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Follicular regulatory T (Tfr) cells with dual Foxp3 and Bcl6 expression suppress germinal center reactions

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Abstract

Foxp3$^+$ regulatory T (T_{reg}) cells suppress different types of immune responses to help maintain homeostasis in the body. How T_{reg} cells regulate humoral immunity, including germinal center reactions, is unclear. Here we identify a subset of T_{reg} cells expressing CXCR5 and Bcl6, and localized in the germinal centers in mouse as well as human. The expression of CXCR5 on T_{reg} cells depends on Bcl6. These CXCR5$^+$Bcl6$^+$ T_{reg} cells are absent in thymus but can be *de novo* generated from CXCR5$^-$Foxp3$^+$ natural T_{reg} precursors. Lack of CXCR5$^+$ T_{reg} cells leads to greater germinal center reactions. These results unveil a Bcl6-CXCR5 axis in T_{reg} cells that undermines the development of follicular regulatory T (Tfr) cells that function to inhibit the germinal center reaction.

The vertebrate immune system contains a unique subset of T cells expressing Foxp3 in order to prevent autoimmunity against self-antigens and exaggerated immune responses. Deficiency of Foxp3 results in a multi-organ autoimmune disease in human, called immune-dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX)[1,2]. Similar multi-organ autoimmune phenotypes are observed in Foxp3 mutant mouse strain *scurfy*; this strain exhibits excessive Th1, Th2 and Th17 responses as well as exacerbated humoral responses [1,2].

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AUTHOR CONTRIBUTIONS

Y.C. and C.D. designed experiments and wrote the manuscript; Y.C., S. T., F. C., R. I. N., G. J. M., S. R., Y.-H. W., H. Y. L., J. M. R., X.Z., H.F. and Z.L. performed experiments and analyzed data; S. S. N designed and analyzed human sample data.

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How do Foxp3$^+$ T_{reg} cells control these diverse pathological responses? A series of recent studies showed that there may be multiple subsets or states of T_{reg} which possess unique regulatory properties required for controlling distinct helper T (Th) cell -mediated responses. For instance, deletion of *Irf4* or *Stat3* in T_{reg} results in dysregulation of Th2 and Th17 responses, respectively[3,4]. Similarly, *Tbx21*$^{-/-}$ T_{reg} fails to control Th1 responses when transferred into *scurfy* mice[5]. Therefore, the activation of these transcription factors in Foxp3$^-$ cells mediates the differentiation of effector Th cells whereas the same transcription factors in Foxp3$^+$ cells are also required for the suppression of the corresponding helper T cell-mediated immunity[6-9].

A distinct subset of Th cells expressing CXCR5 (named T follicular helper or Tfh cells) has been recently shown to mediate germinal center reactions[10,11]. The expression of CXCR5 and the generation of Tfh cells require the transcriptional repressor Bcl6[12-14]. Bcl6 represses the differentiation of naïve T cells into Th1, Th2 or Th17 cells[12-14]. The CXCR5-mediated homing of Tfh cells into the B cell follicles[15-17] and their production of IL-21 likely provide stimuli to mature B cells to form germinal centers[18].

One of the critical functions of T_{reg} on limiting autoimmunity is controlling humoral immune responses. How T_{reg} controls germinal center responses and whether there is a subset of T_{reg} specialized for germinal center responses remain poorly understood. It has been shown that CD69$^-$ T_{reg} in human suppresses the B cell response driven by CD57$^+$ germinal center T cells *in vitro*[19]. Moreover, a recent study identified Qa-1-restricted CD8$^+$ T cells as suppressor T cells of Tfh cells expressing high levels of Qa-1[20]. Nevertheless, the uncontrolled humoral responses exhibited in the *scurfy* mice and the IPEX patients suggested that Foxp3$^+$ T cells are also indispensable for controlling germinal center responses. Our current results demonstrated a subset of T_{reg} express CXCR5 in a Bcl6-dependent manner. These germinal center-specific T_{reg} cells are generated from CXCR5$^-$ Treg cells, and suppress the differentiation of germinal center B cells in the follicles *in vivo*.

Results

A subset of Foxp3$^+$ T cells express Bcl6 and CXCR5

Foxp3$^+$ T cells are critical for preventing autoantibody-mediated autoimmunity[21]. Immunohistochemical analysis of the draining lymph nodes from immunized mice showed that Foxp3$^+$ cells were present in the PNA$^+$ area (Figure 1a). This observation prompted us to hypothesize that there is a specialized subset of T_{reg} responsible for controlling germinal center reactions by localizing in follicular regions. To address this hypothesis, we first asked whether there is a subset of Foxp3$^+$ T cells expressing CXCR5 since the interaction between CXCR5 and CXCL13 mediates homing of lymphocytes to B cell follicle area[15]. In naïve mice, we observed 5-15 % Foxp3$^-$CD4$^+$ T cells were CXCR5$^+$BTLA$^+$ in steady status in the spleen and Peyer's patches with less extent in the lymph nodes (Figure 1b), correlating with spontaneous germinal center reactions in these organs. Of interest, we also observed that a subpopulation of Foxp3$^+$ T_{reg} expressed CXCR5 and BTLA, especially in the spleen (Figure 1b lower panels).

To further characterize the CXCR5$^+$ T$_{reg}$, we analyzed the expression of CXCR5, Foxp3 and Bcl6 in CD4$^+$ T cells in mice immunized with KLH in CFA. In Foxp3$^-$ CD4$^+$ T cells, most of CXCR5$^+$ cells co-express Bcl6, and this population was substantially increased in the draining lymph nodes upon immunization (Figure 1c/d). Notably, we observed that the majority of CXCR5$^+$ T$_{reg}$ was also Bcl6$^+$, and this population was also increased upon immunization (Figure 1c-d).

