Supporting Online Material for

Bcl6 Mediates the Development of T Follicular Helper Cells

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Material and Methods

Mice. Bcl6-deficient mice on 129xC57BL/6 mixed background were kindly provided by Dr. Dalla-Favera at Columbia University (*1*). Bcl6-deficient mice were crossed with OT-II mice and mice at 6-8 weeks of age were used for in vitro differentiation. Rag1-deficient mice on C57BL/6 background and B6.SJL (CD45.1) mice were purchased from Jackson Laboratories. IL-21 KO and IL-21R KO mice on a 129 × C57BL/6 F1 mixed background were obtained from NIH Mutant Mouse Regional Resource Centers. 6 to 8 weeks old mice were used in experiments. Mice were housed in the SPF animal facility at M. D. Anderson Cancer Center and the animal experiments were performed using protocols approved by Institutional Animal Care and Use Committee.

T cell differentiation. Differentiation of OT-II cells was performed as previously described (*2*). $CD62L^{hi}CD44^{lo}CD25^{neg}CD4^{+}$ T cells were isolated and cultured with irradiated splenic APC plus OVA peptide (10 μg/ml) (Th0) and in the presence of polarizing reagents for 5 days: 10 μg/ml of anti-IL-4 (11B11) and 10 ng/ml of IL-12 for Th1; 10 μg/ml of anti-IFNγ (XMG 1.2) and 10 ng/ml of IL-4 for Th2; 30 ng/ml of IL-6, 5 ng/ml of TGFβ, 50 ng/ml IL-23, 10 μg/ml of anti-IL-4 and 10 μg/ml of anti-IFNγ for Th17; 5 ng/ml of TGFβ, 10 μg/ml of anti-IL-4 and 10 μg/ml of anti-IFNγ for Tr; 30 ng/ml of IL-6, 10 μg/ml anti-IFNγ, 10 μg/ml anti-IL-4 and 20 μg/ml anti-TGF-β (1D11) neutralizing Abs for Tfh. IL-4, IL-6, IL-12 and TGFβ were purchased from Peprotech. IL-23 was purchased from R&D. To characterized the in vitro-differentiated CD4 T cells under these conditions, 5 days after activation these cells were restimulated with plate-bound anti-CD3 Ab (5 μg/ml) for 4 hours for real-time PCR analysis, or for 24 hours for cytokine measurement by ELISA. In addition, cells were restimulated with PMA and ionomycin in the presence of Golgi-stop for 5 h, after which Foxp3-, IL-17- and IFNγ-producing cells were analyzed using intracellular staining. Intracellular staining for Foxp3 was performed by using a Foxp3 staining kit (eBioscience).

Retroviral transduction. Naïve $CD4^{+}CD25^{-}CD62L^{hi}CD44^{lo}$ T cells from OT-II mice were FACS-sorted and activated with Ova peptide and irradiated wild-type splenic APCs under neutral (anti-IFNγ, anti-IL-4 and anti-TGFβ), Tfh (IL-6, anti-IFNγ, anti-IL-4 and anti-TGFβ), Th1 (IL-12, anti-IL-4), Th2 (IL-4, anti-IFNγ), Th17 (TGF-β, IL-6, IL-23, anti-IL-4, anti-IFN-γ) conditions. 24 hours after activation, cells were infected by retroviruses expressing Bcl6, Bcl6-ZF3, Bcl6-ZF5 or control empty vector (containing only IRES-GFP). 4 days after infection, GFP^{+} cells were FACS sorted and restimulated with PMA and ionomycin in the presence of Golgi-stop for 4 h, after which IL-17- and/or IFNγ-expressing cells were analyzed using intracellular staining. Also, GFP^{+} cells were restimulated with anti-CD3 for 4 h, and gene expression was determined by real time RT-PCR.

Reporter assay. RORγ, wild-type Bcl6, Bcl6-ZF3 or Bcl6F-Z5 was cloned into bicistronic retroviral vector pGFP-RV provided by Dr. Ken Murphy at Washington University that contains IRES-regulated GFP. The expression vectors were transfected into EL-4 cells with a luciferase construct containing IL-17 minimal promoter with *CNS2* element (*3, 4*). The dual-luciferase reporter system (Promega) was used to assay Firefly and Renilla luciferase activity in each sample. Renilla luciferase was used to normalize transfection efficiency and luciferase activity.

Quantitative real-time PCR. Total RNA was prepared from T cells using TriZol regent (Invitrogen). cDNA were synthesized using Superscript reverse transcriptase and oligo(dT) primers (Invitrogen) and gene expression was examined with a Bio-Rad iCycler Optical System using iQ^{TM} SYBR green real-time PCR kit (Bio-Rad Laboratories, Inc.). The data were normalized to β-actin reference. The primers for IL-21, IL-17, IL-17F, IL-4, IFNγ, RORγ, T-bet, total Bcl-6 (with both primers in the coding sequence), exogenous Bcl-6 (with one primer in the coding sequence and one in the 3' untranslated region that is not included in the retroviral vector), IL-6R, and CXCR5 and β-actin were previously described (*2, 5*).

Keyhole Limpet Hemocyanin (KLH) Immunization. Bcl6-deficient mice and their wild-type controls (6–8 wk old; three per group) were immunized with KLH (0.5 mg/ml) emulsified in CFA (0.5 mg/ml) at the base of the tail (100 μl each mouse, normalized by body weight). Seven days after immunization, these mice were sacrificed and analyzed individually. The germinal center B cells were determined by staining with FITC-labeled GL7, PE-labeled FAS and PerCP-labeled anti-B220 mAb (Pharmingen). The Tfh cell induction was determined by staining with PerCP-labeled anti-CD4 mAb (Pharmingen) and biotinylated anti-CXCR5 mAb (Pharmingen), followed by APC-labeled streptavidin (Jackson ImmunoResearch Laboratories, Inc.). Sera from immunized mice were collected, and antigen-specific IgM, IgG, IgG2a and IgE antibodies were measured by using ELISA. Briefly, serum samples were added in a 3-fold serial dilution onto plates precoated with 100 μg/ml KLH. To analyze the role of Bcl6 in regulation of T cell responses *in vivo,* spleen cells from KLH-immunized mice were stimulated in 96-well plates as triplicates with or without KLH. Effector cytokines (IFN-γ, IL-4, IL-17 and IL-22) were analyzed 4 days later by ELISA (Pharmingen).

