Generation of follicular helper T cells is mediated by IL-21 but independent of TH1, TH2 or TH17 lineages

Roza I. Nurieva[1], **Yeonseok Chung**[1], **Daehee Hwang**[2], **Xuexian O. Yang**[1], **Hong Soon Kang**[3], **Li Ma**[4], **Yi-hong Wang**[1], **Stephanie S. Watowich**[1], **Anton M. Jetten**[3], **Qiang Tian**[4], and **Chen Dong**[1]

1*Department of Immunology, University of Texas M. D. Anderson Cancer Center and Graduate School of Biomedical Sciences, Houston, TX 77030, USA*

2*School of Interdisciplinary Bioscience and Bioengineering & Department of Chemical Engineering, Pohang University of Science and Technology, Kyungbuk 790-784, Republic of Korea*

3*Cell Biology Section, LRB, National Institutes of Health, NIEHS, Research Triangle Park, NC 27709, USA*

4*Institute for Systems Biology, Seattle, WA 98103, USA*

Abstract

After activation, CD4+ helper T (TH) cells differentiate into distinct effector lineages. Although CXCR5+ follicular TH (T$_{FH}$) cells are important in humoral immunity, their developmental regulation is unclear. Here we show that T$_{FH}$ cells have a distinct gene expression profile from other effector T cells and develop in vivo independent of the TH1 or TH2 lineages. T$_{FH}$ cell generation is regulated by B7h expressed on B cells and, similar to TH17 cell development, is dependent on IL-21, IL-6 and STAT3. However, differentiation of T$_{FH}$ cells, unlike TH17 cells, does not require TGFβ signaling or TH17-specific orphan nuclear receptors RORα and RORγ in vivo. Finally, naïve T cells activated in vitro in the presence of IL-21 but not TGFβ signaling preferentially acquire T$_{FH}$ gene expression and function to promote germinal center reactions in vivo. This study thus demonstrates T$_{FH}$ as a distinct lineage of effector TH differentiation.

Introduction

Naïve CD4$^+$ helper T (TH) cells, upon encountering their cognate antigens presented on professional antigen-presenting cells (APC), differentiate into effector cells that are characterized by their distinct cytokine production profiles and immune regulatory functions (Dong and Flavell, 2000; Glimcher and Murphy, 2000). TH1 cells produce IFNγ and regulate antigen presentation and immunity against intracellular pathogens, whereas TH2 cells produce IL-4, IL-5 and IL-13, and mediate humoral responses and immunity against parasites. IFNγ and IL-4 are not only the key effector cytokines but also mediate the differentiation of TH1 and TH2 cells, respectively.

Recently, TH17, a third subset of TH cells, has been identified, which produce IL-17, IL-17F and IL-22 and regulate inflammatory responses by tissue cells (Bettelli et al., 2007; Dong, 2006; Reiner, 2007; Weaver et al., 2006). TH17 differentiation, at least in mouse, is initiated

Correspondence should be addressed to C.D. (cdong@mdanderson.org) or R.I.N. (rnurieva@mdanderson.org) .

by TGFβ and IL-6 (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006a), possibly via regulating the chromatin remodeling of the *Il17-Il17f* locus (Akimzhanov et al., 2007). While IL-6 is necessary for TH17 differentiation (Korn et al., 2007; Yang et al., 2007), IL-21 was recently reported as an autocrine factor induced by IL-6 to regulate TH17 differentiation (Korn et al., 2007; Nurieva et al., 2007a; Zhou et al., 2007). On the other hand, TGFβ signaling has also been clearly demonstrated to mediate TH17 differentiation in vivo (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006b), and activated T cells may serve as an important sources of TGFβ for this regulation (Li et al., 2007). TH17 development is dependent on STAT3 (Laurence et al., 2007; Yang et al., 2007), which functions to upregulate the expression of two TH17-specific orphan nuclear receptors RORγt and RORα that ultimately determines TH17 terminal differentiation (Ivanov et al., 2006; Yang et al.).

A fundamental function of TH cells is to provide "help" to B cells and regulate their proliferation and immunoglobulin class-switching, especially in the germinal center structures. TH1 and TH2 cells have been shown to regulate B cell responses to some extents. For example, IFNγ regulates IgG2a production while IL-4 is critical in IgE class-switching. However, an additional TH subset called follicular helper T (T$_{FH}$) cells are recently found to be present in germinal centers and are characterized by their expression of CXCR5 (Vinuesa et al., 2005b). Although activated T cells may transiently express CXCR5, T$_{FH}$ cells appear to have more stable expression of this chemokine receptor. These cells are thought to regulate humoral immunity, especially germinal center reactions. Consistent with this notion, CXCR5 has been shown to be important for proper T and B cell localization in immune responses and antibody production (Haynes et al., 2007; Junt et al., 2005). In addition to CXCR5, other markers have been also reported for T$_{FH}$ cells, such as ICOS costimulatory receptor, IL-21 cytokine and Bcl-6 transcription factor. ICOS was found essential for generation of T$_{FH}$ cells in vivo (Akiba et al., 2005). In addition to TH17 cells, IL-21 is also expressed in T$_{FH}$ cells and may serve as an important regulator of humoral responses by T$_{FH}$ cells. IL-21 directly regulates B cell proliferation and class-switching; IL-21R deficiency results in defective antibody responses and impaired germinal center formation (Spolski and Leonard, 2008). On the other hand, sanroque mice, which have a mutation in a RING-type E3 ubiquitin ligase, Roquin, developed spontaneous autoantibody production and lupus-like autoimmunity, associated with greatly increased numbers of CXCR5+ CD4 T cells and enhanced expression of IL-21 and ICOS (Vinuesa et al., 2005a).

Despite their potential importance in humoral immunity and immunopathology, the developmental regulation of T$_{FH}$ cells and their relationship with other TH subsets is unclear, particularly considering the fact that IL-21 is expressed by both TH17 and T$_{FH}$ cells. A previous study revealed that human T$_{FH}$ cells express distinct genes from TH1 or TH2 cells (Chtanova et al., 2004). Here we show that mouse T$_{FH}$ cells have a divergent gene expression profile from TH1, TH2 and TH17 cells, and develop in vivo independent of these lineages. IL-21, IL-6 and STAT3 are critical in the generation of T$_{FH}$ cells. Moreover, T cells activated in vitro in the presence of IL-21 but without TGFβ signaling preferentially acquire T$_{FH}$ gene expression and function to promote humoral immunity in vivo. Thus, our data indicate that T$_{FH}$ cells represent a distinct TH lineage and suggest a reciprocal relationship between the T$_{FH}$ and TH17 lineages.

Results

Distinct gene expression profile of T$_{FH}$ cells

As a first step toward understanding T$_{FH}$ cell regulation, we compared the gene expression profiles of TH1, TH2 and TH17 cells differentiated in vitro with in-vivo generated T$_{FH}$ cells. Taking advantage of the co-expression of BTLA by CXCR5$^+$ T$_{FH}$ cells (Supplementary Figure 1), CD4$^+$CD44^{hi}CXCR5$^+$BTLA$^+$ were FACS sorted from splenocytes of C57BL/6 mice 7 days after immunization with KLH. These cells as well as TH1, TH2 and TH17 cells were

restimulated with anti-CD3 for 4 hours and subject to gene profiling analysis in duplicates using Affimetrix gene chips. The microarray data were normalized using GCRMA and the genes whose expression levels were changed across the TH1, TH2, TH17 and T_{FH} cells were then selected using a False Discovery Rate (FDR) estimation method. Then, the expression levels of 8350 probe-sets showing differential expressions among the four types of cells were used for hierarchical clustering, which revealed that T_{FH} cells have a very distinct gene expression profile (Figure 1A).