To examine if the Bcl6$^+$CXCR5$^+$ Foxp3$^+$ cells are also present in human, we stained human tonsillar CD4$^+$ T cells from children undergoing elective tonsillectomy. We observed an evident subpopulation of Foxp3$^+$ cells co-expressed Bcl6 and CXCR5 (Figure 1e, 7.39 ± 1.80 % in Foxp3$^+$ population). Moreover, the Foxp3$^+$ cells were present in the germinal center areas as well as the interfollicular areas in human tonsils (Supplementary Figure 1). Collectively, these data indicate the presence of a subpopulation of T$_{reg}$ expressing Bcl6 and CXCR5 in the germinal centers of mice and human.

An essential role of Bcl6 in the generation of CXCR5$^+$ Treg cells

The co-expression of Bcl6 and CXCR5 prompted us to ask whether Bcl6 mediates the expression of CXCR5 on Foxp3$^+$ T$_{reg}$ as it does in Tfh cells[12-14]. To answer this question, we analyzed the expression of CXCR5 and BTLA on the Foxp3$^-$ and Foxp3$^+$ T cells in *Bcl6*$^{-/-}$ mice after immunization. Consistent with previous reports, the induction of CXCR5$^+$BTLA$^+$ population in conventional CD4$^+$ T cells was almost completely abolished in *Bcl6*$^{-/-}$ mice (Figure 2a). Interestingly, compared with wild-type mice, we also observed a significant reduction of the CXCR5$^+$BTLA$^+$ population in Foxp3$^+$ T cells in the *Bcl6*$^{-/-}$ mice (Figure 2b). These results demonstrate an essential role of Bcl6 in the generation of CXCR5$^+$ T$_{reg}$. On the other hand, we observed more Foxp3$^+$ T cells in the thymi and spleens, but not mesenteric lymph nodes, in *Bcl6*$^{-/-}$ mice than in wild-type littermates (Supplementary Figure 2), suggesting negative regulation by Bcl6 during the generation of natural T$_{reg}$.

Flow cytometric analysis of CXCR5$^+$ T$_{reg}$ showed high expression of Bcl6, BTLA, PD-1, ICOS, CD44, CTLA4, GITR, whereas the expression of CD103 and CXCR3 were low (Figure 3a). Therefore, the Bcl6$^+$CXCR5$^+$ T$_{reg}$ resembles the phenotypes of Tfh but is distinct from CD103$^+$, or CXCR3$^+$ Treg subset. To further characterize CXCR5$^+$ T$_{reg}$, we isolated CXCR5$^+$ T$_{reg}$ and CXCR5$^-$ T$_{reg}$ from the spleen and lymph nodes of immunized Foxp3^{gfp} mice (Supplementary Figure 3a), and determined the suppressive activity by co-culturing them with naïve CD4+ T cells in the presence of irradiated splenocytes and anti-CD3. We observed a comparable suppressive activity between CXCR5$^+$ and CXCR5$^-$ T$_{reg}$ (Supplementary Figure 3b). Quantitative RT-PCR analysis revealed that CXCR5$^+$ T$_{reg}$ expressed lower levels of the *Tbx21*, *Irf4*, and *Gata3* genes compared to CXCR5$^-$ Tregs (Supplementary Figure 3c). To further characterize the role of Bcl6 in T$_{reg}$, we compared the gene expression profiles of the CD25^{hi}CD4$^+$ T cells isolated from *Bcl6*$^{-/-}$ mice or wild-type littermates. *Bcl6*$^{-/-}$ T$_{reg}$ expressed less *Cxcr5*, *Pdcd1* (encoding PD-1) and *Tbx21*, but more *Gata3*, *Irf4* and *Rorc* compared with wild-type T$_{reg}$ (Figure 3b). Bcl6 and Blimp1 reciprocally repress each other's expression in Tfh cells[12]. Moreover, a recent study revealed a critical role of Blimp1 in inducing IL-10 and suppressing CCR6 in Treg cells[22]. Consistent

with these notions, we observed increased levels of *Prdm1* (Blimp1) and *Il10* and a decreased level of *Ccr6* mRNA transcript in the *Bcl6*$^{-/-}$ T_{reg} (Figure 3b). On the other hand, *Bcl6*$^{-/-}$ T_{reg} showed as efficient suppressive activity as wild-type T_{reg} (Figure 3c).

Origin and generation of Bcl6^{+}CXCR5^{+} T_{reg} cells *in vivo*

Foxp3^{+} T cells are generated in thymus (natural T_{reg}) or from naïve CD4^{+} T cells in periphery upon TGFβ signaling (induced T_{reg}). To identify the developmental origin of the CXCR5^{+} T_{reg} subset observed in the spleen, we analyzed the expression of CXCR5 and BTLA on lymphoid cells from thymus and spleen of Foxp3^{gfp} mice. Compared with GFP^{+} T_{reg} in the spleen, few T_{reg} in the thymus expressed CXCR5 and BTLA (Figure 4a).

