STAT5 Protein Negatively Regulates T Follicular Helper (Tfh) Cell Generation and Function*

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Background: Tfh cells regulate B cell-mediated humoral immunity. Results: STAT5 regulated Blimp-1 expression, and STAT5 deficiency in CD4+ T cells resulted in an increase of Tfh generation and an impairment of B cell tolerance.

Conclusion: STAT5 negatively regulates Tfh development by up-regulating Blimp-1 and thus controls the humoral immunity and B cell tolerance.

Significance: These findings may help to find new ways to treat antibody-mediated autoimmune diseases.

Recent work has identified a new subset of CD4+ T cells named as Tfh cells that are localized in germinal centers and critical in germinal center formation. Tfh cell differentiation is regulated by IL-6 and IL-21, possibly via STAT3 factor, and B cell lymphoma 6 (Bcl6) is specifically expressed in Tfh cells and required for their lineage specification. In the current study, we characterized the role of STAT5 in Tfh cell development. We found that a constitutively active form of STAT5 effectively inhibited Tfh differentiation by suppressing the expression of Tfh-associated factors (CXCR5, c-Maf, Bcl6, Batf, and IL-21, and STAT5 deficiency impaired Blimp-1 expression and resulted in elevated expression of Tfh-specific genes. Similarly, inhibition of IL-2 protein tyrosine phosphatase-1B generation, associated with dampened Blimp-1 expression; Blimp-1 overexpression inhibited Tfh gene expression in Stat5-deficient T cells, suggesting that the IL-2/STAT5 axis functions to regulate Blimp-1 expression. In vivo, deletion of STAT5 in CD4+ T cells resulted in enhanced development of Tfh cells and germinal center B cells and led to an impairment of B cell tolerance in a well defined mouse tolerance model. Taken together, this study demonstrates that STAT5 controls Tfh differentiation.

Recently, a new subset of CD4+ T cells, named Tfh4 cells, has emerged as a major player in B cell-mediated humoral immunity, especially in the germinal center reactions (1–4). Tfh cells have been characterized by their expression of chemokine CXCR5, which is induced in T cells following activation and dependent on costimulatory signals, such as CD28, inducible costimulatory molecule, and OX40. Although activated T cells may transiently express CXCR5, Tfh cells exhibit more stable expression of this chemokine receptor (5). In addition to CXCR5, additional Tfh cell markers have also been reported, such as inducible costimulatory molecule, IL-21, and the transcription factors c-Maf, Batf, and Bcl6 (1, 5–12). Moreover, the correlation between an increased number of Tfh and autoimmune has been described in lupus model, suggesting that aberrant Tfh cell expansion can lead to the formation of autoantibodies and a lupus-like phenotype (13, 14). For example, mice homologous for the sanroque allele of Roquin, which encodes the RING-type ubiquitin ligase, developed spontaneous autoantibody production and lupus-like autoimmunity, associated with spontaneous development of germinal centers and excessive number of Tfh cells (13, 15).

Tfh cell development is regulated by cytokines IL-6 and IL-21 and signaling molecule STAT3, but is independent of Th1, Th2, and Th17 effector cell lineages (6). We, as well as others, recently reported that Bcl6, a transcriptional factor selectively

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The abbreviations used are: Tfh, T follicular helper; Bcl6, B cell lymphoma 6; CXCR5, (CXCR5) receptor 5; c-Maf, musculoaponeurotic fibrosarcoma; Batf, basic leucine zipper transcription factor ATF-like; Blimp-1, B lymphocyte-induced maturation protein 1; KLH, keyhole limpet hemocyanin; HEL, hen egg lysozyme; sHEL, soluble HEL.