To confirm the above results, we performed real-time RT-PCR analysis on multiple subset-specific genes. The data indicate that T_{FH} cells did not express the typical markers for TH1 (IFNγ and T-bet) or TH2 (IL-4 and GATA3) cells (Figure 1B), consistent with a previous report on human T_{FH} cells (Chtanova et al., 2004). Although T_{FH} cells shared IL-21 expression with TH17 cells, they did not express IL-17, IL-17F, IL-22 or RORγt (Figure 1B). Instead, similar to their human counterparts, (Chtanova et al., 2004; Kim et al., 2004), mouse T_{FH} cells express mRNAs for CXCR5 as well as Bcl-6 (Figure 1B). In addition, T_{FH} cells preferentially expressed mRNAs for IL-6R and IL-6st (gp130) and also upregulated the expression of IL-21R (Figure 1B), suggesting possible regulation of T_{FH} cells by IL-6 and IL-21. Moreover, consistent with human T_{FH} cells (Chtanova et al., 2004) and a recent report indicating that T_{FH} cells express PD-1 protein (Haynes et al., 2007), PD-1 mRNA was highly upregulated in T_{FH} cells compare to other TH subsets (data not shown).

To substantiate the above results, we also measured cytokine secretion of purified T_{FH} cells after they were activated ex vivo with anti-CD3 and anti-CD28 for 24 hours. High levels of IL-21 but not IL-4, IL-10, IFNγ or IL-17 were observed in T_{FH} cells (Figure 1C). In addition, purified T_{FH} cells were activated with KLH and irradiated splenic APC. Consistent with above results, T_{FH} cells preferentially produced IL-21, but not TH1, TH2 and TH17 cytokines (Supplementary Figure 3). Furthermore, intracellular analysis on CXCR5+ and CXCR5- cells following PMA and ionomycin restimulation also revealed that T_{FH} cells did not express IFNγ or IL-17 (Figure 1D). These results indicate that T_{FH} cells are distinct from TH1, TH2 and TH17 cells in their gene expression and cytokine production.

Naïve T cell differentiation into T_{FH} cells is independent of TH1 and TH2 lineages

Previous (Chtanova et al., 2004) and our current gene expression analysis on human and mouse T_{FH} cells, respectively, suggested that they are distinct from TH1 and TH2 cells. However, it was not clear if generation of T_{FH} cells is distinct from TH1 or TH2 differentiation. To address this question, we transferred FACS sorted naïve CD4+ T cells (CXCR5-) from OT-II TcR transgenic mice (CD45.2+, Figure 2A) into C57BL/6 recipients (CD45.1+) followed by immunization with Ova protein in CFA. 7 days later, we examined splenic CD45.1+ and CD45.2+ CD4 cells, and found a substantial increase in the frequency of CXCR5+CD4+ cells (Figure 2A). Interestingly, treatment of recipient mice with antibodies to IL-4 and IFNγ did not reduce the proportion of CXCR5+CD4+ cells (Figure 2A), suggesting that T_{FH} cell development is independent of TH1 or TH2 development. In addition, donor CD44hiCXCR5+ and CD44hiCXCR5- OT-II cells from immunized mice were purified and real-time RT-PCR analysis of T_{FH} specific genes were performed. CXCR5+ T cells were generated in the presence of blocking antibodies to IL-4 and IFNγ exhibited gene expression patterns comparable to those from control group (Figure 2A), supporting that these CXCR5+ cells were indeed T_{FH} cells.

To substantiate the above finding, we also immunized mice deficient in IL-4, IFNγ STAT6 or STAT4 and their appropriate controls with KLH. 7 days after immunization, we did not detect any defect in T_{FH} cells or PNA+ germinal center B cells in the knockout animals compared to wild-type controls (Figure 2B-D, Supplementary Figure 4A-C). To confirm the above results, we also measured cytokine secretion of purified CXCR5+ cells from IL-4- and IFNγ-deficient

mice and their controls after they were activated ex vivo with anti-CD3 and anti-CD28 for 24 hours. T_{FH} cells IL-4- or IFNγ-deficient mice produced similar level of IL-21, but did not express IL-4, IL-10, IFNγ or IL-17 (Supplementary Figure 4D), supporting that they were T_{FH} cells. Thus, we conclude that CXCR5$^+$CD4$^+$ T_{FH} cells develop independent of the TH1 and TH2 lineages.

B7h expressed on B cells regulates the generation of T_{FH} cells

Inducible costimulator (ICOS) is the third member of the CD28 family with an important role in regulation of T-dependant Ab responses and germinal center reactions (Dong and Nurieva, 2003). ICOS was previously shown to be expressed at high levels on human tonsillular CXCR5 + T cells within the light zone of germinal centers and efficiently supported the immunoglobulin production (Breitfeld et al., 2000; Schaerli et al., 2000). In addition, ICOS deficiency in human and mouse resulted in significantly reduced numbers of T_{FH} cell, indicating an essential role of ICOS in the differentiation of CXCR5+ CD4 T cells (Akiba et al., 2005; Bossaller et al., 2006b). Consistently, we also observed reduced percentages of CXCR5$^+$CD4$^+$ cell and decreased expression of IL-21 but not IFNγ in our B7h germline knockout animals (Nurieva et al., 2003b) following KLH immunization (Figure 3A). T_{FH} cells are regarded as regulators of the germinal center reaction by providing help to activated B cells that also express CXCR5. Since B cells constitutively express B7h, we asked whether the generation of T_{FH} cells may require B cell help via engagement of ICOS receptor on T cells. We thus bred mice carrying the B7h conditional flox (f) allele (Nurieva et al., 2003b) with CD19-cre mice (Rickert et al., 1995). In B7h f/f mice carrying the CD19-cre (B7h BKO), in comparison to those without cre expression, there was efficient deletion of the B7h gene in B cells, similar to those in B7h germline knockout mice (Figure 3B). We then immunized B7h BKO mice with KLH and found that absence of B7h in B cells led to a greatly reduced frequency of CXCR5$^+$CD4$^+$ cells (Figure 3C). Similarly, when CD45.1+ OT-II cells were transferred into B7h BKO mice, the numbers of CD45.1+ T_{FH} cells were also greatly reduced following Ova peptide immunization (data not shown). In addition to CXCR5 expression, we found that the expression of IL-21 was also greatly reduced in B7h BKO mice, whereas IFNγ expression was elevated in these mice (Figure 3C). Moreover, PNA$^+$ germinal center B cells were greatly reduced in these animals (Figure 3C and E). KLH-specific IgG production was also reduced (Figure 3D). Overall, these data indicate that B cell expression of B7h is necessary for IL-21 production and for the generation of T_{FH} cells and appropriate antibody responses, indicating that T_{FH} cell differentiation is regulated by B cells.