We next asked if the Bcl6^{+}CXCR5^{+} T_{reg} cells are generated from naïve CD4^{+} or natural T_{reg} precursors in the periphery. We mixed CD45.1^{+} naïve CD4^{+} T cells (CD25^{-}GITRCD44^{lo}CD62L^{hi}) and CD45.2^{+}CXCR5^{-} T_{reg} from Foxp3^{gfp} mice, intravenously transferred them into *Tcrb*$^{-/-}$ mice, followed by immunizing the recipients with KLH in CFA. As depicted in Figure 4b, more than 98 % of Bcl6^{+}Foxp3^{+} population in the recipient mice was CD45.1^{-}, indicating the origin of Bcl6^{+}CXCR5^{+} T_{reg} is CXCR5^{-} T_{reg}. Helios is a transcription factor exclusively expressed in natural T_{reg}[23,24]. We observed that majority of CXCR5^{+} Foxp3^{+} cells expressed Helios, supporting that they were from natural T_{reg} (Figure 4c). IL-6 and IL-21 have been shown to be required for the generation of Tfh cells[10,11,13]. When CXCR5-negative Foxp3^{+} T cells were stimulated with IL-6 and IL-21, we observed little difference in the levels of *Cxcr5* and *Bcl6* mRNA transcript (supplementary Figure 4). These data overall demonstrated the Bcl6^{+}CXCR5^{+} T_{reg} cells are absent in the thymus but induced in the periphery from CXCR5^{-} Foxp3^{+} natural T_{reg}.

Absence of CXCR5 or Bcl6 in Treg cells enhances germinal center reactions

We finally asked the role of the Bcl6^{+}CXCR5^{+} T_{reg} in controlling germinal center responses. When we analyzed 4-5 weeks-old *scurfy* mice, we observed greatly expanded Bcl6^{+}CXCR5^{+} Tfh cells as well as GL7^{+}CD95^{+} B cells (Figure 5a and b). Of note, the difference was greater in the GL7^{+}CD95^{+} B cell population (> 40-fold increases in the *scurfy* vs wild-type) than in the Bcl6^{+}CXCR5^{+} Tfh cell population (1.94 to 2.4-fold increases). These data indicate that Foxp3^{+} T cells are essential for controlling both Tfh response and germinal center B cells, likely more important for controlling the latter.

To directly address whether Bcl6^{+}CXCR5^{+} T_{reg} control germinal center reactions *in vivo*, we isolated T_{reg} from wild-type and *Cxcr5*$^{-/-}$ mice (CD45.2^{+}), mixed with CD45.1^{+} naïve CD4^{+}T cells (CD25^{-}GITR^{-}CD44^{lo}CD62L^{hi}) at 1:9 ratio, then transferred them into *Tcrb*$^{-/-}$ mice. The recipients were s.c. immunized with KLH in CFA, and the lymphoid cells in the draining lymph nodes were analyzed. We observed comparable percentage of Foxp3^{+} cells among CD4^{+} cells (Figure 6a). Notably, we observed a significant increase in the percentages of GL7^{+}CD95^{+} B cell population in the recipients of *Cxcr5*$^{-/-}$ T_{reg} compared with that in the recipients of wild-type T_{reg} (Figure 6b/c; 4.13 ± 0.45 vs 2.07 ± 0.25, p=0.0014). We also observed a small but statistically insignificant increase in the CXCR5^{+} BTLA^{+} CD4^{+} T cells among CD45.1^{+} population (originally naïve T cells) in the *Cxcr5*$^{-/-}$ T_{reg} recipients compared with that in the wild-type T_{reg} recipients (Figure 6d; 26.85 ± 3.19

vs 20.54 ± 2.11, p=0.121). Wild-type T_{reg} distributed through the whole lymph node including PNA^+ areas, whereas the *Cxcr5*$^{-/-}$ T_{reg} showed more restricted distribution in the T cell area (Figure 6e). Immunohistochemical analysis with anti-CD4 and PNA showed comparable numbers of $CD4^+$ T cells in the germinal centers between two groups (112.9 ± 14.3 vs 117.2 ± 18.56; cells per germinal center), while less $Foxp3^+$ cells in the germinal centers in *Cxcr5*$^{-/-}$ T_{reg} recipients (20.25 ± 6.30 vs 7.83 ± 2.09).

To further address the function of $Bcl6^+$ T_{reg} *in vivo*, we performed a similar adoptive transfer experiment with T_{reg} from wild-type or *Bcl6*$^{-/-}$ mice. Of interest, we observed a great increase in the percentages of $GL7^+CD95^+$ B cell population in the recipients of *Bcl6*$^{-/-}$ T_{reg} compared with that in the recipients of wild-type T_{reg} (Supplementary Figure 5a/b/e). However, $CXCR5^+$ $BTLA^+$ $CD4^+$ T cell population among $CD45.1^+$ cells was comparable between two groups (Supplementary Figure 5c). Consistent with data in Figure 2b, $CXCR5^+BTLA^+$ population among donor T_{reg} ($CD45.2^+CD4^+$) was significantly decreased in the recipient of *Bcl6*$^{-/-}$ T_{reg} (supplementary Figure 5d), indicating that Bcl6 expression in T_{reg} is required for the generation of $CXCR5^+BTLA^+$ T_{reg}.

Affinity maturation of antibodies is a hall mark of T cell-dependent humoral responses. To examine whether the $Bcl6^+CXCR5^+$ T_{reg} also regulates affinity maturation, we performed a similar adoptive transfer experiment, except that the recipient mice were immunized with 4-hydroxy-3-nitrophenyl (NP)-conjugated KLH in CFA. The levels of NP-specific IgM at day 5 post immunization were comparable between two groups (Supplementary Figure 6a). At this time point, we observed a weak induction of global affinity IgG1 and IgG2b in the *Bcl6*$^{-/-}$ T_{reg} recipients, but not in wild-type T_{reg} recipients. By day10, we observed that the *Bcl6*$^{-/-}$ T_{reg} recipients produced significantly higher levels of NP-specific global affinity IgG1, IgG2a and IgG2b than wild-type T_{reg} recipients, with comparable levels of IgM (Figure 6f, upper panels). More importantly, the production of high affinity IgG1, IgG2a and IgG2b were also greater in the *Bcl6*$^{-/-}$ T_{reg} recipients (Figure 6f, lower panels). Furthermore, both NP-specific B cells and $B220^-CD138^+$ plasma cells were significantly higher in the *Bcl6*$^{-/-}$ T_{reg} recipients (Figure 6g). The *Bcl6*$^{-/-}$ T_{reg} recipients showed slight increases in antibody-forming cells and $IgD^-IgM^-B220^+$ memory B cell population (Supplementary Figure 6b/c). Collectively, these data demonstrate that the $Bcl6^+CXCR5^+$ subset of T_{reg} plays a crucial role in controlling germinal center B cell responses *in vivo* including affinity maturation and plasma cell differentiation.

