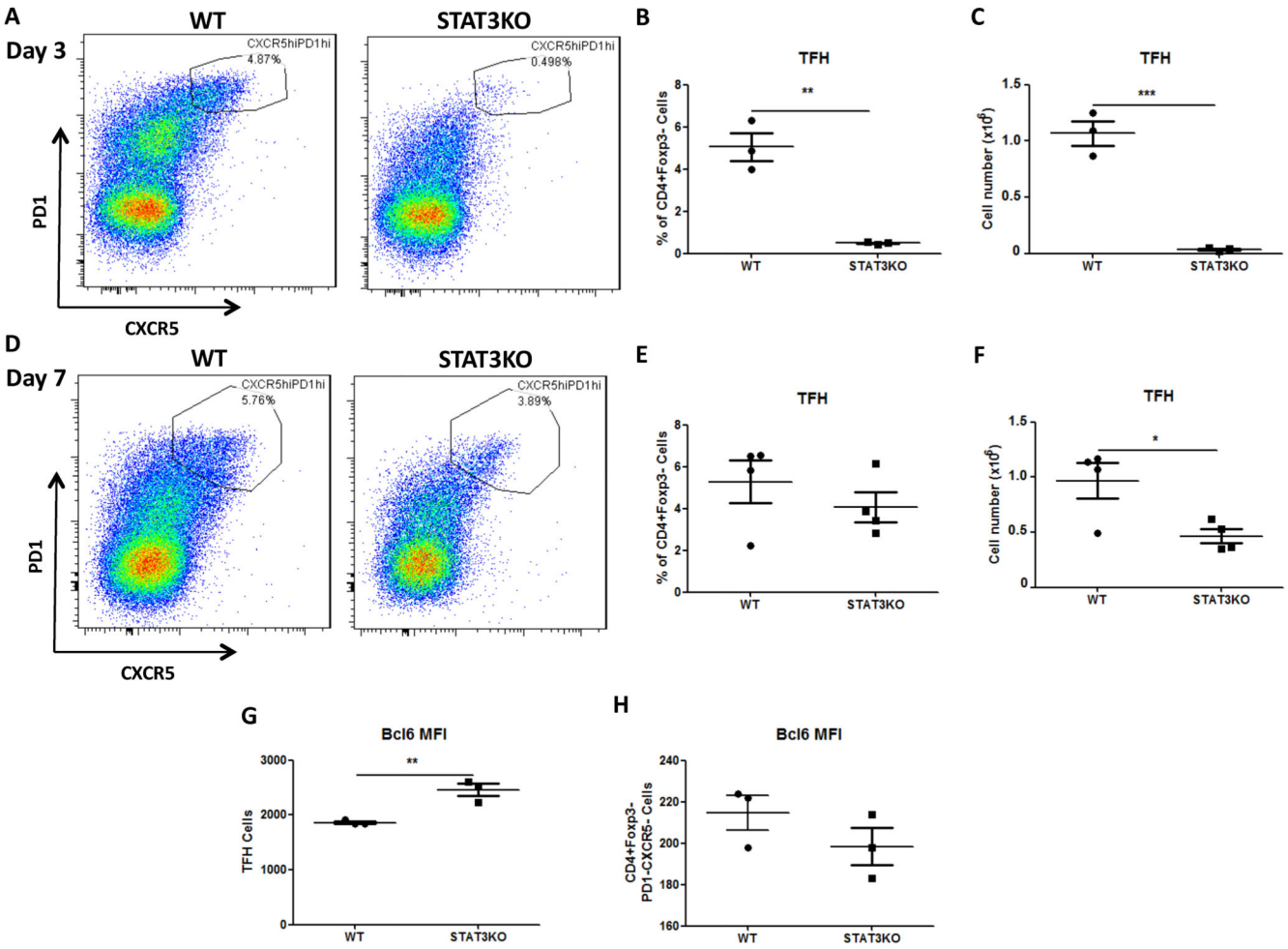

Figure 4. Delayed T_{FH} cell responses in the spleen of Stat3-deficient conditional knockout mice after immunization