IL-21 and IL-6 are required for generation of T_{FH} cells, which is dependent on STAT3

IL-21 has been recently shown to be induced by IL-6 and to autoregulate its own expression during TH17 differentiation (Nurieva et al., 2007a). In addition, T_{FH} T cells produced significantly greatrt amount of IL-21 compared to TH1 and TH2 subsets (Chtanova et al., 2004), and induced the differentiation of autologous B cells into Ig-secreted plasma cells through IL-21 (Bryant et al., 2007). Since IL-21 is also expressed in T_{FH} cells, we assessed if IL-21 is important for T_{FH} cell generation. IL-21+/+, +/- and -/- mice were immunized with KLH and splenic T_{FH} cells were analyzed in these mice. IL-21+/- mice exhibited reduced number of T_{FH} cells, which was further reduced in IL-21-/- mice (Figure 4A). In addition, PNA$^+$ germinal center B cells were also greatly reduced in IL-21-/- mice (Figure 4B-C). In contrast, CD4 T cells from IL-21+/- and -/- mice showed normal proliferation and IFNγ expression after re-stimulation with KLH ex vivo. (Figure 5D). Thus, these results indicate that IL-21 is necessary for T_{FH} cell development.

Since IL-6 induces IL-21 expression, we also tested T_{FH} cell generation in mice lacking IL-6. It has been previously that IL-6 deficient mice showed reduced germinal centers and antigen specific Ig-production (IgG) (Kopf et al., 1998). Compared to the wild-type controls, IL-6-/-

mice exhibited greatly reduced numbers of T_{FH} cells and germinal center B cells (Figure 5A), indicating that IL-6 is also necessary for T_{FH} cell generation.

Since T_{FH} cells expressed high levels of IL-21R, IL-6R and IL-6st (Figure 1B), we also examined if IL-6 and IL-21 signaling regulates T_{FH} cells. C57BL/6 mice were immunized with KLH and $CD4^+CD44^{hi}$ T cells with or without CXCR5 expression were FACS sorted and restimulated with anti-CD3 and anti-CD28 in the absence or presence of IL-21 or IL-6. Compared to non-T_{FH} cells, $CXCR5^+$ T cells showed a significant reduction in proliferation when activated with anti-CD3 and anti-CD28. Treatment of $CXCR5^+$ T cells with IL-6 or IL-21 significantly enhanced their proliferation (Figure 5B), suggesting that IL-6 and IL-21 preferentially regulate T_{FH} cells. Furthermore, we stained restimulated $CXCR5^+$ and $CXCR5^-$ cells with Annexin V. We found that a significant portion of CXCR5+ cells underwent apoptosis compare to CXCR5- (Figure 5B). However, IL-6 or IL-21 treatment reduced apoptosis of T_{FH} cells.

Since both IL-6 and IL-21 signal through STAT3, we analyzed T_{FH} cell generation in STAT3 flox/flox mice (Takeda et al., 1999) bred with CD4-cre mice (Lee et al., 2001). The deletion of STAT3 gene in $CD4^+$ thymocytes was found to be complete (data not shown). When we immunized these mice as well as their controls with KLH, the numbers of CXCR5+ T_{FH} cells were found to be greatly reduced in the absence of STAT3 (Figure 5C). Moreover, STAT3 deficiency in T cells also led to defective germinal center B cell generation (Figure 5C). KLH-specific IgG and IgM production was also reduced in the absence of STAT3 in T cells (Supplementary Figure 5).

T_{FH} cell generation is independent of TH17 differentiation or function

The above results indicate that the IL-6-IL-21 axis and STAT3 transcription factor are necessary for T_{FH} cell generation, which is similar to the regulation of TH17 differentiation. To test whether the development of T_{FH} cells is dependent or independent upon the TH17 lineage, we utilized Rag1-/- mice reconstituted with bone marrow cells from wild-type, *ROR α* $^{st/st}$ or *ROR* $α^{st/st}ROR$ $α^{-/-}$ mice. We recently reported that the latter mice were completely impaired in TH17 differentiation in vitro and in vivo (Yang et al., 2008b). However, upon KLH immunization, these mice deficient in both RORα and RORγ in lymphocytes developed more CXCR5+ cells in spleen (Figure 6A), indicating that T_{FH} cells can be generated in the absence of TH17 development. Moreover, PNA$^+$ germinal center B cells were not affected in these animals (Supplementary Figure 6B).

Since TH17 differentiation requires also TGFβ in addition to IL-6 or IL-21, we examined whether TGFβ signaling is required for T_{FH} cell generation. C57BL/6 mice were immunized with KLH in the absence or presence of anti-TGFβ blocking antibody as previously described (Veldhoen et al., 2006b). While IL-17 expression was substantially decreased in anti-TGFβ-treated mice, CXCR5 expression was not (Figure 6B), indicating that TGFβ signaling is not essential for T_{FH} cell generation. Moreover, PNA expression on B cells was not affected either (Figure 6B).

To understand if T_{FH} cell generation requires TH17 function, we also immunized mice deficient in IL-17 or IL-17F. Lack of IL-17 or IL-17F did not significantly reduce the number of T_{FH} cells in spleen or the number of germinal center B cells (Figure 6C, Supplementary Figure 6B). IL-17 or IL-17F is thus not essential in the generation of T_{FH} cells in vivo.

IL-21 in the absence of TGFβ initiates T_{FH} cell differentiation

Our results thus far suggest that although IL-6 and IL-21 are required for both T_{FH} and TH17 differentiation, these two subsets appear to have distinct genetic program and differ in their

dependency on TGFβ signaling. Since T_{FH} cells have never been generated in vitro, we next assessed whether IL-21 is sufficient to drive T_{FH} cell development in the absence of TGFβ signaling. Naïve OT-II cells were activated by Ova peptide and splenic APC in the absence (neutral condition) or presence of IL-21, TGFβ and antibodies to IL-4 and IFNγ (TH17 condition) or IL-21 plus antibodies to IL-4, IFNγ and TGFβ. 5 days later, the activated T cells were extensively washed and restimulated with anti-CD3 for 4 hours and their gene expression was assessed by real-time RT-PCR. As expected, cells cultured under TH17 condition highly expressed TH17-specific genes, including IL-17, IL-17F, IL-22, RORα and RORγt (Figure 7A). In contrast, T cells treated with IL-21 in the absence of TGFβ signaling upregulated genes that are specifically expressed in T_{FH} cells, including CXCR5, Bcl-6, IL-6R and IL-6st (Figure 7A). They also upregualted IL-21R expression but did not express TH17 genes (Figure 7A). PD-1 was also highly expressed in these cells (data not shown). To confirm this result, cell supernatants were measured 24 hours after restimulation for cytokine secretion by ELISA. IL-21 in the absence of TGFβ signaling did not support IL-17 expression, but the resulting cells expressed high levels of IL-21 (Figure 7B), supporting that IL-21 expression is independent of TGFβ signaling.

To test the function of these T_{FH}-like cells in vivo, we transferred OT-II cells (CD45.1+) activated under neutral condition or with IL-21 plus blocking antibodies to IFNγ, IL-4 and TGFβ into recipient mice (CD45.2+) followed by immunization with Ova protein. Compared to mice receiving no cells or T cells activated under neutral condition, the recipients of IL-21-treated cells exhibited greatly increased CD45.1+ T_{FH} cells (Figure 7C). Interestingly, host T cells in these mice also had approximately 4 times T_{FH} cells compared to those receiving T cells activated under neutral condition (Figure 7C). The transferred cells also promoted Ova-specific antibody production and germinal center reactions (Figure 7D-F). As a control for the above experiment, we sorted CD45.1^{+} CXC5^{+}CD44^{hi} cells from KLH immunized mice and transferred them into recipient mice (CD45.2+) followed by immunization with KLH. Similar to in vitro generated T_{FH} cells, in vivo generated CXCR5+ cells also promoted a significant increase in PNA[+] germinal center B cells (Figure 7G). These results indicate that IL-21, in the absence of TH1, TH2 and TH17 differentiation, initiates T_{FH} cell development.