Adoptive transfer and mixed bone marrow chimera studies. FACS sorted naïve CD4 T cells from wild-type or Bcl6 knockout mice and B cells from wild type mice were mixed at 1:1 ratio. These cells were intravenously transferred into syngenic *Rag1*$^{-/-}$ mice (10×10^6 cells/mouse). The recipient mice were immunized subcutaneously with 100 μg of KLH protein emulsified in CFA. In Figure 4B, recipient mice were treated with a 300 μg of anti-IL-4 mAbs at the time of immunization (day 0) and on days 2 and 4. Seven days after immunization, spleen cells from the recipient mice were isolated and stained with PerCP-labeled anti-CD4 mAb plus biotinylated anti-CXCR5 mAb, followed by APC-labeled streptavidin, or stained with FITC-labeled GL7, PE labeled FAS and PerCP-labeled anti-B220 mAb. Sera from immunized mice were collected, and antigen-specific IgM, IgG, IgG2a and IgE antibodies were measured by using ELISA. Effector cytokines (IFN-γ, IL-4, IL-17 and IL-22) were analyzed 4 days later by ELISA (Pharmingen).

To generate mixed bone marrow chimera, T cell-depleted bone marrow cells were obtained from B6.SJL (CD45.1) or Bcl6 KO mice and mixed 1:1 ratio before being transferred into irradiated *Rag1*$^{-/-}$ mice (10^7 cells/transfer, 750 rad). Eight weeks later, the recipient mice were immunized with KLH and analyzed as above.

Immunohistochemical analysis. Fresh mouse spleen tissues were embedded in OCT and frozen with isopentane in Histobath. Tissue blocks were sliced 6 μm with cryotome. Slides were fixed with acetone cold. Biotin-labeled anti-mouse PNA were applied as primary Ab followed by biotinylated anti-rat secondary Ab and avidin-peroxidase complex reagent. Novared was used as

substrate. PNA and other reagent are from Vector Laboratary. Slides for PNA staining were count stained with Hematoxlin.

Supplementary References

1. B. H. Ye *et al.*, *Nat Genet* **16**, 161 (Jun, 1997).
2. R. I. Nurieva *et al.*, *Immunity* **29**, 138 (Jul, 2008).
3. A. M. Akimzhanov, X. O. Yang, C. Dong, *J Biol Chem* **282**, 5969 (Mar 2, 2007).
4. X. O. Yang *et al.*, *Immunity* **28**, 29 (Jan, 2008).
5. R. J. Lund, E. K. Ylikoski, T. Aittokallio, O. Nevalainen, R. Lahesmaa, *Eur J Immunol* **33**, 1105 (Apr, 2003).

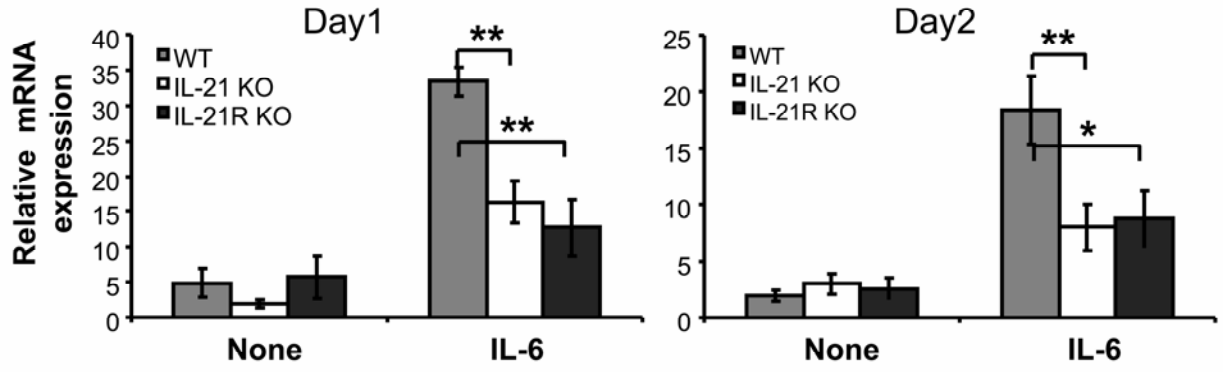

Figure S1. IL-21 signaling is involved in the regulation of Tfh differentiation by IL-6. Naïve $CD4^+$ T cells from wild-type, IL-21 KO and IL-21R KO mice were activated under neutral conditions or together with IL-6 (both in the presence of antibodies against IL-4, IFNγ and TGFβ) for 1 and 2 days. Bcl6 mRNA expression was analyzed by real-time RT- PCR. The data shown were normalized to the expression of a reference gene *Actb*. The graph shows means ± standard deviation (SD). *, $p < 0.005$; **, $p < 0.001$, t-test.