A) T_{FH} cells are gated on CD4+Foxp3^{-} CXCR5^{hi}PD1^{hi} cells within the splenocyte population. Average T_{FH} cell percentages (B) and absolute numbers (C) in WT and STAT3KO mice (n=4, mean ± SEM), 3 days post-immunization (dpi) with Sheep Red Blood Cells (SRBC). D) Day 7 T_{FH} cells are gated as in (A). Average T_{FH} cell percentages (E) and absolute numbers (F) in WT and STAT3KO mice (n=4, mean ± SEM), 7 dpi with SRBC. G) Bcl6 MFI of T_{FH} cells at 7 dpi (n=3, mean ± SEM). H) Bcl6 MFI of non-T_{FH} 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.

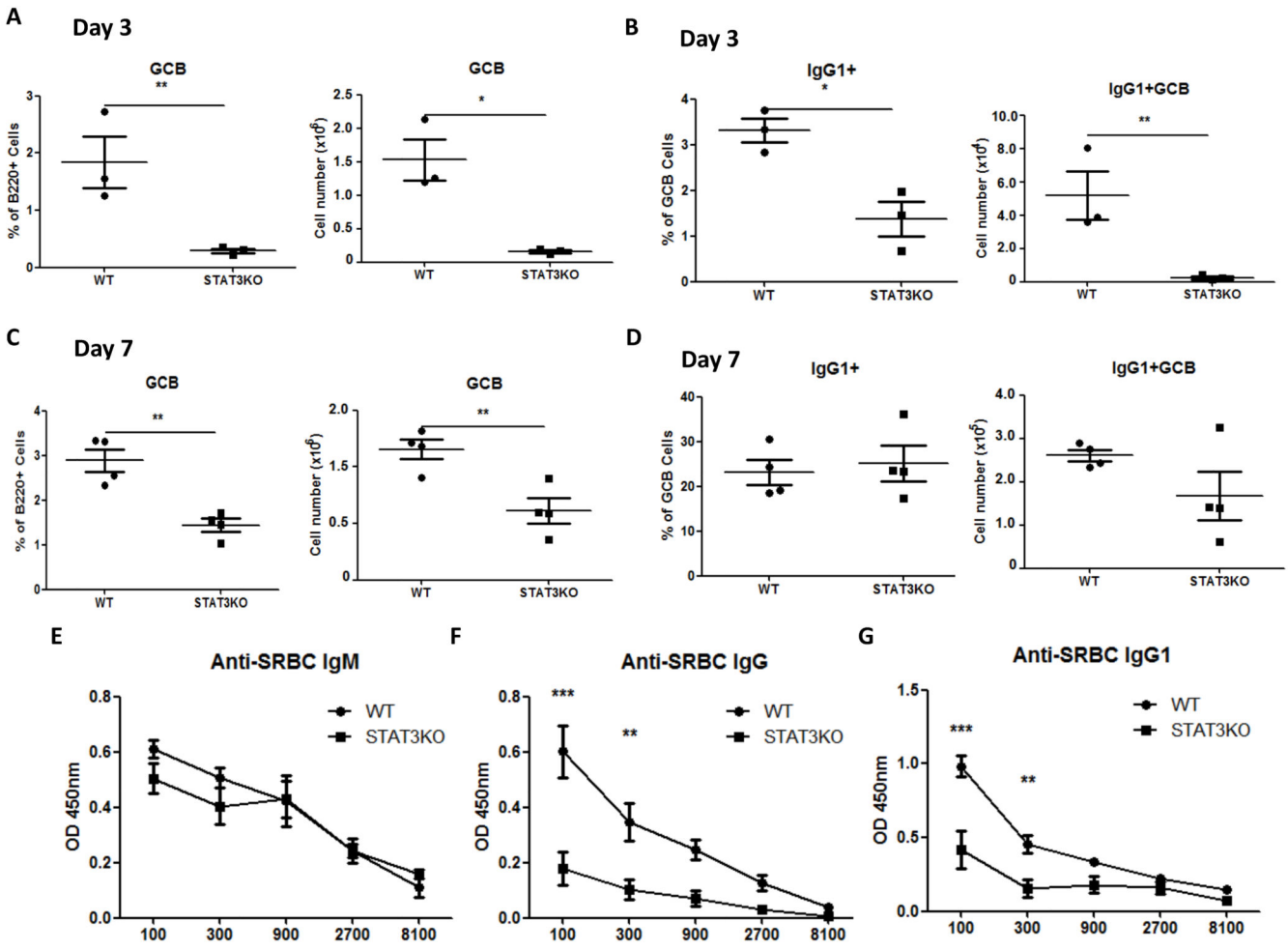

Figure 5. Weak GCB cell responses and antibody responses following SRBC immunization when Stat3 is absent in T cells

Mice were immunized with SRBC and spleen analyzed 3 or 7 dpi. **(**A, C) GCB cells and (B, D) IgG1+ GCB cells were gated as in Figure 1. Average GCB cell populations in terms of percentage of B220+ cells and absolute number at 3 dpi (A, B) and 7 dpi (C, 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