Discussion

Following antigenic activation, naïve TH cells differentiate into distinct effector subsets. A fundamental function of TH cells is to "help" humoral immunity. Although strongly implicated in antibody production and germinal center reactions, the ontogeny of T_{FH} cells has been unclear. In the current study, we find that T_{FH} cells are distinct from TH1, TH2 or TH17 cells in their gene expression and developmental regulation. Generation of T_{FH} cells requires IL-21, IL-6 and STAT3.

Although implicated in humoral immunity and antibody-mediated autoimmunity, the regulation of T_{FH} cell development is unclear. ICOS-B7h interaction has been well established in the literature to regulate humoral immunity and germinal center reactions (Dong and Nurieva, 2003). This costimulatory pathway was also found important in generation of T_{FH} cells in mouse (Akiba et al., 2005). Interestingly, ICOS deficiency in human patients also causes a severe reduction of T_{FH} cells (Bossaller et al., 2006a). Conversely, impaired negative regulation of ICOS by Roquin E3 ubiquitin ligase led to increased numbers of CXCR5+ T_{FH} cells and IL-21 hyperproduction (Vinuesa et al., 2005a; Yu et al., 2007). However, the mechanism by which ICOS/B7h regulates T_{FH} cell development has not been clear. In our current study, we find that ICOS-B7h interaction is necessary for IL-21 expression by T cells. ICOS may thus regulate T_{FH} cells through production of IL-21. A recent study by Kim et al has revealed IL-21 regulation by calcium signaling and NFAT factors (Kim et al., 2005). We previously showed that ICOS, together with TcR and CD28, increases the expression of

NFATc1 through a PI-3 kinase-Itk-calcium pathway (Nurieva et al., 2007b; Nurieva et al., 2003a). ICOS may act through NFATc1 to regulate IL-21 expression. Since IL-21 also regulates TH17 differentiation, an IL-21 defect may account for the impairment in IL-17 expression in the ICOS-deficient animals (Dong and Nurieva, 2003). Moreover, using a B cell-specific B7h knockout mouse, we show that B7h expression on B cells is required for the generation of T_{FH} cells and IL-21 expression. This result not only substantiates the importance of ICOS-B7h interaction in T_{FH} cell development, but also suggests an important function of B cells in vivo as APCs in the generation or maintenance of T_{FH} cells. Consistent with our findings, Ebert et al previously showed that human B cells regulate T_{FH} cell phenotypes (Ebert et al., 2004).

Previous gene expression analysis has revealed that human T_{FH} cells are distinct from TH1 and TH2 cells (Chtanova et al., 2004; Kim et al., 2004). Our current study also reveals that T_{FH} cells do not produce TH1 or TH2 cytokines. Interestingly, although human T_{FH} cells appear to express IL-10 and CXCL13, a ligand for CXCR5 (Chtanova et al., 2004; Ebert et al., 2004; Kim et al., 2004; Kim et al., 2001), mouse T_{FH} cells do not. What accounts for this species difference would need further investigation. Moreover, we found that naïve T cells differentiate into T_{FH} cells in vivo, which is independent of IFNγ, IL-4 and STAT6. From these data, we conclude that T_{FH} cells develop independent of TH1 and TH2 lineages.

On the other hand, T_{FH} cells share common regulators with TH17 cells. Both subsets express IL-21 and their development depends similarly on IL-6, IL-21 and STAT3. However, T_{FH} cells differ from TH17 cells in the following aspects. First, they are distinct in their gene expression profiles. Second, T_{FH} cells do not produce IL-17, IL-17F or IL-22. Most importantly, T_{FH} cell development does not require RORα or RORγt. Thus, we believe that T_{FH} cells develop independent of the TH17 lineage. TH17 development in mouse is not only mediated by the IL-6-IL-21 axis, but also by TGFβ. We show here that IL-21 can be induced in T cells independent of TGFβ signaling. T cells activated in the presence of IL-21 but in the absence of IL-4, IFNγ and TGFβ signaling produced IL-21 but not IL-4, IFNγ, IL-17, IL-17F or IL-22. Furthermore, these cells acquired expression of CXCR5, Bcl-6, IL-6R and IL-6st, genes that expressed by in vivo generated T_{FH} cells, suggesting that T_{FH} cells may be generated in vitro under the above condition. Moreover, these T_{FH}-like cells generated in vitro preferentially expressed CXCR5 in vivo and functioned to promote humoral immunity, similar to in vivo-generated T_{FH} cells. Furthermore, TGFβ signaling, although required for IL-17 expression in vivo, is not essential for T_{FH} cells, indicating a reciprocal relationship of T_{FH} and TH17 cells. Interestingly, Bcl-6 has recently been shown as a repressor of TGFβ-SMAD signaling (Wang et al., 2008), suggesting that lack of TGFβ signaling may favor T_{FH} cell development. These results further support that T_{FH} cells development is independent of TH1, TH2 and TH17 cells, and IL-21 serves as critical factor for generation of this lineage. Interestingly, in vitro generated T_{FH} cells also enhanced the T_{FH} cell generation in recipient mice, suggesting that IL-21 may function in a paracrine fashion to regulate T_{FH} cell development.

In summary, our current study has extensively characterized the developmental regulation of T_{FH} cells. Our data indicate that T_{FH} cells are distinct in their gene expression and immune function and develop via a pathway that is dependent on IL-21 or IL-6 but independent of TH1, TH2 or TH17 lineages (Supplemental Figure 7). In mice defective in T_{FH} cells, there existed still detectable amounts of antigen-specific antibodies, suggesting that other TH subsets may independently regulate the humoral immunity. This knowledge may help us to find ways to treat antibody-mediated autoimmune diseases.

Methods

Mice

IL-4-, IFNγ- and IL-6-deficient mice on C57BL/6 background and STAT6 and STAT4-deficient mice on BALB/c background were purchased from Jackson Laboratories and C57BL/6, B6.SJL (CD45.1) and BALB/c mice were used as controls. RORα$^{st/st}$, RORα$^{st/st}$ RORγ$^{-/-}$ and wild-type bone marrow chimeras were generated as described (Yang et al., 2008b). Stat3 fl/fl mice (Takeda et al., 1999) were bred with CD4-Cre mice provided by Dr. Christopher Wilson (Lee et al., 2001). IL-21 knockout mice on 129×C57BL/6 F1 mixed background were obtained from NIH Mutant Mouse Regional Resource Centers (MMRRC) (Nurieva et al., 2007a). B7h BKO mice were created by breeding B7H flox mice (Nurieva et al., 2003b) with CD19-cre mice (Rickert et al., 1995). IL-17- and IL-17F- mice were recently generated in our lab (Yang et al., 2008a). Mice were housed in the SPF animal facility at M. D. Anderson Cancer Center and the animal experiments were performed at the age of 6-10 weeks using protocols approved by Institutional Animal Care and Use Committee.