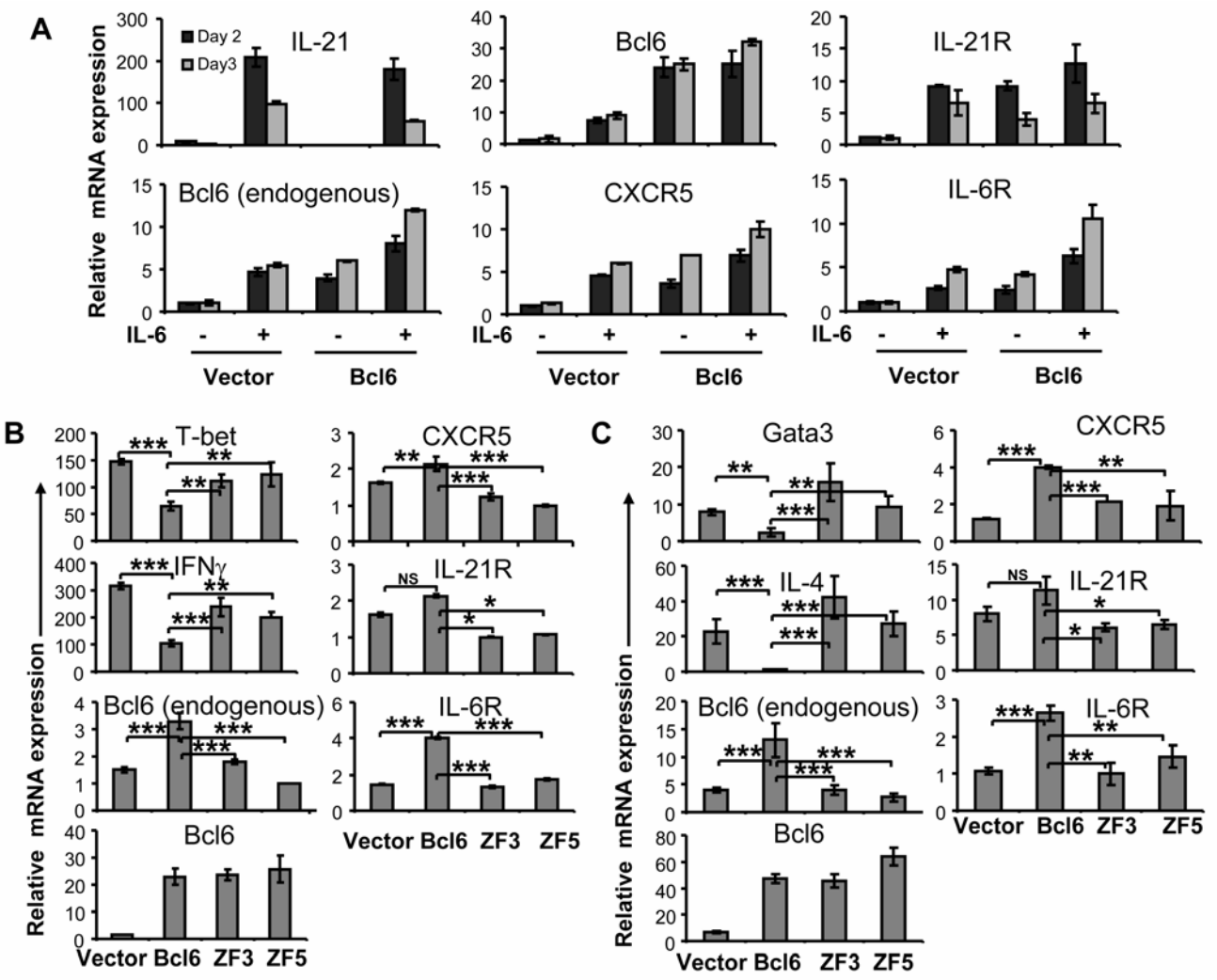

Figure S2. Regulation of Tfh, Th1 and Th2 differentiation by Bcl6. (A) Naïve OT-II T cells were activated under neutral conditions or together with IL-6 (both in the presence of antibodies against IL-4, IFNγ and TGFβ) and infected with an IRES-GFP-containing bicistronic retrovirus expressing Bcl6 or a vector control virus. GFP^+ cells were sorted on day 2 and 3 after infection and restimulated for 4 hours with anti-CD3. mRNA expression of the indicated genes was analyzed by real-time RT–PCR. The data shown were normalized to the expression of a reference gene *Actb*. The graph shows means ± standard deviation (SD). (B-C) Naïve OT-II T cells were activated with Ova peptide and irradiated wild-type APC under Th1 (B) or Th2 (C) conditions and infected with retroviruses expressing Bcl6, Bcl6 mutants, or vector alone. FACS-sorted GFP^+ cells were restimulated for 4 hours with anti-CD3. mRNA expression of indicated genes was analyzed by real-time RT–PCR. The data shown were normalized to the expression of a reference gene *Actb*. The graph shows means ± standard deviation (SD). *, $p < 0.05$; **, $p < 0.005$, ***$p<0.001$, t-test. The experiments were repeated two times with consistent results.

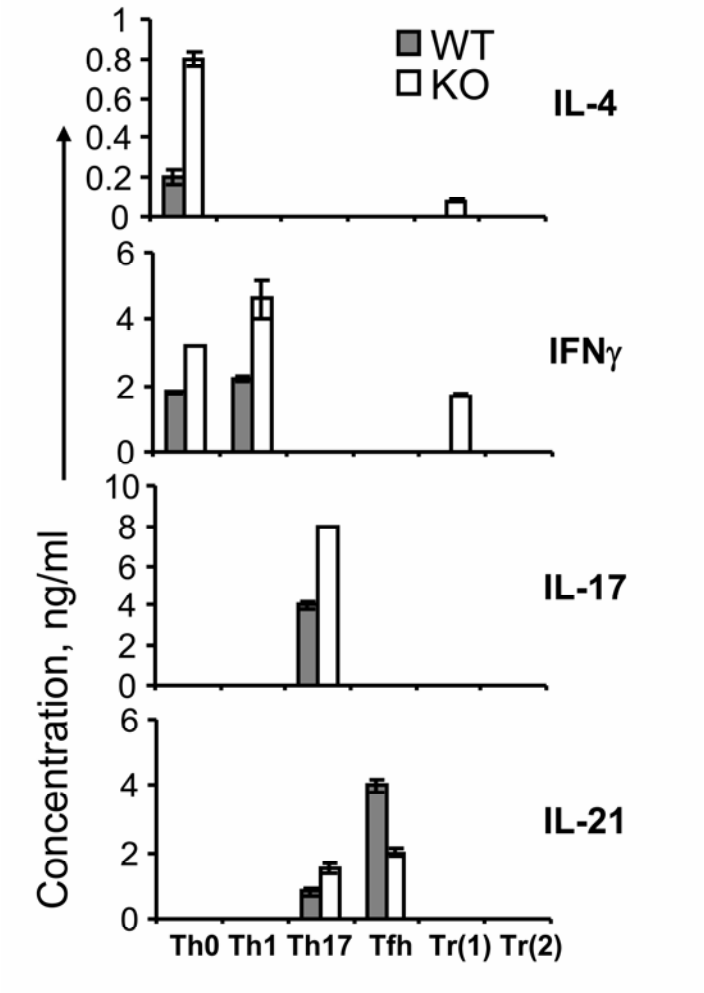

Figure S3. Regulation of Th cell differentiation by Bcl6. Naïve Th cells from Bcl6 KO OT-II mice and their littermate control mice were activated under neutral, Th1, Tfh, Th17, and Tr [Tr(1), TGFβ; Tr(2), TGFβ, anti-IL-4 and anti-IFNγ] conditions. Seven days later, cells were restimulated with plate-bound anti-CD3 for 24 hrs, and cytokines were measured by ELISA. The graph shows means ± standard deviation (SD). The experiments were repeated three times with consistent results.