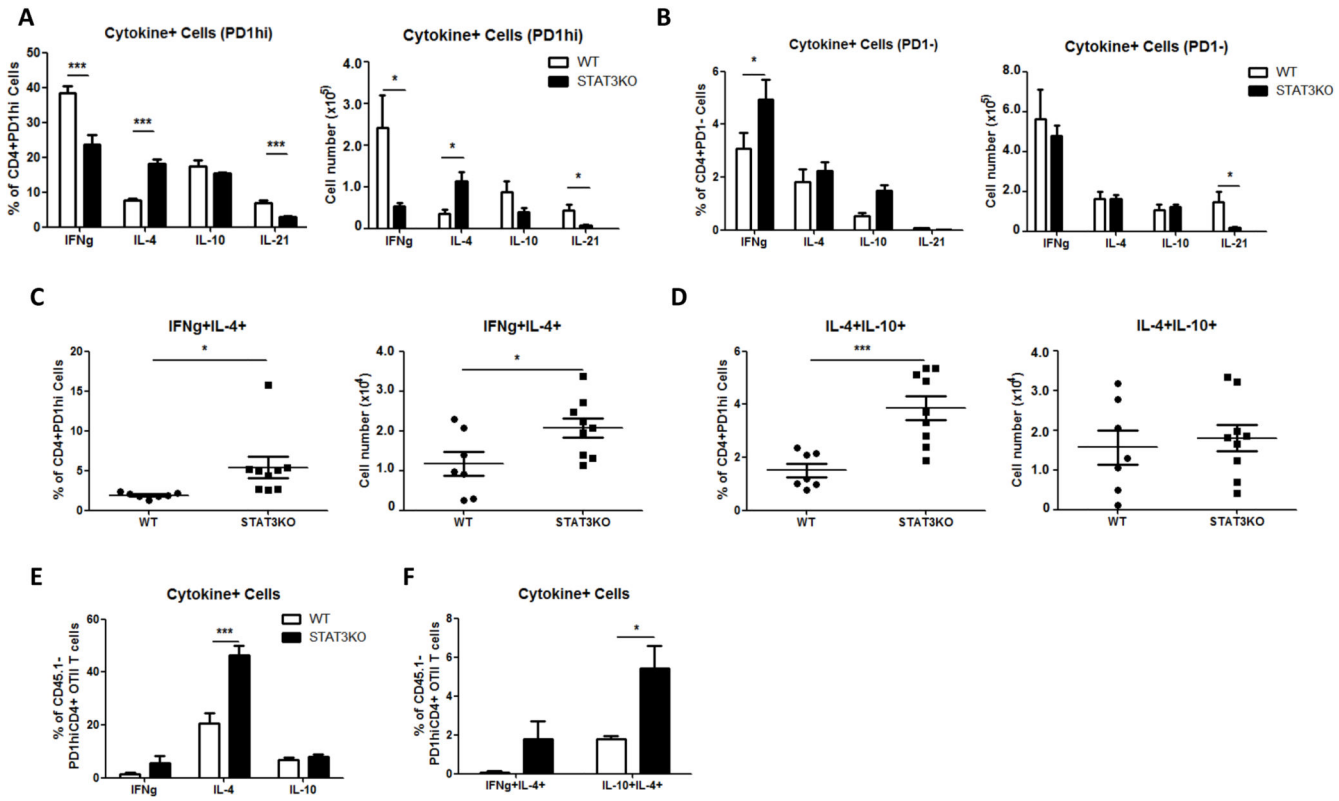

Figure 6. Stat3 controls IL-4 expression in T_{FH} cells under multiple conditions

Cytokines expressed by T_{FH} cells were measured by ICS and T_{FH} cells were gated as in Figure 2. A) Average percentage and absolute number of cytokine-producing (IFNg+ (IFNγ+), IL-4+, IL-10+ and IL-21+) T_{FH} cells in the spleens of WT and STAT3KO mice 7 dpi with SRBC (n=7-9, mean ± SEM). B) Average percentage and absolute number of cytokine-producing (IFNg+ (IFNγ+), IL-4+, IL-10+ and IL-21+) non-T_{FH} cells in the spleens of WT and STAT3KO mice 7 dpi with SRBC (n=7-9, mean ± SEM). Average percentage and absolute number of double cytokine-producing IL-10+IL-4+ (C) and IFNg+IL-4+ (D) cells within the populations assayed in (A). Each symbol represents one mouse (n=7-9, mean ± SEM). (E-F) 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_{FH} cells were analyzed for cytokine expression at 6 dpi. Graphs show average percent of cells expressing single cytokines (IFNg, IL-4 and IL-10) and double cytokine-producing cells in the CD45.1-PD1^{hi}CD4+ T cell population (n=3, mean ± SEM). *P <0.0, ***P <0.001.