When the splenocytes were restimulated with KLH, we observed less, but not significant, levels of IFNγ and IL-17 (Supplementary Figure 7), indicating the *Bcl6*$^{-/-}$ T_{reg} is at least as efficient as wild-type T_{reg} in regulating Th1, Th2 and Th17 responses *in vivo*.

Discussion

In the present study, we identified the $Bcl6^+CXCR5^+$ $Foxp3^+$ T cell as a distinct effector T_{reg} subset specialized for controlling germinal center reactions. $Bcl6^+CXCR5^+$ T_{reg} resembles follicular helper T cells by expressing BTLA, PD-1 and ICOS, but are distinct from $CD103^+$ and $CXCR3^+$ T_{reg} subsets. This $CXCR5^+$ subset of T_{reg} was absent in thymus but can be differentiated from $CXCR5^-$ natural T_{reg} in the periphery upon antigenic

stimulation, which is completely dependent on Bcl6. Therefore, we propose an analogous differentiation pathway between Tfh cells and follicular regulatory T (Tfr) cells to balance B cell responses *in vivo* (Supplementary Figure 8). Compared with wild-type Treg cells, both *Cxcr5*$^{-/-}$ T_{reg} and *Bcl6*$^{-/-}$ T_{reg} were significantly less efficient in suppressing the generation of germinal center B cells, affinity maturation of antibodies, and the differentiation of plasma cells. Our study thus unveiled Bcl6-mediated CXCR5 expression in Foxp3^{+} T cells as a novel mechanism that underlies specialization of effector T_{reg} that suppresses the germinal center formation and subsequent humoral responses.

The humoral immunity seem not completely to be dependent on Tfh cells; rather Th1, Th2 and Th17 cells partially contribute to the germinal center formation. For instance, IFNγ-producing Th1 cells and IL-4-producing Th2 cells in the follicles play critical roles in IgG2a and IgG1 class-switching in mice[25], even though the generation of CXCR5^{+} Tfh cells is known to be independent of these cytokines[10]. In addition, IL-17 was reported to trigger B cell proliferation and IgG2a isotype-switching in mice[26,27], and Th17 cells might be responsible for the generation of spontaneous germinal centers in the autoimmune BXD2 mice[28]. Therefore, it is not surprising to observe that mice specifically ablated for *Irf4* in Foxp3^{+} cells and consequently exhibit uncontrolled Th2 responses, showed enhanced germinal center formations[4]. We believe that the Bcl6^{+}CXCR5^{+} T_{reg} described in this study are not solely responsible for controlling humoral immunity, but rather cooperate with the other subsets of T_{reg}. In this aspect, we propose that, due to their homing property to the B cell area, the Bcl6^{+}CXCR5^{+} subset of T_{reg} controls the later stage of germinal center reactions, such as the differentiation of GL7^{+}CD95^{+} B cells, antibody affinity maturation, and the differentiation of plasma cells. The mechanism underlying Bcl6-mediated CXCR5^{+} Tfr generation is not clear at this stage. Moreover, a recent study by Ise *et al* demonstrated that BATF is required for the expression of Bcl6 as well as c-Maf[29]. Transcription factor c-Maf is also known to be important for the generation of Tfh cells[30]. It would be interesting to examine whether BATF and c-Maf are also required for the specialization of Tfr subset.

Kim *et al* recently reported that Qa-1-restricted CD8^{+} regulatory T cells directly inhibit Qa-1^{+} Tfh cells, which is dependent on IL-15[20]. These Tfh-specific CD8^{+} regulatory T cells express high ICOSL and CXCR5, but not Foxp3. Qa-1 mutant mice show more than 5-fold increase in Tfh population and 15-fold increase in GL7^{+}CD95^{+} B cells at the age of 6 months [20]. We observed that *scurfy* mice have a moderate increase in Tfh population (Figure 5a; about 2-fold increase vs littermates) but a dramatic increase in GL7^{+}CD95^{+} B cells (Figure 5b; about 40-fold increase), despite presumably intact numbers of Qa-1-restricted CD8^{+} T cells. These observations together suggest non-redundant roles of the CD8^{+} T regulatory cells and Foxp3^{+} T_{reg} *in vivo* in controlling the follicular and germinal center reactions. It is feasible to surmise that the CD8^{+} regulatory T cells are more specialized to control the generation of Tfh cells whereas Tfr cells are more specialized to control the generation of germinal center B cells. Further study will be needed to dissect how diverse Foxp3^{+} or Foxp3^{-} regulatory T cells serve as suppressors for humoral response and germinal center reactions.