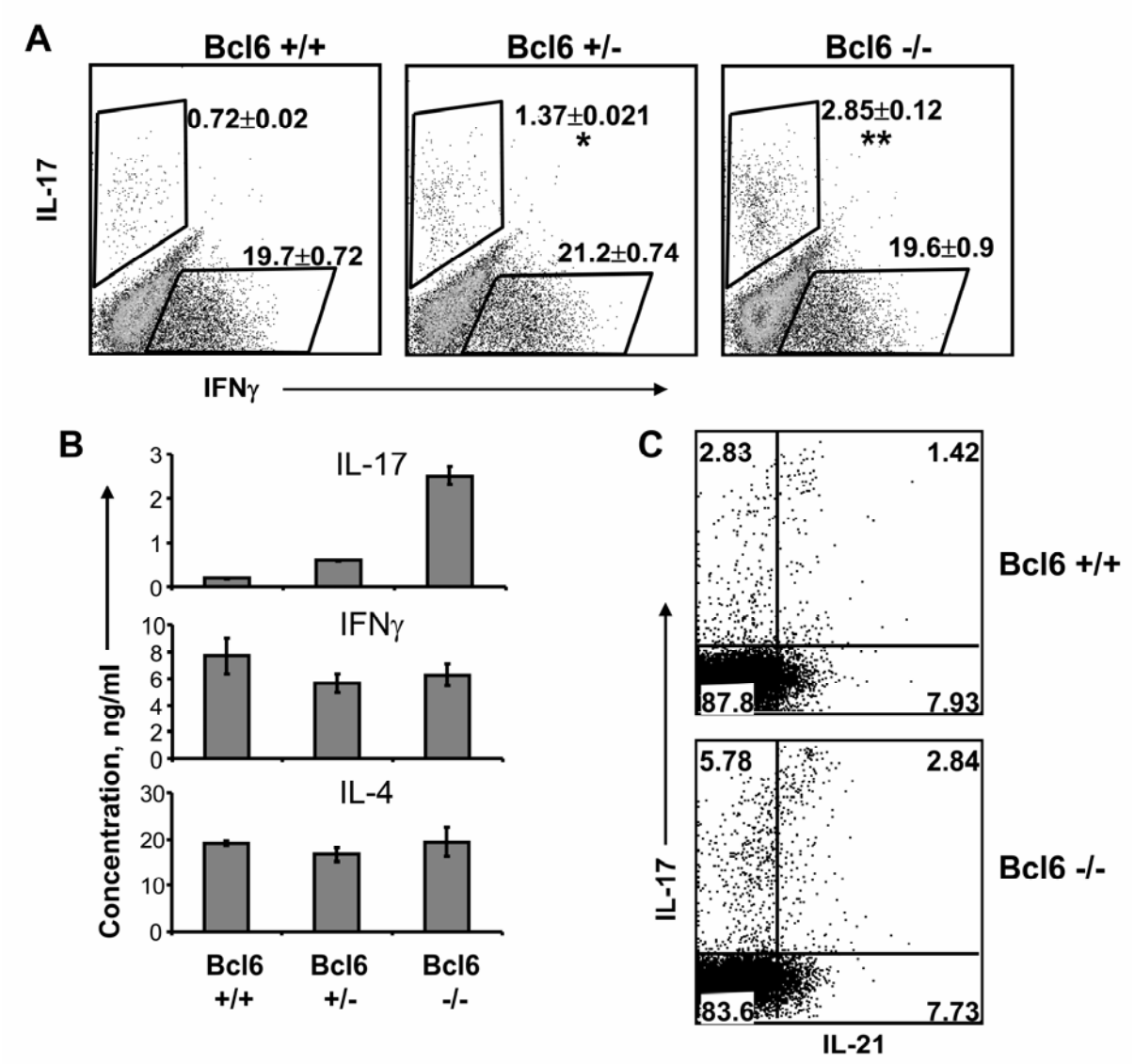

Figure S4. Bcl6 deficiency increases IL-17 expression in vivo. $CD4^{+}CD25^{-}CD44^{hi}CD62L^{lo}$ cells were sorted from 4 months old Bcl6 KO mice and their littermate control mice. (A) Cells were assessed for IFNγ and IL-17 production after PMA/ionomycin restimulation through intracellular staining. Numbers in dot-plot quadrants represent the percentages. p values were calculated using t test comparing $IL\text{-}17^{+}$ $CD4^{+}$T cells between wild-type and Bcl6 knockout mice and are indicated as follows: *, $p < 0.005$, **, $p < 0.001$. (B) $CD4^{+}CD25^{-}CD44^{hi}CD62^{lo}$ cells were restimulated with plate-bound anti-CD3 for 48 hrs, and cytokines were measured by ELISA. The graph shows means ± standard deviation (SD). The experiments were repeated two times with consistent results. (C) Total $CD4^{+}$ T cells from spleen of wild-type and Bcl6 KO mice (n=2 per group) were assessed for IL-21 and IL-17 production by intracellular staining after PMA/ionomycin restimulation. Numbers in dot-plot quadrants represent the percentages of cells.