T cell differentiation

Differentiation of OT-II cells in Figure 1A-B was performed as previously described (Chung et al., 2006). CD62L^{hi}CD44^{lo}CD25^{neg}CD4$^+$ T cells were isolated and cultured with irradiated splenic APC plus OVA$_{323-339}$ peptide (10 μg/ml) (TH0 condition) and in the presence of polarizing reagents as the following 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. In Figure 7A, the cytokine stimuli for TH17 differentiation were 100 ng/ml of IL-21, 5 ng/ml of TGFβ, and 10 μg/ml of anti-IL-4 and 10 μg/ml of anti-IFNγ, and for generation of T$_{FH}$ cells were 50 ng/ml of IL-21, 10 μg/ml anti-IFNγ, 10 μg/ml anti-IL-4 and 20 μg/ml anti-TGF-β (1D11) neutralizing Abs. IL-4, IL-6, IL-12 and TGFβ were purchased from Peprotech. IL-21, IL-23 and anti-TGF-β (1D11) neutralizing Abs were purchased from R&D. To characterized the in vitro-differentiated CD4 T cells under these conditions, these cells were restimulated with plate-bound anti-CD3 Ab (5 μg/ml) for 4hours for real-time PCR analysis, or for 24 hours for cytokine measurement by ELISA.

Keyhole Limpet Hemocyanin (KLH) Immunization

Various strains of mice (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). In Figure 6 for local blockade of TGF-β, 100 μg anti-TGFβ (1D11) was included in the emulsion; the control group received 100 μg isotype control antibodies. Seven days after immunization, these mice were sacrificed and analyzed individually. The germinal center B cells were determined by staining with FITC-labeled PNA (Pharmingen) and PerCP-labeled anti-B220 mAb (Pharmingen). The T$_{FH}$ 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.). In some experiments, sera from immunized mice were collected, and antigen-specific IgM, and IgG antibodies were measured by using ELISA. Briefly, serum samples were added in a 3-fold serial dilution onto plates precoated with 10 μg/ml KLH or Ova protein. Antigen-specific antibodies were detected with biotinylated goat antimouse IgM or rat anti-mouse IgG antibodies (Southern Biotechnology Associates). To analyze the role of B7h in regulation of T cell responses *in vivo* (Fig. 3A and C), spleen cells from KLH-immunized mice were stimulated in 96-well plates as triplicates with or without KLH. Effector cytokines (IFN-γ and IL-21) were analyzed 4 days later by ELISA (Pharmingen). In Figure 6 B, spleen cells from immunized mice were restimulated with 50 μg KLH for 24 h. In the final 5 h, Golgi-stop (BD Bioscience) was added and IL-17- and

IFNγ-producing cells were analyzed using a BD CytoFix/CytoPerm intracellular staining kit (BD Bioscience).

Statistical analysis of microarray data

The DNA microarray analysis was carried out at the Institute for Systems Biology microarray core facility using Affymetrix Mouse 430 2.0 chips. The total RNA samples were labeled according to manufacturer's instruction using One-Cycle Target Labeling method, which consists of oligo-dT primed cDNA synthesis followed by in vitro transcription that incorporates biotinylated nucleotides. The microarray data were normalized using GCRMA (Zhijin et al., 2004). We then selected the genes whose expression levels were changed across the TH1, TH2, TH17, and T_{FH} cells using a False Discovery Rate (FDR) estimation method (Storey and Tibshirani, 2001): 1) one-way ANOVA was performed to compute unadjusted p-value for the four classes of cells; 2) the number of non-differentially expressed probe-sets (m_0) was estimated as $2 \times$ the number of the probe-sets with p-value>0.5 ($m0= 25088$); 3) the expected number of false positives under the complement null hypothesis $E(V_0)$ was estimated for a given ANOVA F-statistic value by performing 500 times of resampling; and 4) FDR was finally estimated as $m_0/m \times E(V_0)/R$ where m is the total number of the probe-sets ($m=45037$) and R is the number of the genes being selected using the given ANOVA F-statistic value (FDR estimated for various F-statistic value is shown in Supplementary Figure 2). Then, the expression levels of 8350 probe-sets showing differential expressions among the four types of cells (FDR = 0.1, corresponding to an unadjusted p-value = 0.033 from one-way ANOVA) were used for hierarchical clustering (Euclidian distance metric and Ward Minimum Variance Linkage) and PCA.

Adoptive transfer study

CD4+ T cells from OT-II mice (CD45.2) were intravenously transferred into C57BL/6 (CD45.1±) mice (3×10^6 cells/mouse) (3 groups; 3 mice per group). 2 groups of recipient mice were immunized subcutaneously with 100 μg Ova protein emulsified in CFA and treated with a 300 μg of control rat Ig or anti-IFNγ and anti-IL-4 mAbs at the time of immunization (day 0) and on days 2 and 4. Seven days after the immunization, experimental mice were sacrificed and splenic CD45.1+ and CD45.2+ CD4 cells were stained with biotinylated anti-CXCR5 mAb, followed by APC-labeled streptavidin. In Figure 7C-E, FACS-sorted naïve (CD4^{+}CD62L^{hi}CD44^{-}CD25^{-}) T cells from CD45.1+ OT-II mice were activated under TH0 or T_{FH} condition, washed and intravenously transferred into C57BL/6 (CD45.2^{+}) mice (4×10^6 cells/mouse) and the recipient mice were subcutaneously immunized with 100 μg OVA protein emulsified in IFA. A group of C57BL/6 mice that did not receive T cells was used as a control (No transfer). In Fig. 7G, CXCR5+CD44^{hi} cells were sorted from B6.SJL (CD45.1) mice immunized with KLH. These cells were transferred into C57BL/6 (CD45.2^{+}) mice (5×10^6 cells/mouse) (3 mice per group). Second group did not receive cells. All mice were immunized subcutaneously with 1000 μg KLH. Seven days after immunzation, lymphoid cells from the draining lymph nodes of the recipient mice were isolated and stained with FITC-labeled anti-CD45.1 mAb and PerCP-labeled anti-CD4 mAb plus biotinylated anti-CXCR5 mAb, followed by APC-labeled streptavidin, or stained with FITC-labeled PNA and PerCP-labeled anti-B220 mAb.

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™ SYBR green real-time PCR kit (Bio-Rad Laboratories, Inc.). The data were normalized to β-actin reference. The following primer pair for Bcl-6, IL-6R, IL-6st, and CXCR5 was used: Bcl6 forward:

CACACCCGTCCATCATTGAA, reverse: TGTCCTCACGGTGCCTTTTT; IL6R forward: GGTGGCCCAGTACCAATGC, reverse: GGACCTGGACCACGTGCT; CXCR5 forward: ACTCCTTACCACAGTGCACCTT, reverse: GGAAACGGGAGGTGAACCA; IL-6st forward: ATT TGT GTG CTG AAG GAG GC, reverse: AAA GGA CAG GAT GTT GCA GG. The primers for IL-21, IL-17, IL-17F, IL-4, IFNγ, RORγ, RORα, T-bet, GATA3, and β-actin were previously described (Yang et al., 2007).