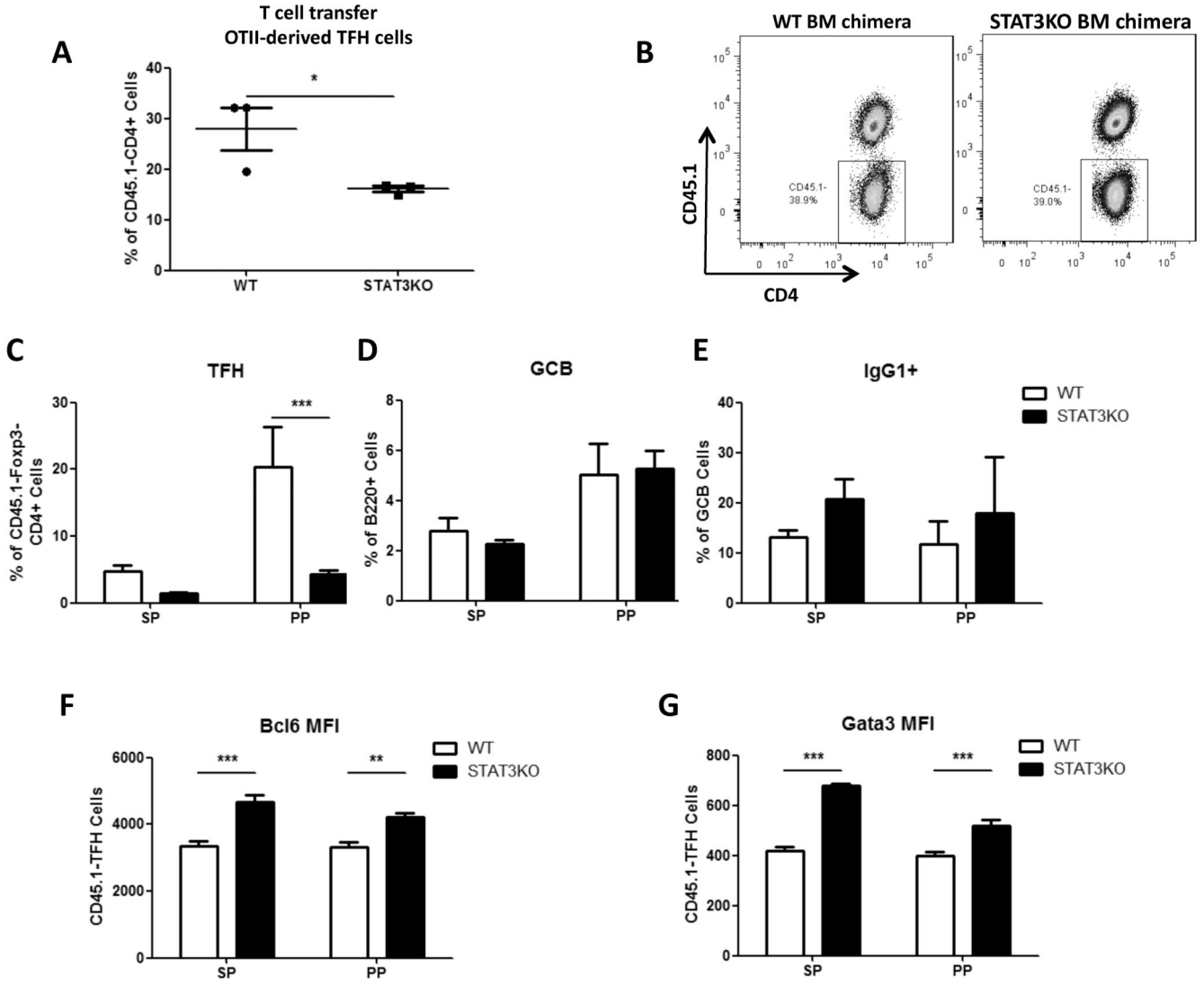

Figure 7. Intrinsic defect of Stat3-deficient T_{FH} cell development

(A) WT OTII TCR transgenic and STAT3KO OTII TCR transgenic CD45.2+ CD4+ T cells were transferred to separate cohorts of CD45.1+ BoyJ mice, and recipient BoyJ mice were immunized with OVA-Alum. Graph shows average percent T_{FH} cells within the transferred T cell population**,** analyzed at 6 dpi. (B-G) CD45.1+ BoyJ mice were lethally irradiated and divided into two groups. The WT group was given a mixture of bone marrow (BM) from BoyJ mice and CD45.2+ $Stat3^{fl/fl}$ (WT) mice. The STAT3KO group was given a mixture of bone marrow (BM) from BoyJ mice and CD45.2+ CD4-cre $Stat3^{fl/fl}$ (STAT3KO) mice. After 90 days, the mice were immunized with SRBC. At 7 dpi, the mice were analyzed for percentages of transferred BM-derived CD45.1− CD4 T cells in spleen (B), and percent of T_{FH} cells (C) and GCB (D) in spleen (SP) and PP. Graphs show average percentage of CD45.1− (CD45.2+) cells in the WT and STAT3KO groups. T_{FH} cells were analyzed by flow cytometry as in Figures 1 and 4. E) IgG1+ GCB cell percentages in spleen and PP (n=4, mean ± SEM). ***P <0.05 (t-test). D-E) MFI of CD45.1− T_{FH} cells in spleen (SP) and

PP for Bcl6 (F) and Gata3 (G). (n=4, mean ± SEM). *P <0.05, **P <0.01, ***P <0.001 (*t*-test). Data are representative of two independent experiments with similar results.