Human CD69^{-}CD25^{+} CD4^{+} T cells have been reported to suppress germinal center T cell and B cell responses [19]. It is not clear if the CD69^{-}CD25^{+} CD4^{+} T cells express Bcl6;

however, it is possible that the CD69$^-$ T_{reg} is the precursor of Bcl6$^+$CXCR5$^+$ T_{reg}. The existence of Bcl6$^+$CXCR5$^+$ T_{reg} in the spleen of healthy naive mice indicates stable expression of Bcl6 and CXCR5 in this subset of T_{reg}. It has been recently reported that Foxp3$^+$ T cells can differentiate into Tfh cells in Peyer's patches[31]. On the other hand, Foxp3$^+$ T_{reg} appears to be quite stable *in vivo* even under inflammatory conditions[32]. When sorted CXCR5$^+$ T_{reg} cells were transferred into *Tcrb*$^{-/-}$ mice, we observed a decreased population of CXCR5-expressing donor T_{reg} upon immunization (data not shown). Further studies by using fate-mapping genetic tools and Bcl6-reporter mice will be needed to examine the stability of Bcl6$^+$CXCR5$^+$ T_{reg}.

In summary, the present study unveiled that the 'Bcl6-CXCR5' axis in T_{reg} as a novel mechanism for controlling germinal center responses. It is feasible to surmise that the adoptive transfer of the Tfr cells might be a promising approach to treat the autoantibody-mediated inflammatory disorders such as lupus and rheumatoid arthritis[33,34]. Our study supports the notion that Foxp3$^+$ T_{reg} utilizes the same mechanism as conventional CD4$^+$ T cells to promote inflammation in order to repress different types of inflammation. This novel subset of T_{reg} adds to the complexity of immune-suppressive mechanisms mediated by T_{reg}.

Methods

Mice

C57BL/6, B6SJL (CD45.1), *Tcrb*$^{-/-}$, *Cxcr5*$^{-/-}$, and *scurfy* mice were purchased from Jackson Laboratory. *Bcl6*$^{-/-}$ mice were kindly provided by Dr. Dalla-Favera (Columbia University)[35]. Foxp3^{gfp} reporter mice were generously provided by Dr. Rudensky (Memorial Sloan-Kettering Cancer Center). All mice were housed in the SPF animal facility at MD Anderson Cancer Center. All animal experiments were performed using protocols approved by Institutional Animal Care and Use Committee of MD Anderson Cancer Center.

Keyhole Limpet Hemocyanin (KLH) Immunization

C57BL/6 , *Bcl6*$^{-/-}$ and their littermate, or the recipient mice in the adoptive transfer studies were immunized with 100 μl of KLH or NP_{15}-KLH (0.5 mg/ml) emulsified in CFA at tail base[13]. Seven to ten days later, lymphoid cells from the spleens and draining lymph nodes of immunized mice were obtained. For T cell analysis, the cells were stained with PerCP-Cy5.5-conjugated anti-CD4, PE-conjugated anti-BTLA, and biotinylated anti-CXCR5 followed by APC-conjugated streptavidin. Pacific blue-conjugated anti-CD45.1 or anti-CD45.2 were additionally used for surface staining. These cells were permeabilized with Foxp3 staining kit (eBioscience), and further stained with Alexa488- or Alexa 450-conjugated anti-Foxp3 and PE-conjugated anti-Bcl6 (a kind gift from BD Bioscience). For phenotypic analysis, PE-conjugated anti-BTLA, PE-conjugated anti-PD-1, PECy5.5-conjuated anti-ICOS, FITC-conjugated anti-CD44, PE-conjugated anti-GITR, PE-conjugated anti-CTLA4, FITC-conjugated anti-CD103, APC-conjugated anti-CXCR3 and APC-conjugated anti-CD39 were used. Anti-Helios Ab was kindly provided by Dr. Dat Tran (University of Texas at Houston).

For B cell analysis, the cells were stained with PerCP-conjugated anti-B220, PE-conjugated anti-CD95, FITC-conjugated anti-PNA, APC-conjugated anti-CD138, FITC-conjugated anti-IgM, PE-conjugated anti-IgD, and PE-conjugated NP_{32}. These cells were analyzed by using LSRII® and Flowjo® software. Ab forming cell ELISPOT assay was performed by culturing serially diluted splenocytes in KLH-precoated filter plates, followed by adding HRP-conjugated detection Abs. Spots were formed by incubation with ECL substrate and enumerated by using CTL Immunospot® Reader (Cellular Technology Ltd.).

NP-specific IgM and IgG Abs in sera obtained 5 or 10 days after immunization were measured with ELISA. Serum samples were added in a 3-fold serial dilution onto plates pre-coated with 10μg/ml NP_4-BSA (for high affinity) or NP_{26}-BSA (for global affinity), followed with HRP conjugated goat anti-mouse IgM or rat anti-mouse IgG Abs.

Adoptive transfer studies

$CD25^-GITR^-CD44^{lo}CD62L^{hi}$ naïve $CD4^+$ T cells from $CD45.1^+$ congenic mice were isolated by using FACS Aria® (BD Bioscience)[36]. In some experiments, $CD25^{hi}CD4^+$ T cells were isolated from C57BL/6 mice, *Cxcr5*$^{-/-}$ mice, or *Bcl6*$^{-/-}$ mice, and used as T_{reg}. The FACS sorted naïve $CD4^+$ T cells ($3\text{-}4\times10^6$ cells/transfer) and T_{reg} cells ($0.3\text{-}0.4\times10^6$ cells/transfer) were mixed at 9:1 ratio before adoptively transferred into *Tcrb*$^{-/-}$ mice. Next day, the recipient mice were subcutaneously immunized with KLH in CFA and the induction of Tfh cells and germinal center B cells were analyzed as described[13].

Analysis of human tonsils

Immunohistochemical analysis was performed as previously described[13]. Tissue block was sliced 6 μm, air dried and fixed with acetone cold. Tissue slides were stained with primary antibodies against PNA, CD4 (BD Bioscience), and Foxp3 (eBioscence) followed with Alexa 594- and Alexa 488-conjugated secondary antibodies (Invitrogen) .