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STAT5 Controls Tfh Development

STAT5 controls Tfh development in vitro. Previously, we have shown that T cells activated in vitro in the presence of IL-6 or IL-21 but without TGFB, IL-4, and IFN-γ signaling preferentially acquire Tfh gene expression and function to promote humoral immunity in vivo. To determine the role of STAT5 in Tfh generation, naive CD4+ T cells from OT-II mice activated with Ova peptide and irradiated antigen-presenting cells under neutral (anti-TGFβ, anti-IL-4, and anti-IFN-γ) conditions were infected with a constitutively active Tfh-specific retrovirus expressing Cre or a vector control virus and in the presence of the indicated cytokines. FACS-sorted GFP+ cells were restimulated for 4 h with anti-CD3 for real-time RTPCR analysis. In Fig. 1D, naive CD4+ T cells from Stat5fl/+ and Stat5fl/+ mice coinfected with two bicistronic retroviruses expressing Cre-GFP or GFP vector and Blimp-1-hCD2 or hCD2 were further activated under neutral conditions (anti-IL-4, anti-IFNγ, and anti-TGFB) in the presence or absence of IL-6. GFP+ hCD2+ cells were sorted and restimulated for 4 h with anti-CD3 for real-time RTPCR analysis.

Adaptive Transfer Study—Poly(I:C) was administered into Stat5fl/+ and Stat5fl/+ Mx1Cre/YFP mice two times at 2-day intervals. Three weeks after the last injection, mice were sacrificed, and YFP+ CD4+ (CD45.2) cells were FACS-sorted for adoptive transfer into B6.SJL (CD45.1+) mice (3 x 10^6 cells/mouse) (two groups; three mice per group). Two groups of recipient mice were immunized subcutaneously with keyhole limpet hemocyanin (KLH) protein emulsified in complete Freund’s adjuvant. Seven days after the immunization, experimental mice were sacrificed, and splenic YFP+ CD45.2+ CD4+ T cells were stained with biotinylated anti-CCXCR5 mAb followed by allophycocyanin-labeled streptavidin and Phycoerythrin-conjugated anti-B and T lymphocyte attenuator (BTLA) mAbs (BD Biosciences). Germinal center B cells were detected by staining with FITC-labeled anti-GL7 mAbs, PE-labeled anti-Fas mAbs, and Peridinin-Chlorophyll-protein-labeled anti-B220 mAb (BD Biosciences). Sera from immunized mice were collected, and antigen-specific IgM, IgG, IgG1, and IgG2a antibodies were measured by ELISA. Briefly, serum samples were added in a 3-fold serial dilution onto plates pre-coated with 10 μg/ml KLH protein. Antigen-specific antibodies were detected with biotinylated anti-mouse IgG, HRP-conjugated anti-mouse IgG1, and HRP-conjugated anti-mouse IgG2a antibodies (Southern Biotechnology Associates). In addition, FACS-sorted CD45.2+ CD4+ CD44+ YFP+ T cells were restimulated with anti-CD3 for 4 h, and gene expression was determined by real-time RT-PCR.

Quantitative Real-time RTPCR—Total RNA was prepared from T cells using TRIzol regent (Invitrogen). cDNAs were synthesized using the SuperScript reverse transcriptase and oligo(dT) primers (Invitrogen), and gene expression was examined with a Bio-Rad iCycler optical system using a iQ™ SYBR Green real-time PCR kit (Bio-Rad Laboratories). The data were normalized to β-actin reference. The following primer pair for c-Maf was used: forward, GCAGAGACAGCTCTGAGGTCG, and reverse, CGAGCTTGGCCTGCAACTAGC. The primers for IL-21, CXCR5, Bcl6, Batf, c-Maf, Blimp-1, and β-actin were previously described (8, 18).