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. Purified anti-mouse CD4 or biotin labeled anti-mouse PNA were applied as primary Ab following with biotinylated anti-rat secondary Ab and avidin-peroxidase complex reagent. Novared was used as substrate. For double staining, biotin-labeled B220 was applied and following with avidin-aklinphosphotase complex reagent. Vector blue was used as substrate. CD4 and B220 are from BD Pharmagen. PNA and other reagent are from Vector Laboratary. Slides for PNA staining were count stained with Hematoxlin.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

References

Akiba H, Takeda K, Kojima Y, Usui Y, Harada N, Yamazaki T, Ma J, Tezuka K, Yagita H, Okumura K. The Role of ICOS in the CXCR5+ Follicular B Helper T Cell Maintenance In Vivo. J Immunol 2005;175:2340–2348. [PubMed: 16081804]

Akimzhanov AM, Yang XO, Dong C. Chromatin remodeling of interleukin-17 (IL-17)-IL-17F cytokine gene locus during inflammatory helper T cell differentiation. J Biol Chem 2007;282:5969–5972. [PubMed: 17218320]

Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature 2006;441:235–238. [PubMed: 16648838]

Bettelli E, Oukka M, Kuchroo VK. T(H)-17 cells in the circle of immunity and autoimmunity. Nat Immunol 2007;8:345–350. [PubMed: 17375096]

Bossaller L, Burger J, Draeger R, Grimbacher B, Knoth R, Plebani A, Durandy A, Baumann U, Schlesier M, Welcher AA, et al. ICOS Deficiency Is Associated with a Severe Reduction of CXCR5+CD4 Germinal Center Th Cells. J Immunol 2006a;177:4927–4932. [PubMed: 16982935]

Bossaller L, Burger J, Draeger R, Grimbacher B, Knoth R, Plebani A, Durandy A, Baumann U, Schlesier M, Welcher AA, et al. ICOS deficiency is associated with a severe reduction of CXCR5+CD4 germinal center Th cells. J Immunol 2006b;177:4927–4932. [PubMed: 16982935]

Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, Forster R. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. J Exp Med 2000;192:1545–1552. [PubMed: 11104797]

Bryant VL, Ma CS, Avery DT, Li Y, Good KL, Corcoran LM, de Waal Malefyt R, Tangye SG. Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. J Immunol 2007;179:8180–8190. [PubMed: 18056361]

Chtanova T, Tangye SG, Newton R, Frank N, Hodge MR, Rolph MS, Mackay CR. T Follicular Helper Cells Express a Distinctive Transcriptional Profile, Reflecting Their Role as Non-Th1/Th2 Effector Cells That Provide Help for B Cells. J Immunol 2004;173:68–78. [PubMed: 15210760]

Chung Y, Yang X, Chang SH, Ma L, Tian Q, Dong C. Expression and regulation of IL-22 in the IL-17-producing CD4+ T lymphocytes. Cell Res 2006;16:902–907. [PubMed: 17088898]

Dong C. Diversification of T-helper-cell lineages: finding the family root of IL-17-producing cells. Nat Rev Immunol 2006;6:329–334. [PubMed: 16557264]

Dong C, Flavell RA. Cell fate decision: T-helper 1 and 2 subsets in immune responses. Arthritis Res 2000;2:179–188. [PubMed: 11094427]

Dong C, Nurieva RI. Regulation of immune and autoimmune responses by ICOS. J Autoimmun 2003;21:255–260. [PubMed: 14599850]

Ebert, Lisa M.; Horn, Michael P.; Lang, Alois B.; Moser, B. B cells alter the phenotype and function of follicular-homing CXCR5+ T cells. Eur J Immunol 2004;34:3562–3571. [PubMed: 15549776]

Glimcher LH, Murphy KM. Lineage commitment in the immune system: the T helper lymphocyte grows up. Genes Dev 2000;14:1693–1711. [PubMed: 10898785]

Haynes NM, Allen CDC, Lesley R, Ansel KM, Killeen N, Cyster JG. Role of CXCR5 and CCR7 in Follicular Th Cell Positioning and Appearance of a Programmed Cell Death Gene-1High Germinal Center-Associated Subpopulation. J Immunol 2007;179:5099–5108. [PubMed: 17911595]

Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell 2006;126:1121–1133. [PubMed: 16990136]

Junt T, Fink K, Forster R, Senn B, Lipp M, Muramatsu M, Zinkernagel RM, Ludewig B, Hengartner H. CXCR5-Dependent Seeding of Follicular Niches by B and Th Cells Augments Antiviral B Cell Responses. J Immunol 2005;175:7109–7116. [PubMed: 16301613]

Kim CH, Lim HW, Kim JR, Rott L, Hillsamer P, Butcher EC. Unique gene expression program of human germinal center T helper cells. Blood 2004;104:1952–1960. [PubMed: 15213097]

Kim CH, Rott LS, Clark-Lewis I, Campbell DJ, Wu L, Butcher EC. Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. J Exp Med 2001;193:1373–1381. [PubMed: 11413192]

Kim H-P, Korn LL, Gamero AM, Leonard WJ. Calcium-dependent Activation of Interleukin-21 Gene Expression in T Cells. J Biol Chem 2005;280:25291–25297. [PubMed: 15879595]

Kopf M, Herren S, Wiles MV, Pepys MB, Kosco-Vilbois MH. Interleukin 6 influences germinal center development and antibody production via a contribution of C3 complement component. J Exp Med 1998;188:1895–1906. [PubMed: 9815267]

Korn T, Bettelli E, Gao W, Awasthi A, Jager A, Strom TB, Oukka M, Kuchroo VK. IL-21 initiates an alternative pathway to induce proinflammatory TH17 cells. Nature 2007;448:484–487. [PubMed: 17581588]

Laurence A, Tato CM, Davidson TS, Kanno Y, Chen Z, Yao Z, Blank RB, Meylan F, Siegel R, Hennighausen L, et al. Interleukin-2 Signaling via STAT5 Constrains T Helper 17 Cell Generation. Immunity 2007;26:371–381. [PubMed: 17363300]

Lee PP, Fitzpatrick DR, Beard C, Jessup HK, Lehar S, Makar KW, Perez-Melgosa M, Sweetser MT, Schlissel MS, Nguyen S, et al. A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. Immunity 2001;15:763–774. [PubMed: 11728338]

Li MO, Wan YY, Flavell RA. T Cell-Produced Transforming Growth Factor-[beta]1 Controls T Cell Tolerance and Regulates Th1- and Th17-Cell Differentiation. Immunity 2007;26:579–591. [PubMed: 17481928]

Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, Hatton RD, Wahl SM, Schoeb TR, Weaver CT. Transforming growth factor-beta induces development of the T(H)17 lineage. Nature 2006;441:231–234. [PubMed: 16648837]

Nurieva R, Yang XO, Martinez G, Zhang Y, Panopoulos AD, Ma L, Schluns K, Tian Q, Watowich SS, Jetten AM, Dong C. Essential autocrine regulation by IL-21 in the generation of inflammatory T cells. Nature 2007a;448:480–483. [PubMed: 17581589]

Nurieva RI, Chuvpilo S, Wieder ED, Elkon KB, Locksley R, Serfling E, Dong C. A Costimulation-Initiated Signaling Pathway Regulates NFATc1 Transcription in T Lymphocytes. J Immunol 2007b; 179:1096–1103. [PubMed: 17617602]

Nurieva RI, Duong J, Kishikawa H, Dianzani U, Rojo JM, Ho I, Flavell RA, Dong C. Transcriptional regulation of th2 differentiation by inducible costimulator. Immunity 2003a;18:801–811. [PubMed: 12818161]

Nurieva RI, Mai XM, Forbush K, Bevan MJ, Dong C. B7h is required for T cell activation, differentiation, and effector function. Proc Natl Acad Sci U S A 2003b;100:14163–14168. [PubMed: 14615582]

Reiner SL. Development in motion: helper T cells at work. Cell 2007;129:33–36. [PubMed: 17418783]

Rickert RC, Rajewsky K, Roes J. Impairment of T-cell-dependent B-cell responses and B-l cell development in CD19-deficient mice. Nature 1995;376:352–355. [PubMed: 7543183]

Schaerli P, Willimann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. J Exp Med 2000;192:1553–1562. [PubMed: 11104798]

Spolski R, Leonard WJ. Interleukin-21: Basic Biology and Implications for Cancer and Autoimmunity. Annu Rev Immunol 2008;26

Storey, JD.; Tibshirani, R. Estimating the Positive False Discovery Rate Under Dependence, with Applications to DNA Microarrays. 2001. Department of Statistics, Stanford University; 2001.