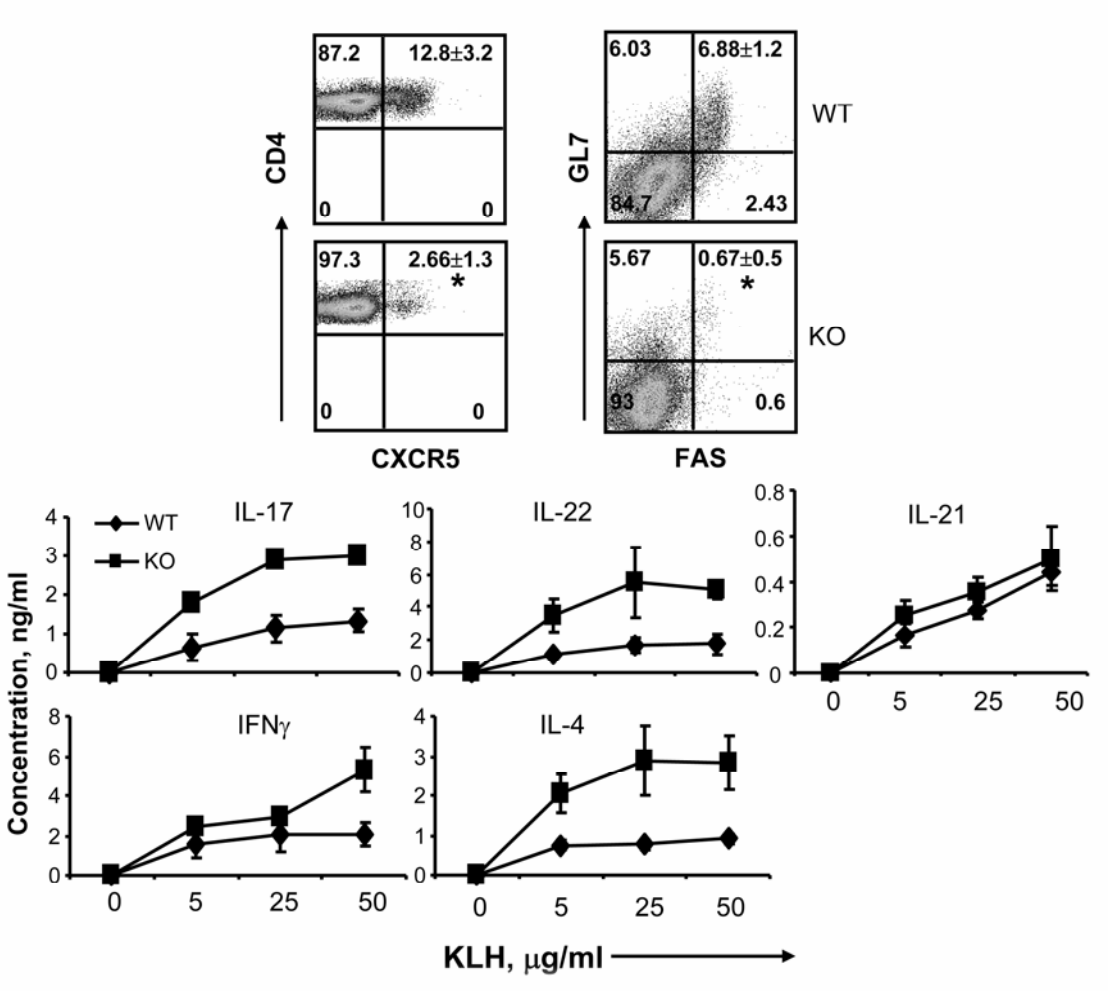

Figure S5. Bcl6 is required for generation of Tfh cells. Bcl6+/+ and Bcl6-/- mice were immunized with KLH in CFA (3 mice per group). Seven days after the immunization, experimental mice were sacrificed and the germinal center B cells were determined by staining with FITC-labeled GL-7, PE-labeled FAS and PerCP-labeled anti-B220 mAb. The Tfh cells were analyzed by staining with PerCP-labeled anti-CD4 mAb and biotinylated anti-CXCR5 mAb, followed by APC-labeled streptavidin. Numbers in dot plot quadrants represent the percentages. Spleen cells from immunized mice were stimulated in 96-well plates as triplicates with the indicated concentration of KLH. Effector cytokines (IL-17, -22, -4, -21 and IFNγ) were measured after 4 days of treatment. p values were calculated using t test comparing $CXCR5^+$ cells and $GL7^+Fas^+$ cells between wild-type and Bcl6 knockout mice and are indicated as follows: *, $p < 0.005$. The data represent at least three independent experiments with consistent results.

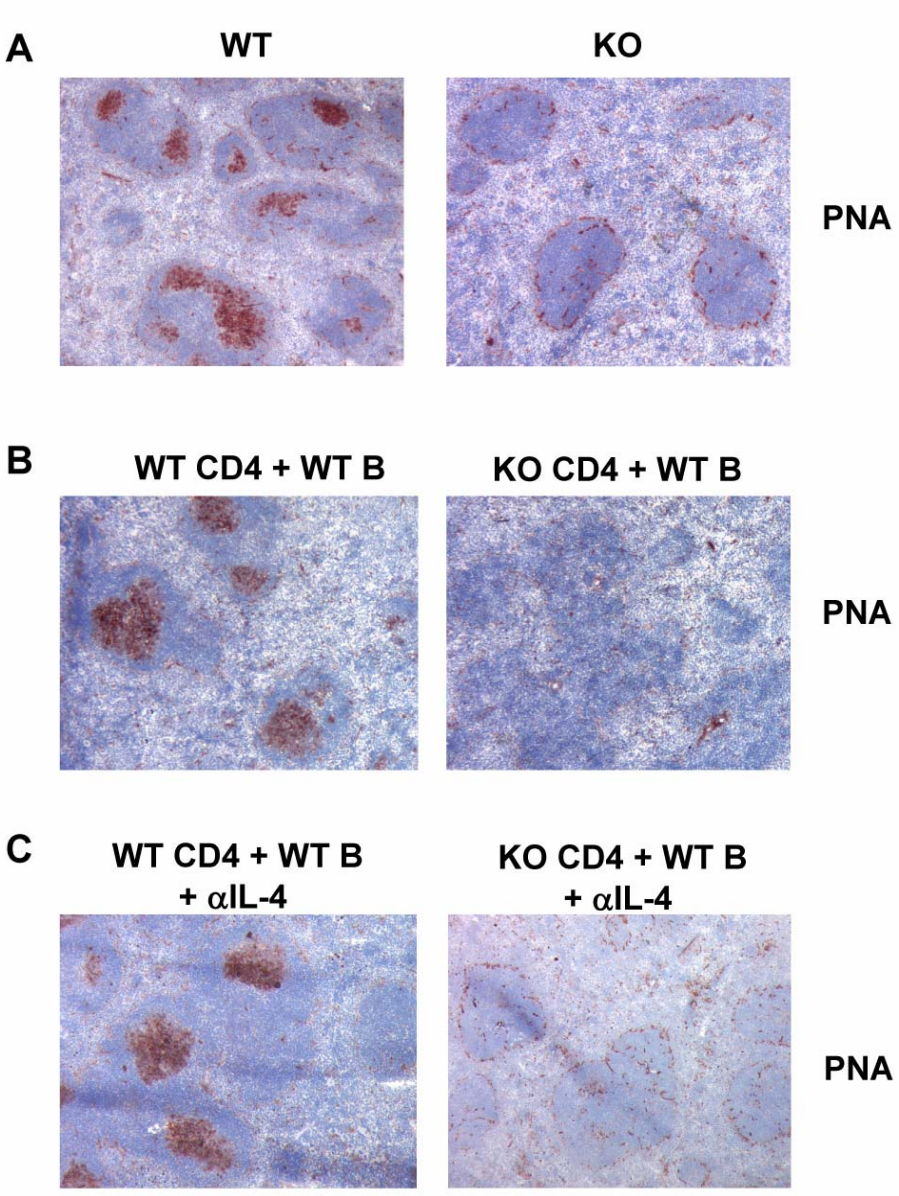

Figure S6. Regulation of germinal center formation by Bcl6. (A) Bcl6+/+ and Bcl6-/- mice were immunized with KLH in CFA (3 mice per group). (B-C) Naïve $CD4^+$ cells from WT or Bcl6 KO mice were mixed with wild-type B cells before being intravenously transferred into *Rag1*$^{-/-}$ mice (3 mice per group). The recipient mice were immunized subcutaneously with KLH emulsified in CFA, without (B) or with (C) treatment of an anti-IL-4. Seven days later, germinal centers in the spleens of KLH-immunized (A-B) mice were identified by PNA staining (brown).