Reactive tonsils were obtained from children undergoing elective tonsillectomy after informed consent on an institutional review board-approved protocol. Formalin-fixed paraffin-embedded tissue sections of human tonsils were processed according to manufacturer's protocol (Vector Laboratories). Sections were incubated with Foxp3 antibody (Abcam), followed with anti-mouse IgG antibody (BA-2000, Vector Laboratories). The sections were stained using DAB solution.

To analyze the expression of Foxp3 and Bcl6 in human $CD4^+$ T cells, single cell suspensions from human tonsils were first stained for surface antigens (CD3, CD4, CXCR5) and then fixed and permeabilized for staining intracellular antigens. The following antibodies were obtained from BD Biosciences: Pacific blue-conjugated anti-CD3 (UCHT1), APC-conjugated anti-CD4 (SK3), Alexa488-conjugated anti-CXCR5 (RF8b2), and PE-conjugated anti-Bcl6. PerCPCy5.5-conjugated anti-FoxP3 (PCH101) was obtained from eBioscience.

Treg suppression assay

Naïve $CD4^+$ T cells from C57BL/6 mice were cultured with irradiated T cell-depleted splenocytes in the presence of soluble anti-CD3 (2 μg/ml). In some wells, FACS-sorted $CD25^{hi}CD4^+$ T cells isolated from the lymphoid cells of $Bcl6^{-/-}$ mice, wild-type littermates were added. $CD25^{hi}CD4^+$ T cells were added at a ratio of 1:0.5 (Naïve T cell:T_{reg}). In some experiments, $CXCR5^+GFP^+$ or $CXCR5^-GFP^+$ cells from $Foxp3^{gfp}$ reporter mice were added. Three days later, the proliferation of T cells was measured by [H-3]-thymidine incorporation for the last 8 hours.

Quantitative real-time PCR assay

$CD25^{hi}CD4^+$ T cells were isolated from C57BL/6 mice (wild-type) and *Cxcr5*$^{-/-}$ mice, or $CXCR5^+$ and $CXCR5^-$ among GFP^+ cells from the $Foxp3^{gfp}$ reporter mice were sorted by FACS Aria. Total RNA was prepared from the T_{reg} using Trizol (Invitrogen), and cDNA were synthesized and analyzed with a Bio-Rad iCycler Optical System (Bio-Rad Laboratories), as previously described [9,10,12,13]. The levels of gene expression were calculated after normalized to β-actin reference.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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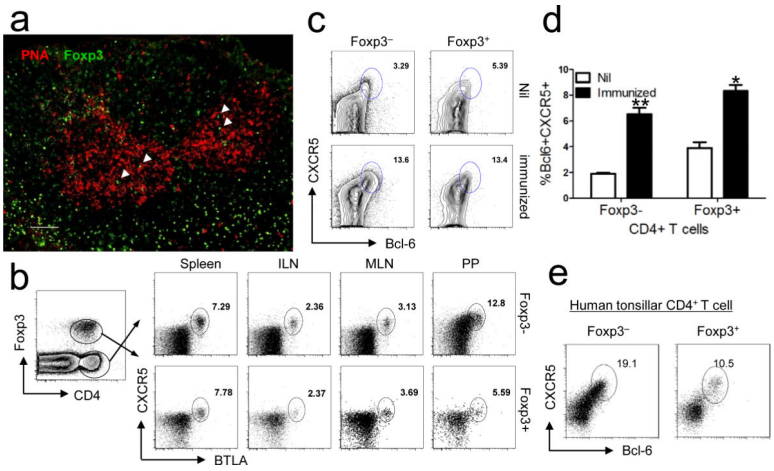

Figure 1. CXCR5$^+$ T$_{reg}$ cells co-express Bcl6 and expand upon immunization

a, Foxp3$^+$ cells (green) in the PNA$^+$ (red) germinal center were stained in the draining LNs. White arrow heads indicate Foxp3$^+$ cells in PNA$^+$ area. Scale bar shown is 50 μm. **b**, Lymphoid cells from the indicated secondary lymphoid tissues from naïve mice were analyzed for the expression of CXCR5, BTLA and Foxp3 in CD4$^+$ T cells by flow cytometry. **c-d**, Expression of Bcl6 and CXCR5 in Foxp3$^+$ and Foxp3$^-$ CD4$^+$ T cells were analyzed with draining lymph node cells obtained 7 days after s.c. immunization with KLH in CFA ('Immunized') or without immunization (Nil). Values in **d** are mean ± SE. *, $p<0.05$; **, $p<0.01$ in comparison with 'Nil' group. **a-d**, Data represent two or three independent experiments. **e**, Expression of CXCR5, Foxp3 and Bcl6 in human tonsillar CD4$^+$ T cells were analyzed. The dot plots represent the T cell staining from three donors.

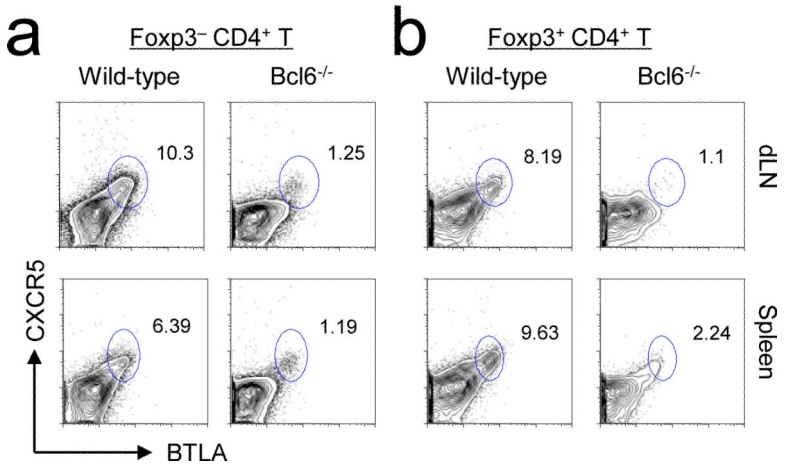

Figure 2. Generation of CXCR5^{+}Foxp3^{+} T cells requires Bcl6

a and **b**, Expression of CXCR5 and BTLA in Foxp3^{-}(**a**) and Foxp3^{+}(**b**) CD4^{+} T cells were analyzed with lymphoid cells from the draining lymph nodes or spleen of wild-type littermates or *Bcl6*$^{-/-}$ mice at 7 days after s.c. immunization with KLH in CFA. Data represent two independent experiments.