Takeda K, Clausen BE, Kaisho T, Tsujimura T, Terada N, Forster I, Akira S. Enhanced Th1 Activity and Development of Chronic Enterocolitis in Mice Devoid of Stat3 in Macrophages and Neutrophils. Immunity 1999;10:39–49. [PubMed: 10023769]

Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM, Stockinger B. TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity 2006a;24:179–189. [PubMed: 16473830]

Veldhoen M, Hocking RJ, Flavell RA, Stockinger B. Signals mediated by transforming growth factor-beta initiate autoimmune encephalomyelitis, but chronic inflammation is needed to sustain disease. Nat Immunol 2006b;7:1151–1156. [PubMed: 16998492]

Vinuesa CG, Cook MC, Angelucci C, Athanasopoulos V, Rui L, Hill KM, Yu D, Domaschenz H, Whittle B, Lambe T, et al. A RING-type ubiquitin ligase family member required to repress follicular helper T cells and autoimmunity. Nature 2005a;435:452–458. [PubMed: 15917799]

Vinuesa CG, Tangye SG, Moser B, Mackay CR. FOLLICULAR B HELPER T CELLS IN ANTIBODY RESPONSES AND AUTOIMMUNITY. Nature Reviews Immunology Nat Rev Immunol 2005b; 5:853–865.

Wang D, Long J, Dai F, Liang M, Feng X-H, Lin X. BCL6 Represses Smad Signaling in Transforming Growth Factor-B Resistance. Cancer Research. 2008

Weaver CT, Harrington LE, Mangan PR, Gavrieli M, Murphy KM. Th17: an effector CD4 T cell lineage with regulatory T cell ties. Immunity 2006;24:677–688. [PubMed: 16782025]

Yang X, Chang SH, Park H, Nurieva R, Shah B, Acero L, Wang Y, Schluns KS, Broaddus RR, Zhu Z, Dong C. Regulation of inflammatory responses by IL-17F. J Exp Med. 2008aIn press

Yang XO, Panopoulos AD, Nurieva R, Chang SH, Wang D, Watowich SS, Dong C. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. J Biol Chem 2007;282:9358–9363. [PubMed: 17277312]

Yang XO, Pappu BP, Nurieva R, Akimzhanov A, Kang HS, Chung Y, Ma L, Shah B, Panopoulos AD, Schluns KS, et al. T Helper 17 Lineage Differentiation Is Programmed by Orphan Nuclear Receptors ROR[alpha] and ROR[gamma]. Immunity 2008b;28:29–39. [PubMed: 18164222]

Yu D, Tan AH, Hu X, Athanasopoulos V, Simpson N, Silva DG, Hutloff A, Giles KM, Leedman PJ, Lam KP, et al. Roquin represses autoimmunity by limiting inducible T-cell co-stimulator messenger RNA. Nature 2007;450:299–303. [PubMed: 18172933]

Zhijin W, Rafael AI, Robert G, Francisco M-M, Forrest S. A Model-Based Background Adjustment for Oligonucleotide Expression Arrays. Journal of the American Statistical Association 2004;99:909–917.

Zhou L, Ivanov II, Spolski R, Min R, Shenderov K, Egawa T, Levy DE, Leonard WJ, Littman DR. IL-6 programs TH-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat Immunol 2007;8:967–974. [PubMed: 17581537]
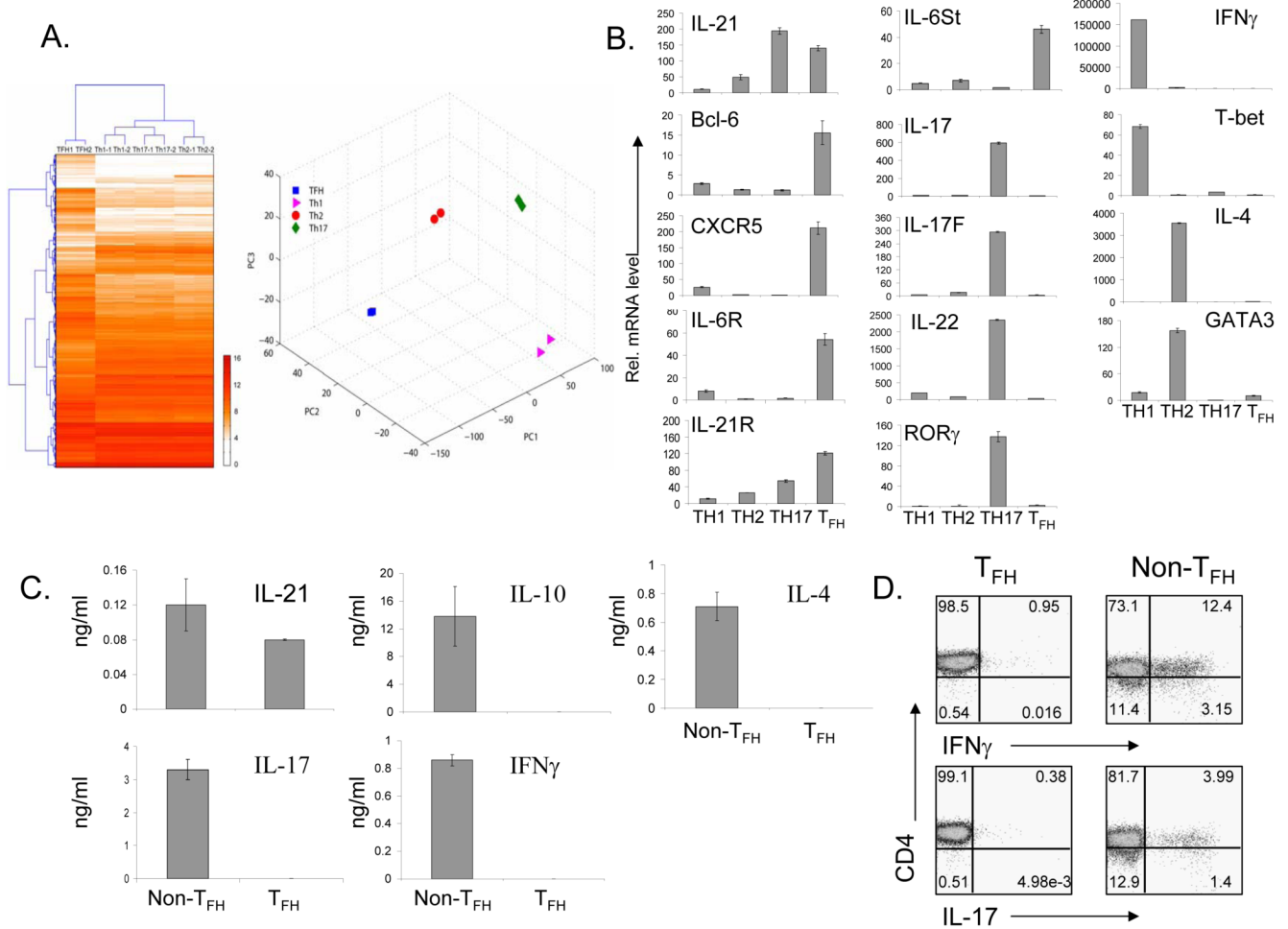
Figure1. T_{FH} cells express distinct genes from TH1, TH2 and TH17 cells