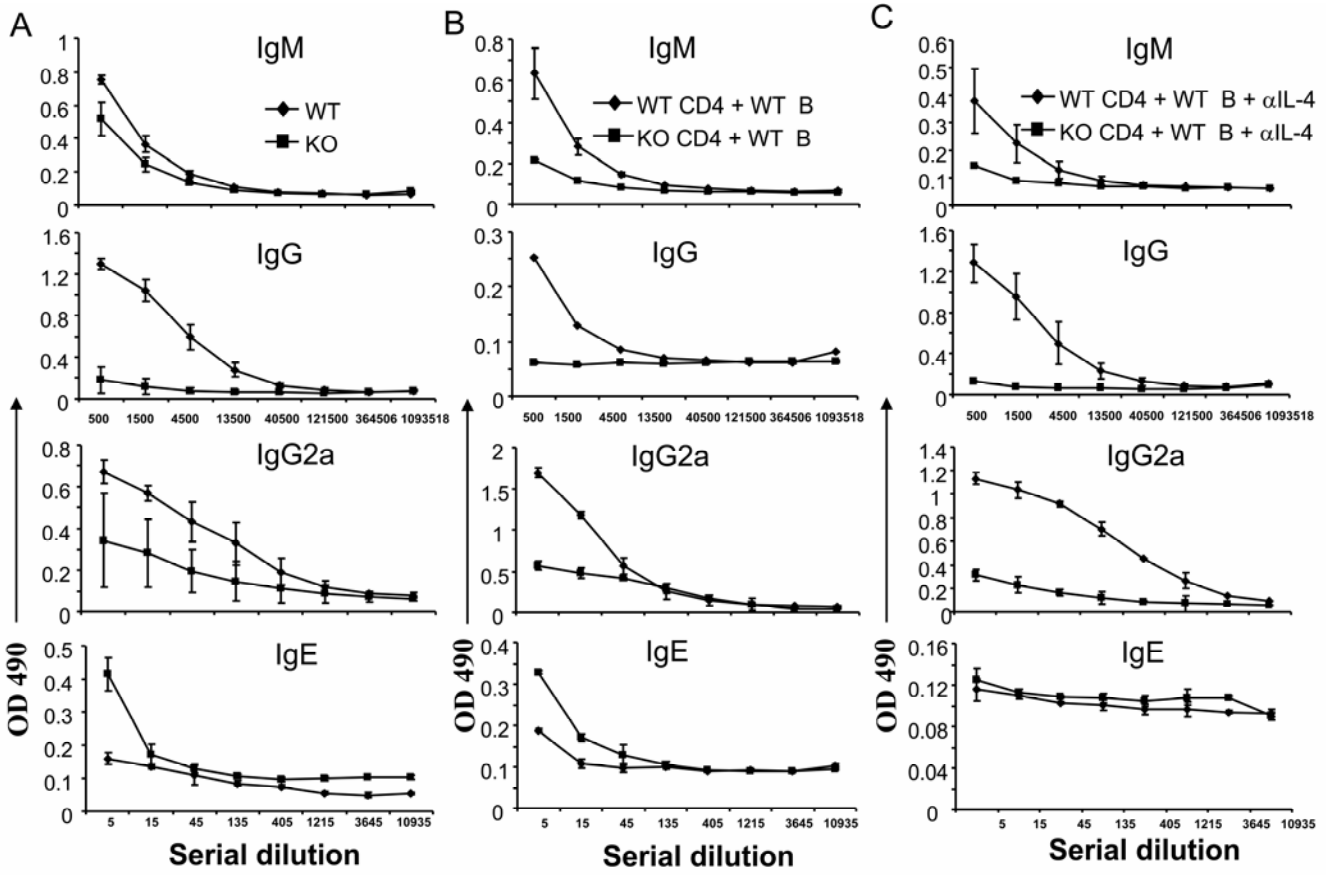

Figure S7. Regulation of antibody responses by Bcl6. (A). Bcl6 KO mice and littermate controls were immunized with KLH in CFA. (B-C) Naïve $CD4^+$ cells from WT or Bcl6 KO mice were mixed with wild-type B cells before being intravenously transferred into *Rag1*$^{-/-}$ mice. The recipient mice were then immunized subcutaneously with KLH emulsified in CFA, without (B) or with treatment of anti-IL-4 (C). Seven days after the immunization, anti-KLH antibodies (IgM, IgG and IgG2a) were measured in the sera by ELISA. The graph shows means ± standard deviation (SD). The experiments were repeated two times with consistent results.