RESULTS AND DISCUSSION

STAT5 Inhibits Tfh Differentiation—We first examined the role of STAT5 in Tfh generation in vitro. Previously, we have shown that T cells activated in vitro in the presence of IL-6 or IL-21 but without TGFB, IL-4, and IFN-γ signaling preferentially acquire Tfh gene expression and function to promote humoral immunity in vivo. To determine the role of STAT5 in Tfh generation, naive CD4+ T cells from OT-II mice activated with Ova peptide and irradiated antigen-presenting cells under neutral (anti-TGFβ, anti-IL-4, and anti-IFN-γ) or IL-6 treatment (IL-6, anti-TGFB, anti-IL-4, and anti-IFN-γ) conditions were infected with a constitutively active form of STAT5 or a vector control retrovirus that contains an IRES-GFP. Four days after infection, we sorted the retrovirus-transduced cells based on GFP expression and analyzed for their gene expression by real-time RTPCR. Expression of constitutively active STAT5 dramatically decreased the expression of Tfh-specific genes, such as CXCR5, Bcl6, c-Maf, Batf, and IL-21 (Fig. 1A). Interestingly, we found that the expression of transcription factor Blimp-1 (encoded by Prdm1) was strongly enhanced by constitutively active STAT5 under IL-6 treatment condition. This finding correlates with the previously published observation that IL-2Ra hi cells exhibit strong Blimp-1 expression, which represses Bcl6 (16). Thus, constitutively active STAT5 inhibits Tfh differentiation, which is sufficient to suppress most of the Tfh-specific genes through induction of Blimp-1.
STAT5 Deficiency Enhances Tfh Development—Our data indicated a potential role of STAT5 in regulation of Tfh development. We further examined the role of STAT5 in Tfh development by using Stat5-deficient CD4$^+$ T cells. C57BL/6 Stat5-deficient mice with deletion of the entire Stat5A/5B locus (Stat5$^{-/-}$) and mice with the entire Stat5A/5B locus gene flanked with loxP sites (Stat5$^{fl/fl}$) were previously described (17). The Stat5$^{fl/fl}$ mice were generated by breeding Stat5$^{-/-}$ and Stat5$^{fl/fl}$ mice. We used a GFP-containing bicistronic retrovirus expressing Cre or a vector control virus and activated in the presence or absence (none) of the indicated cytokines. The data shown were normalized by the expression of a reference gene Actb. C, naive OT-II cells were activated under neutral conditions in the presence or absence (none) of IL-6/IL-21 and neutralizing antibodies to IL-2 (all-2) for 4 days. mRNA expression of Tfh-related genes was analyzed by real-time RT-PCR analysis (upper). The data shown were normalized by the expression of a reference gene Actb. CXCR5- and Bcl6-expressing cells were also assessed by FACS staining (lower). The numbers in the dot plot quadrants represent the percentages. D, naive CD4$^+$ T cells from Stat5$^{fl/fl}$ and Stat5$^{-/-}$ mice were activated with plate-bound anti-CD3 and anti-CD28, coinfected with two bicistronic retroviruses expressing Cre-GFP or GFP vector and Blimp-hCD2 or hCD2, and further activated under neutral conditions (anti-IL-4, anti-IFN$\gamma$, and anti-TGF$\beta$) in the presence or absence (none) of IL-6. GFP$^+$ hCD2$^+$ cells were sorted and restimulated for 4 h with anti-CD3 for real-time RT-PCR analysis. The data shown were normalized by the expression of a reference gene Actb. The experiments were performed two times with consistent results. Error bars in panels A–D indicate S.D.

**FIGURE 1. Stat5 negatively regulates Tfh-specific gene expression.** A, naive OT-II T cells were activated under neutral conditions (in the presence of antibodies against IL-4, IFN$\gamma$, and TGF$\beta$) in the presence or absence of IL-6 and infected with a GFP-containing bicistronic retrovirus expressing constitutively active STAT5 (STAT5) or a vector control virus (C). GFP$^+$ cells were sorted and restimulated for 4 h with anti-CD3 for real-time RT-PCR analysis. The data shown were normalized by the expression of a reference gene Actb. B, naive CD4$^+$ T cells from Stat5$^{fl/fl}$ and Stat5$^{-/-}$ mice were infected with a GFP-containing bicistronic retrovirus expressing Cre or a vector control virus and activated in the presence or absence (none) of the indicated cytokines. The data shown were normalized by the expression of a reference gene Actb. C, naive OT-II cells were activated under neutral conditions in the presence or absence (none) of IL-6/IL-21 and neutralizing antibodies to IL-2 (all-2) for 4 days. mRNA expression of Tfh-related genes was analyzed by real-time RT-PCR analysis (upper). The data shown were normalized by the expression of a reference gene Actb. CXCR5- and Bcl6-expressing cells were also assessed by FACS staining (lower). The numbers in the dot plot quadrants represent the percentages. D, naive CD4$^+$ T cells from Stat5$^{fl/fl}$ and Stat5$^{-/-}$ mice were activated with plate-bound anti-CD3 and anti-CD28, infected on day 2, and subsequently further differentiated under IL-6 treatment, Th17 (IL-6 and TGF$\beta$), and regulatory T cell (TGF$\beta$) conditions. Four days after infection, GFP-positive cells were analyzed for their gene expression by real-time RT-PCR analysis. The data shown were normalized by the expression of a reference gene Actb. The experiments were performed two times with consistent results. Error bars in panels A–D indicate S.D.
IL-6-treated cells dramatically decreased the mRNA level of Bcl6 repressor, Blimp-1 (Fig. 1B). These results suggest that STAT5 controls Tfh development through Blimp-1.