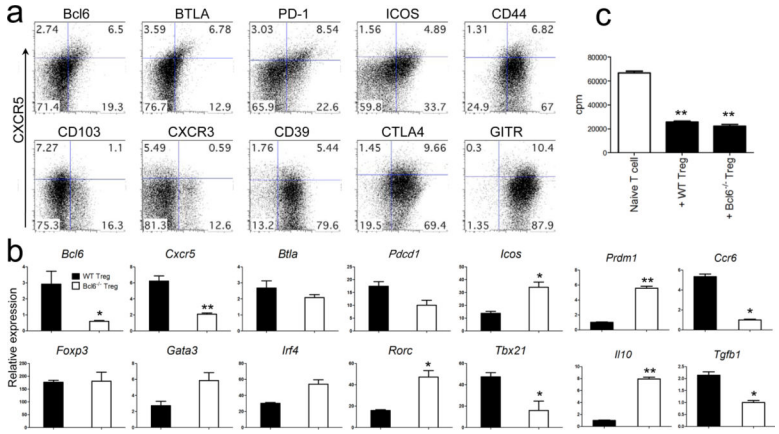

Figure 3. Characterization of CXCR5^{+}Foxp3^{+} T cells

a, Surface expression of the indicated molecules on CXCR5^{+} Foxp3^{+} T cells. Data shown are gated on CD4^{+} Foxp3^{+} cells, and represent two independent experiments. **b**, The relative expression levels of the indicated genes in FACS-sorted CD25^{hi}CD4^{+} T cells from *Bcl6*$^{-/-}$ mice or wild-type littermates. Data were normalized with expression amounts of *Actb*. *, p<0.05; **, p<0.01 in comparison with wild-type T_{reg}. **c**, Suppressive activity of CD25^{hi}CD4^{+} T cells from *Bcl6*$^{-/-}$ mice or wild-type littermates. Data shown are mean ± SE. **, *p*<0.01 in comparison with 'naïve T cell alone' condition. Data represent two independent experiments.

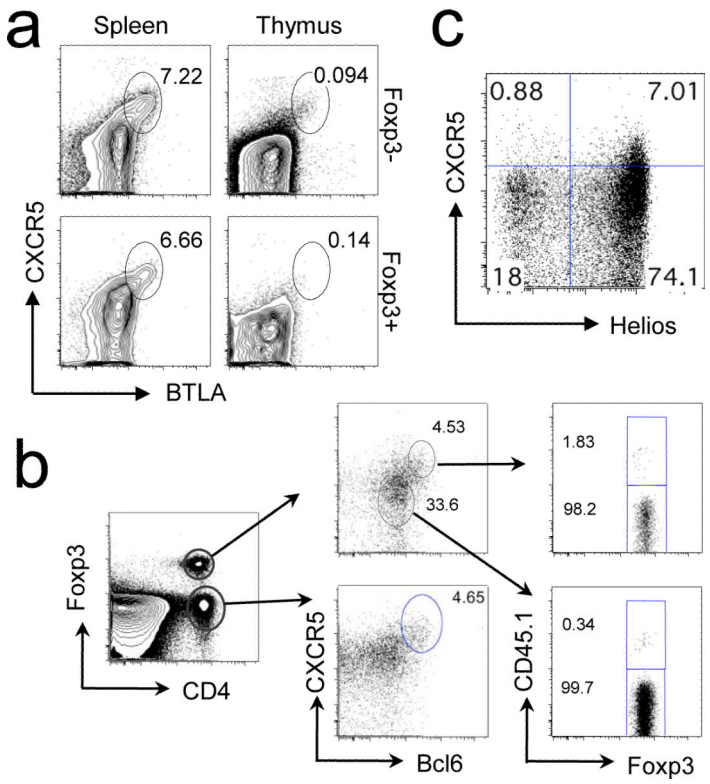

Figure 4. Bcl6^{+}CXCR5^{+} T$_{reg}$ cells are generated from CXCR5^{-} natural T$_{reg}$ in the periphery

a, Expression of CXCR5, BTLA and Foxp3 in CD4^{+} T cells from spleen or thymus (CD4^{+}CD8^{-}). **b**, Analysis of Foxp3^{+} and Foxp3^{-} CD4^{+} T cells in the draining lymph nodes of *Tcrb*$^{-/-}$ recipients given mixture of CD25^{-}GITR^{-}CD44^{lo}CD62L^{hi} naïve CD4^{+} T cells (CD45.1^{+}) and CXCR5-negative Foxp3^{+} T cells (CD45.2^{+}; CXCR5^{-}gfp^{+} cells from Foxp3^{gfp} mice) after immunization with KLH in CFA (left panel). The expression of CXCR5 and Bcl6 in Foxp3^{+} or Foxp3^{-} CD4^{+} T cells (middle panels). CD45.1/CD45.2 expression in CXCR5^{+}Bcl6^{+} or CXCR5^{-}Bcl6^{-} population among Foxp3^{+} T$_{reg}$ cells (right panels). **c**, Helios expression in CXCR5^{+} T$_{reg}$ cells. Data shown are gated on CD4^{+} Foxp3^{+} cells. Data shown represent two independent experiments.