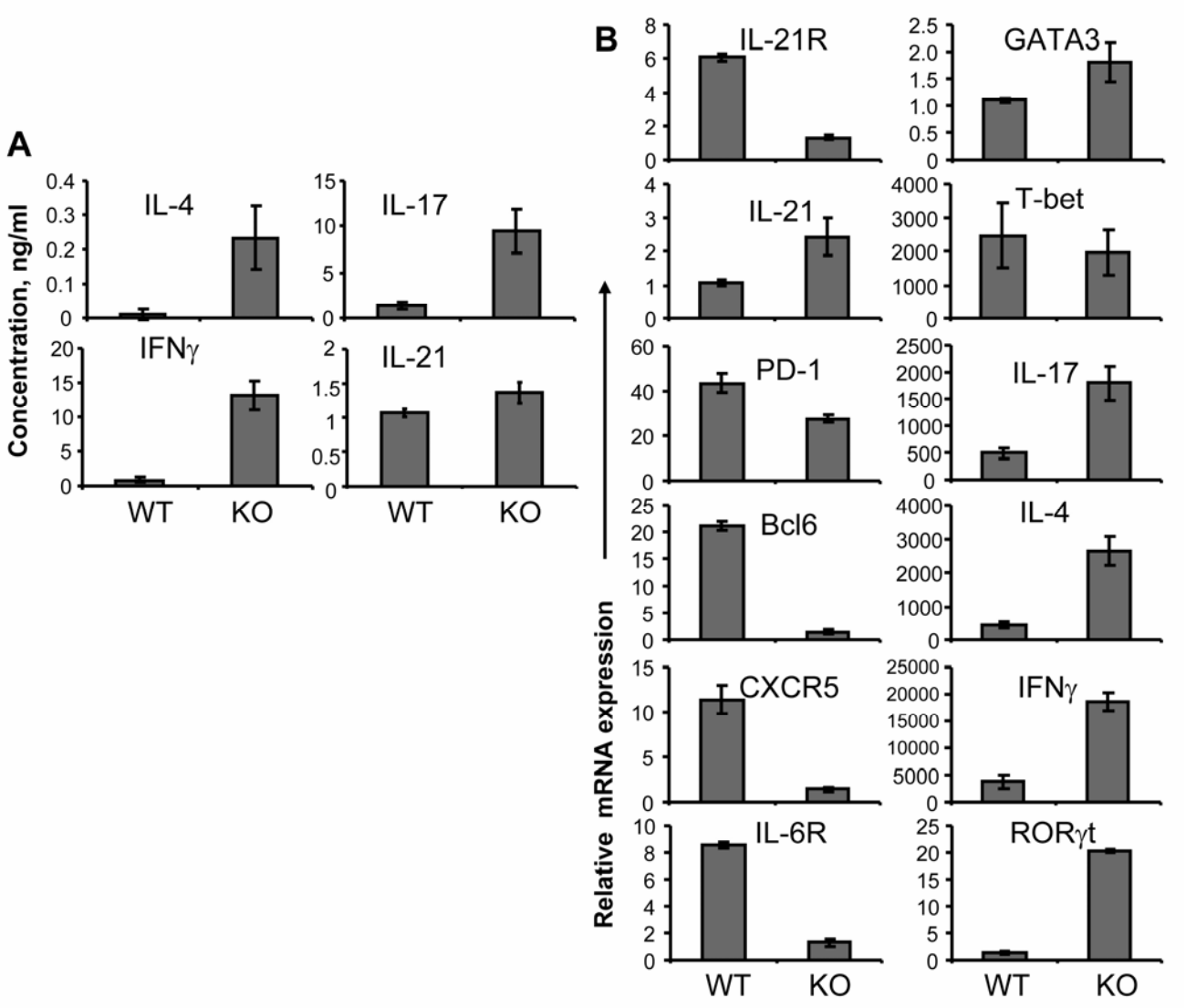

Figure S8. Bcl6 expression in T cells regulates Th1, Th2 and Th17 differentiation. (A-B) Bcl6+/+ and Bcl6-/- mice were immunized with KLH in CFA (3 mice per group). Seven days after immunization $CD4^{+}CD44^{hi}$ cells were sorted and restimulated with KLH and irradiated wild-type APC for 48 hr for cytokine measurement by ELISA (A), or with anti-CD3 for 6 hr for analysis of Th1, Th2, Th17 and Tfh specific genes by real-time PCR (B). The graph shows means ± SD.

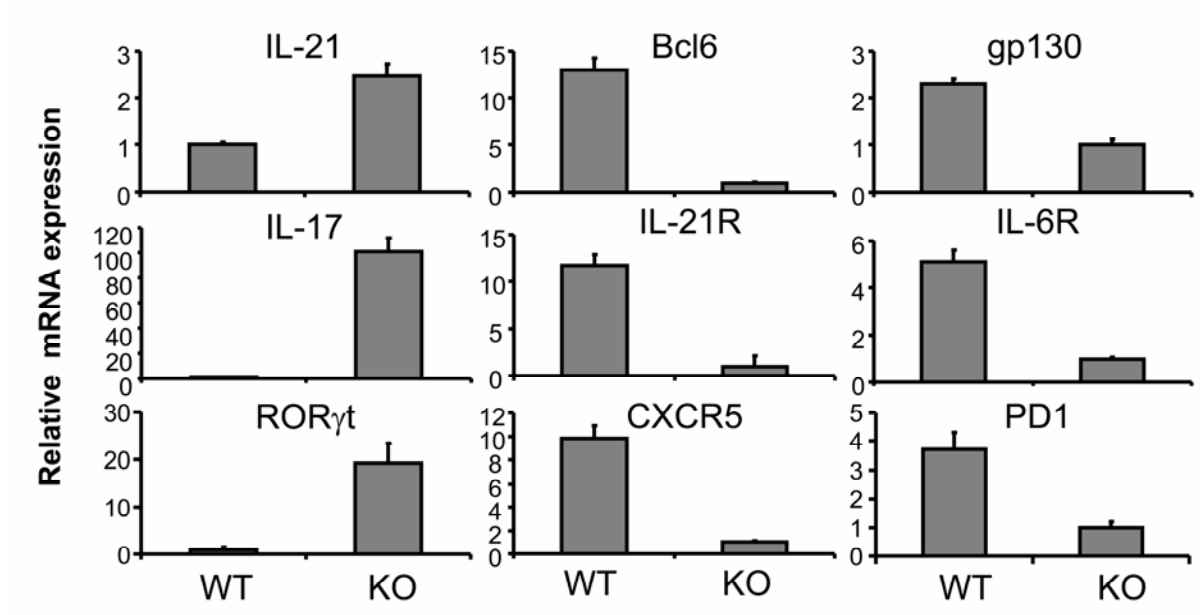

Figure S9. Bcl6 is essential for Tfh cell differentiation in vivo. $CD4^+$ cells from WT or Bcl6 KO mice were mixed with wild-type B cells before being intravenously transferred into *Rag1*$^{-/-}$ mice. The recipient mice were then immunized subcutaneously with KLH emulsified in CFA. Seven days later, $CD4^+CD44^{hi}$ cells were sorted and following anti-CD3 restimulation, mRNA expression of Tfh specific genes was analyzed by real-time RT- PCR. The data shown were normalized to the expression of a reference gene *Actb*. The graph shows means ± standard deviation (SD). The experiments were repeated two times with consistent results.