Because IL-2 is important for Blimp-1 expression (16) and largely functions through STAT5, we next activated naive CD4+ T cells under neutral or IL-6/IL-21 treatment conditions in the presence or absence of IL-2-neutralizing antibodies (Fig. 1C). Activation of cells under IL-6/IL-21 treatment condition significantly increased the mRNA expression of Tfh-specific genes and up-regulated CXCR5 and Bcl6 protein expression (Fig. 1C), which were further enhanced by blocking of IL-2. In addition, blockade of IL-2 resulted in suppression of Blimp-1 expression. Thus, our data indicate that IL-2 signaling controls Blimp-1 expression and Tfh differentiation.

To further determine whether STAT5 negatively regulates Tfh differentiation through Blimp-1, we overexpressed Blimp-1 in Stat5-deficient CD4+ T cells (Fig. 1D). Naive CD4+ T cells from Stat5fl/+ and Stat5fl/- mice were differentiated in vitro with plate-bound anti-CD3 and anti-CD28, infected on day 2 with two viruses expressing Cre-GFP or GFP vector and Blimp-1-hCD2 or hCD2 vector, and subsequently further differentiated under neutral or IL-6 treatment conditions. Four
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Days after infection, we sorted GFP\(^+\)hCD2\(^+\) T cells and analyzed for their gene expression by real-time RT-PCR. Under IL-6 treatment condition, Blimp-1 overexpression significantly decreased the expression of Tfh-associated genes such as CXCR5, Bcl6, c-Maf, Batf, and IL-21 in the presence of STAT5 or even in the absence of STAT5 (Fig. 1D). Thus, altogether our in vitro data suggest that STAT5-mediated Blimp-1 expression is sufficient to antagonize Tfh program.

Next, we examined the effect of STAT5 deficiency on Tfh generation and function in vivo. Stat5\(^{fl/+}\) and Stat5\(^{fl/-}\)/Mx1Cre/YFP mice were treated with poly(I:C), which can induce Cre expression, resulting in deletion of a "floxed" Rosa26 locus and subsequent YFP expression to track floxed Stat5 floxed/yellow fluorescent protein (YFP) mice (data not shown). Importantly, the population of Tfh cells within CD4\(^+\) YFP\(^+\) splenic T cells was markedly increased in naive Stat5\(^{fl/-}\) relative to Stat5\(^{fl/+}\) (Fig. 3, A, upper). In contrast, the population of CD4\(^+\)CXCR5\(^+\) Tfh cells within CD4\(^+\) YFP\(^+\) splenic T cells was comparable between naive Stat5\(^{fl/-}\) and Stat5\(^{fl/+}\) (Fig. 3, A, middle). In addition, the population of germinal center B cells (Fas\(^+\)GL7\(^+\)) was greatly increased in Stat5\(^{fl/-}\) relative to Stat5\(^{fl/+}\) CD4Cre/YFP mice (Fig. 3, A, lower). Therefore, STAT5 deficiency intrinsically results in an increase of Tfh cells in vivo, leading to an increase of germinal center B cells.

Furthermore, we investigated the effect of Stat5 deficiency-induced elevation of Tfh cells on B cell tolerance. Stat5\(^{fl/-}\)/CD4Cre/YFP mice were crossed with Ig\(^{HEL}\)SHEL transgenic mice, which bear rearranged heavy (H) and Ig\(_\lambda\) L chain genes encoding a B cell receptor specific for hen egg lysozyme (HEL) and express soluble HEL (sHEL). Due to self-antigen-induced B cell tolerance, there is little production of HEL-specific IgM in wild-type Ig\(^{HEL}\)SHEL mice (21). As in nontransgenic mice (Fig. 3A), the population of CD4\(^+\)CXCR5\(^+\) Tfh cells within CD4\(^+\) YFP\(^+\), but not CD4\(^+\) YFP\(^-\), splenic T cells was markedly increased in Stat5\(^{fl/-}\) relative to Stat5\(^{fl/+}\) CD4Cre/YFP/Ig\(^{HEL}\)SHEL mice (Fig. 3B, upper and middle). The population of