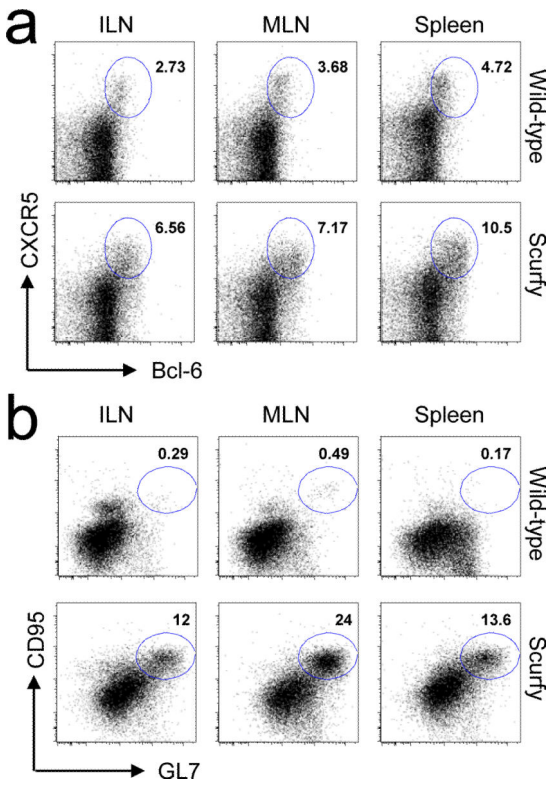

Figure 5. Uncontrolled germinal center reactions in *scurfy* mice

Expression of Bcl6 and CXCR5 on $CD4^+$ T cells (**a**) or GL7 and CD95 on B cells (**b**) from the indicated secondary lymphoid tissues of scurfy mice and littermates (4-5 weeks old). Data are shown on CD4 gate (**a**) or B220 gate (**b**).

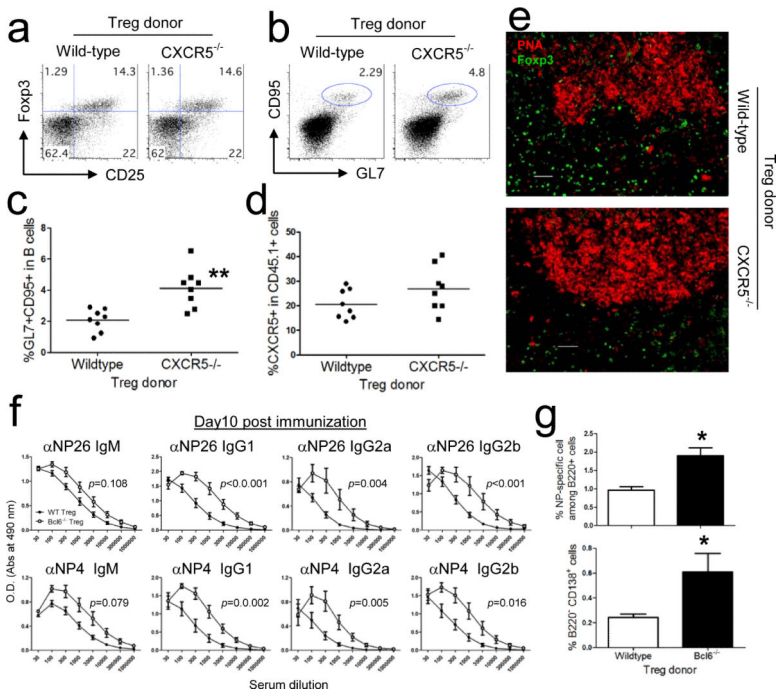

Figure 6. Both *Cxcr5*$^{-/-}$ T_{reg} and *Bcl6*$^{-/-}$ T_{reg} were inefficient in controlling germinal center reactions

Expression of CD25 and Foxp3 among CD4$^+$ T cells (**a**), GL7$^+$CD95$^+$ cells among B220$^+$ B cells (**b, c**), and CXCR5$^+$BTLA$^+$ cells among CD45.1$^+$ population (**d**) in the draining lymph nodes of *Tcrb*$^{-/-}$ recipients given the mixture of naïve CD4$^+$ T cells (CD45.1$^+$) and regulatory T cells (CD25^{hi}; CD45.2$^+$) from wild-type or *Cxcr5*$^{-/-}$ mice, followed by s.c. immunization with KLH in CFA. **e**, The draining LNs were stained with PE-labeled PNA to visualize germinal center together with FITC-labeled anti-Foxp3. Scale bars shown are 25 μm. Data shown were obtained 9 days after immunization and represent three independent experiments. Data in **c** and **d** are pooled from two independent experiments. **f-g**, Mixture of naïve CD4$^+$ T cells (CD45.1$^+$) and T_{reg} (CD25^{hi}; CD45.2$^+$) from wild-type or *Bcl6*$^{-/-}$ mice were transferred into *Tcrb*$^{-/-}$ mice, followed by s.c. immunization with KLH-NP_{15} in CFA. Levels of immunoglobulin specific for NP_4 or NP_{26} in sera obtained 10 days post immunization (**f**). Population of NP-specific B220$^+$ cells or B220$^-$CD138$^+$ plasma cells in the spleens of the recipients (**g**). Data shown are mean ± SE (n=5). *, p<0.05 in comparison with wild-type Treg recipients. p-values in '**f**' were analyzed by two-way ANOVA. Data represent two independent experiments.