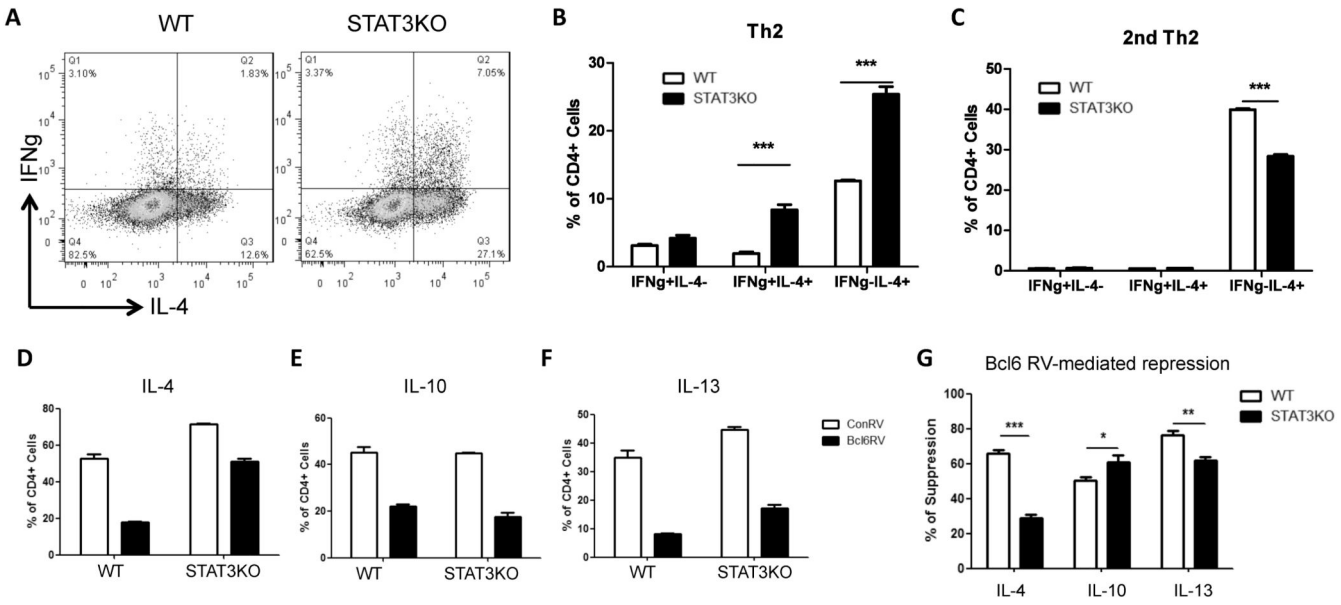

Figure 8. Defective Suppression of IL-4 by Bcl6 in Stat3-deficient T cells

(A-B) WT and STAT3KO OTII TCR transgenic CD4+ T cells were cultured under Th2 conditions with APC and OVA peptide *in vitro* for 3 days. Cells were then stimulated with PMA plus ionomycin and analyzed for IFNg and IL-4 ICS by flow cytometry. A) Sample staining of WT versus STAT3KO T cells after one round of stimulation and Th2 culture (n=3, mean ± SEM). B) Average percentages of IFNg+IL-4−, IFNg+IL-4+ and IFNg−IL-4+ cell CD4+ T cells after one round of stimulation and culture under Th2 conditions for 3 days (n=3, mean ± SEM). C) Average percentages of IFNg+IL-4−, IFNg+IL-4+ and IFNg-IL-4+ cell CD4+ T cells after an additional stimulation and 5 days of Th2 culture ("2nd Th2", n=3, mean ± SEM). (D-G) WT and STAT3KO CD4+ T cells under Th2 condition and infected with Bcl6-expressing and control retroviruses (RVs). Average percentages of T cells expressing (D) IL-4, (E) IL-10, (F) IL-13 assayed by ICS, after PMA plus ionomycin stimulation of WT + control RV, WT + Bcl6 RV, STAT3KO + control RV and STAT3KO + Bcl6 RV CD4+ T cells (n=3, mean ± SEM). G) Analysis of data in (D-F) showing average percent suppression by Bcl6 RV in WT and STAT3KO CD4 T cells. Percent suppression = ((% cytokine$^+$ cells with control RV minus % cytokine$^+$ cells with Bcl6 RV) divided by % cytokine$^+$ cells with control RV) × 100. Data are representative of two independent experiments with similar results. **P* <0.05, ***P* <0.01, ****P* <0.001 (*ANOVA*).