FIGURE 3. Increase of Tfh and germinal center B cells and impairment of B cell tolerance in mice lacking STAT5 specifically in T cells. A, increase of Tfh and germinal center B cells in Stat5\(^{fl/-}\)/CD4Cre/YFP mice. Splenocytes from 2–3-month-old Stat5\(^{fl/+}\) and Stat5\(^{fl/-}\)/CD4Cre/YFP mice were stained with antibodies to CD4 and CXCR5 or B220, Fas, and GL7. Percentages indicate CD4\(^+\) CXCR5\(^+\) cells in the gated CD4\(^+\) YFP\(^+\) (upper) or CD4\(^+\) YFP\(^-\) (middle) cells and Fas\(^+\) GL7\(^+\) cells in the gated B220\(^-\) cells (lower). B, increase of Tfh and germinal center B cells in Stat5\(^{fl/-}\)/CD4Cre/YFP/Ig\(^{HEL}\)SHEL mice. Splenocytes from Stat5\(^{fl/+}\) and Stat5\(^{fl/-}\)/CD4Cre/YFP/Ig\(^{HEL}\)SHEL mice were stained with antibodies to CD4 and CXCR5 or B220, Fas and GL7. Percentages indicate CD4\(^+\) CXCR5\(^+\) cells in the gated CD4\(^+\) YFP\(^+\) (upper) or CD4\(^+\) YFP\(^-\) (middle) cells and Fas\(^+\) GL7\(^+\) cells in the gated B220\(^-\) cells (lower). C, increased levels of HEL-specific antibodies in Stat5\(^{fl/-}\)/CD4Cre/YFP/Ig\(^{HEL}\)SHEL mice. Sera from 2–3-month-old control (Stat5\(^{fl/+}\), Stat5\(^{fl/-}\), Stat5\(^{fl/-}\)) and Stat5\(^{fl/-}\)/CD4Cre/YFP/Ig\(^{HEL}\)SHEL mice were obtained. HEL-specific antibody levels in the serum of each individual mouse were determined by ELISA. Data shown were obtained from five mice of each genotype. Error bars indicate S.D.
Fas+GL7+ germlinal center B cells was also greatly increased in Stat5β/− relative to Stat5β/+ CD4Cre/YFP/IgHELsHEL mice (Fig. 3B, lower). Importantly, serum levels of HEL-specific IgM were markedly increased in Stat5β/− relative to control CD4Cre/YFP/IgHELsHEL mice (Fig. 3C). These data demonstrate that Stat5 deficiency in CD4+ T cells causes an increase of Tfh cells and germlinal center B cells in IgHELsHEL transgenic mice, resulting in an impairment of B cell tolerance.

Significant advances in the understanding of Tfh cell differentiation and involvement in immune responses have been made over the past few years. However, the signaling events required for commitment of CD4+ T cells to the Tfh subset are only beginning to be elucidated. IL-21/IL-6, STAT3, and Bcl6 are required for Tfh cell generation, although the precise mechanisms controlling the expression of these major Tfh factors have yet to be resolved. We have analyzed the role of Stat5 in developmental regulation of Tfh cells. We found that Stat5, which is known as a critical regulator of regulatory T cell and Th17 reciprocal development, also played a critical role in Tfh development and in B cell-mediated humoral immune responses. IL-2/STAT5 promotes the expression of Bcl6 repressor Blimp-1 and inhibits the differentiation of Tfh cells. Mice with Stat5 deficiency in CD4+ T cells exhibited an increase of Tfh and germlinal center B cells and impairment of B cell tolerance, suggesting that Stat5 signaling controls the Tfh generation and humoral immunity. This finding highlights a negative cross-talk between the IL-2/STAT5/Blimp-1 and the IL-6/IL-21/Bcl6 pathways in Tfh development and may help us to find ways to treat antibody-mediated autoimmune diseases associated with expansion of Tfh cells.

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