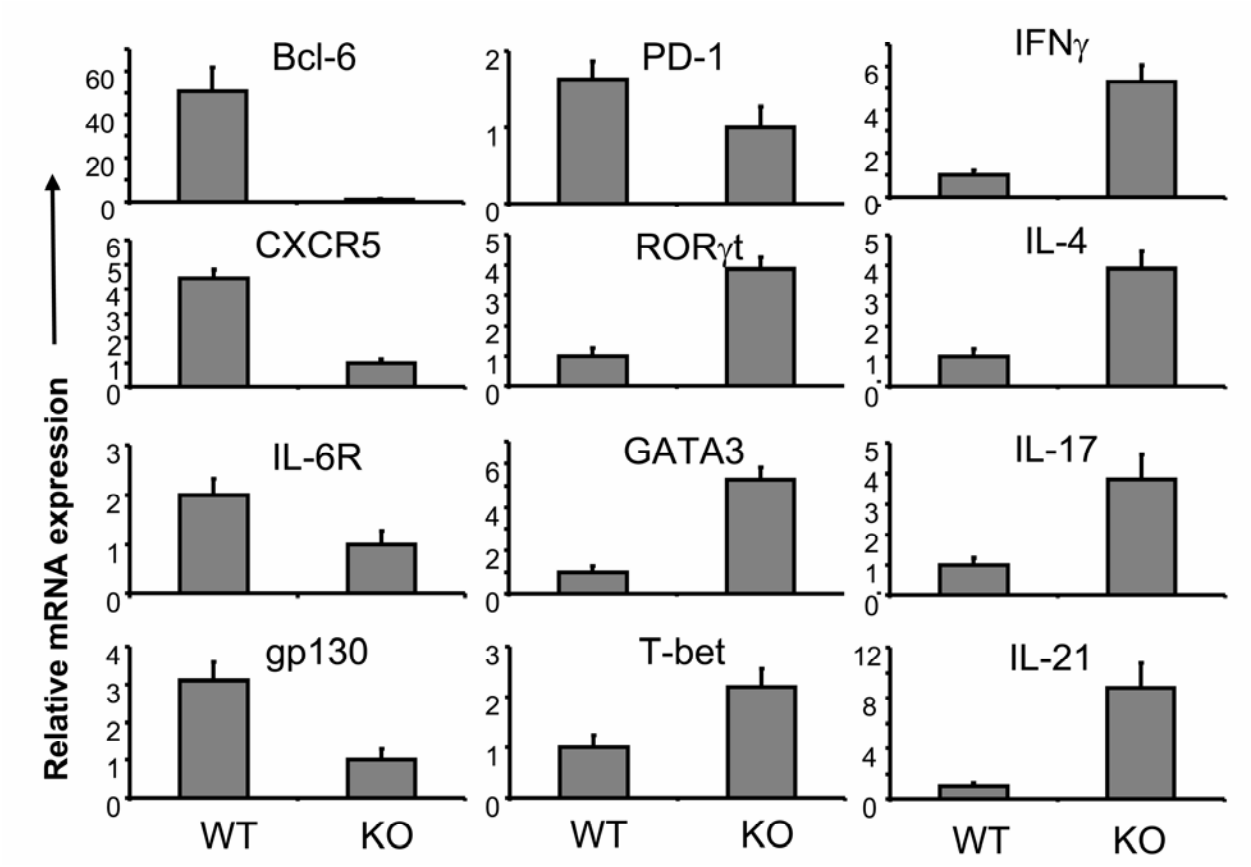

Figure S10. Bcl6 regulation of Th cell differentiation in vivo. To generate mixed bone-marrow chimera, T cell-depleted bone-marrow cells were obtained from wild-type (CD45.1) or Bcl6-deficient (CD45.2) mice and mixed 1:1 ratio before transferred into irradiated *Rag1*$^{-/-}$ mice (10×10^6 cells/transfer, 750 rad). Eight weeks later, the recipient mice (n=5) were subcutaneously immunized with KLH in CFA. Mononuclear cells in the draining lymph nodes and spleen were stained with anti-CD45.1, anti-CD4 and anti-CD44, then CD45.1+CD44hiCD4+ T cells (WT) or CD45.1-CD44hiCD4+ T cells (KO) were FACS-sorted. mRNA expression was assessed by real-time RT-PCR after restimulation of T cells by anti-CD3 restimulation. Data shown are normalized with expression amounts of *Actb*. In the graph shows means ± SD.

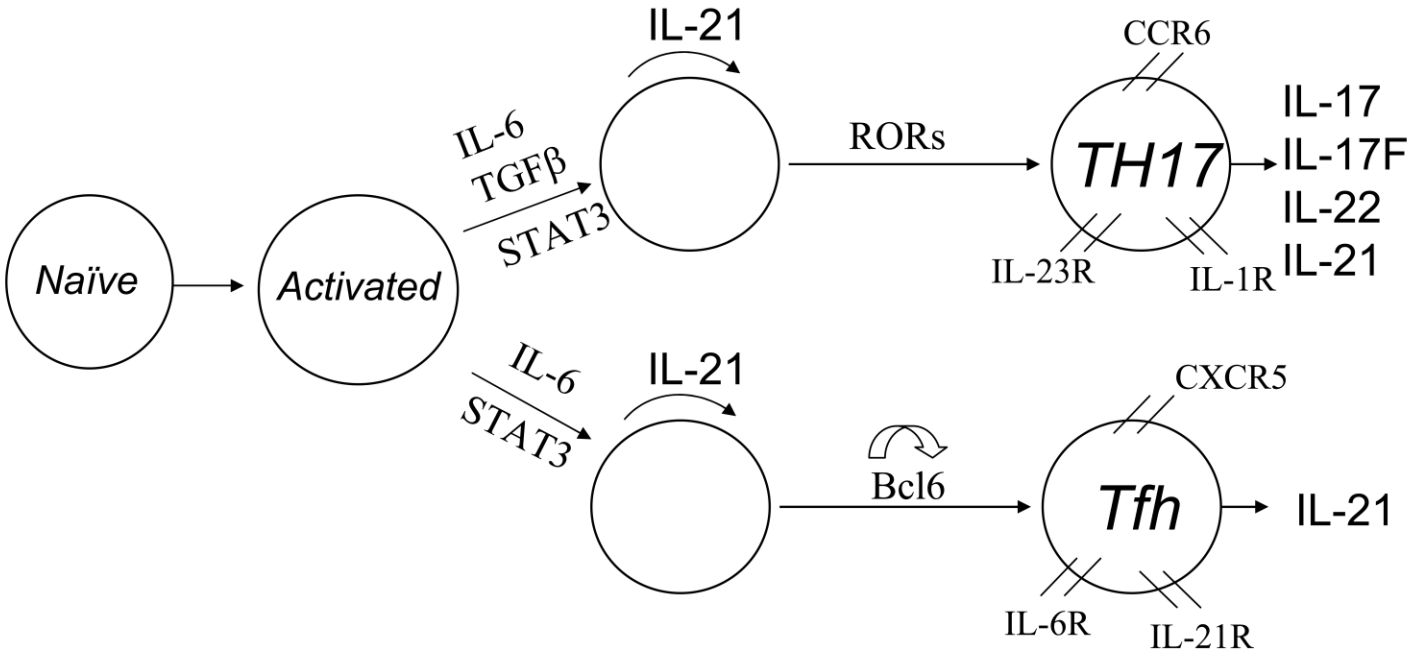

Figure S11. Schematic illustration of the reciprocal development of Tfh and Th17 cells. IL-6 induces IL-21 expression in a STAT3-dependent manner. IL-6 and IL-21, in the presence of TGFβ, induce ROR expression and Th17 differentiation, whereas Bcl6 is induced by IL-6 or IL-21 in the absence of TGFβ to program Tfh cell development.