C57BL/6 mice were immunized with KLH in CFA. 7 days later CD4$^+$CD44^{hi}CXCR5$^+$ BTLA$^+$ (T$_{FH}$) cells were sorted and restimulated with anti-CD3 for 4 hours for gene profiling analysis (A) and for real-time PCR analysis (B). OT-II T cells differentiated under various conditions were restimulated with anti-CD3 for 4 hours and were analyzed together with T$_{FH}$ cells. (A). A distinct gene expression profile of T$_{FH}$ cells from TH1, TH2, and TH17 cells. Hierarchical clustering and PCA were applied to the expression levels of 8350 probe-sets showing differential expressions among the four types of cells (FDR = 0.1, corresponding to an unadjusted p-value = 0.033 from one-way ANOVA). (B). Real-time RT-PCR analysis of TH specific genes. (C-D). CD4$^+$CD44^{hi}CXCR5$^+$ BTLA$^+$ and CD4$^+$CD44^{hi}CXCR5$^-$ cells were restimulated with anti-CD3/anti-CD28 for 48 hours for cytokine measurement by ELISA (C) and with PMA/Ion for 6 hours for detection of IL-17 and IFNγ expression by intracellular cytokine staining (D). The data represent one of two independent experiments with similar results.
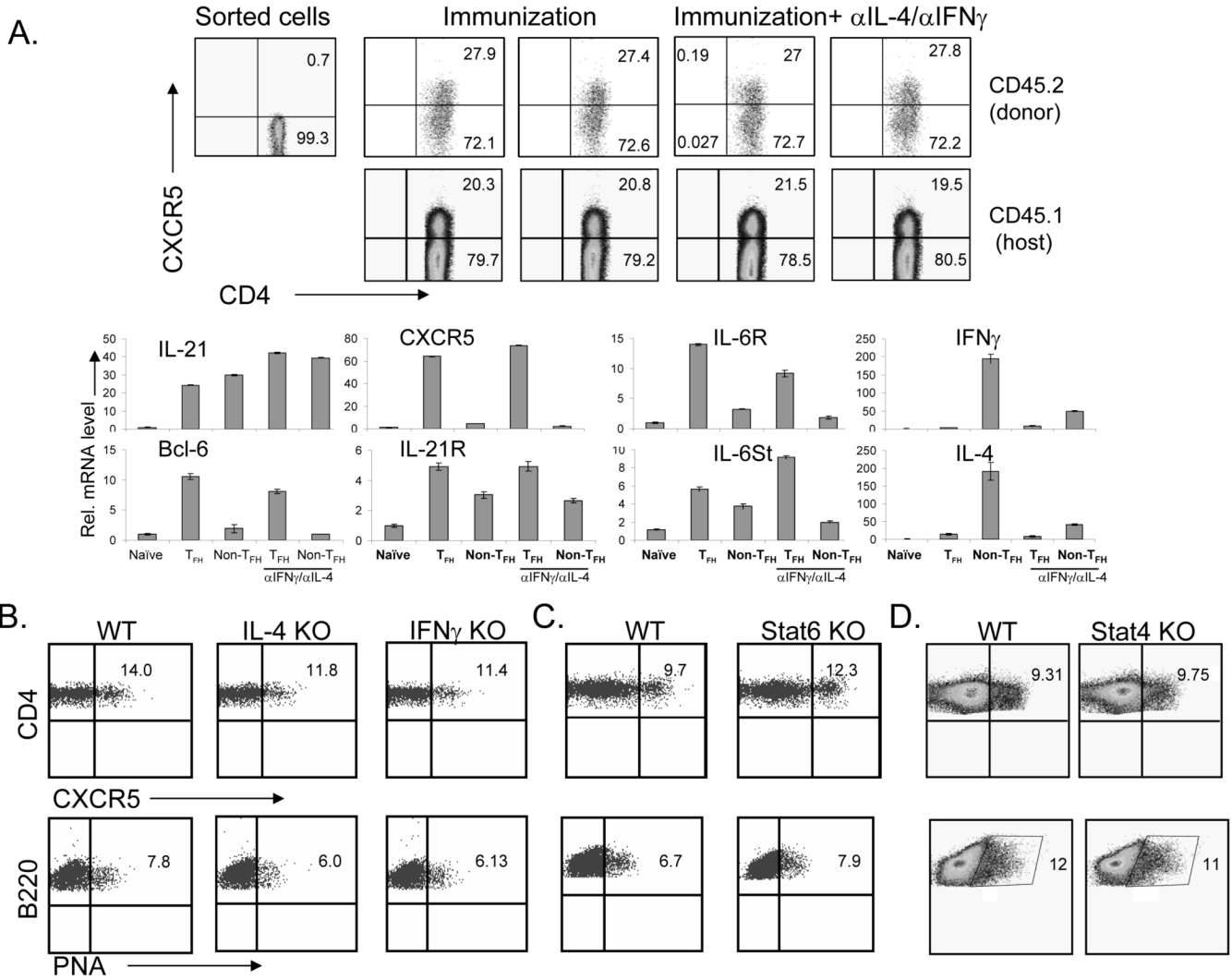
Figure 2. Generation of T_FH cells is independent of TH1 and TH2 lineages

(A). CD4+ T cells from <u>CD45.2/OT-II</u> mice were transferred into C57BL/6 (CD45.<u>1</u>) mice which were subsequently divided <u>into 2</u> groups (3 mice per group). Mice were immunized subcutaneously with Ova <u>protein</u> emulsified in CFA and treated with a 300 μg of control rat Ig or anti-IFNγand anti-IL-4 mAbs. Seven days after the immunization, experimental mice were sacrificed and splenic CD45.1+ and CD45.2+ CD4 cells were stained with biotinylated anti-CXCR5 mAb, followed by APC-labeled streptavidin. Numbers in dot plot quadrants represent the percentages. CD44hiCXCR5$^+$ and CD44hiCXCR5$^-$ cells from immunized mice were purified and real-time RT-PCR analysis of T_FH specific genes were performed. (B-C). IL-4 KO, IFNγ KO (B), STAT6 KO (C) <u>and STAT4 KO (D)</u> and their appropriate controls (WT, 3 mice per group) were immunized with KLH emulsified in CFA. Seven days after the immunization, experimental mice were sacrificed and the germinal center B cells were determined by staining with FITC-labeled PNA and PerCP-labeled anti-B220 mAb. The T_FH 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. The experiments were repeated three times with consistent results.
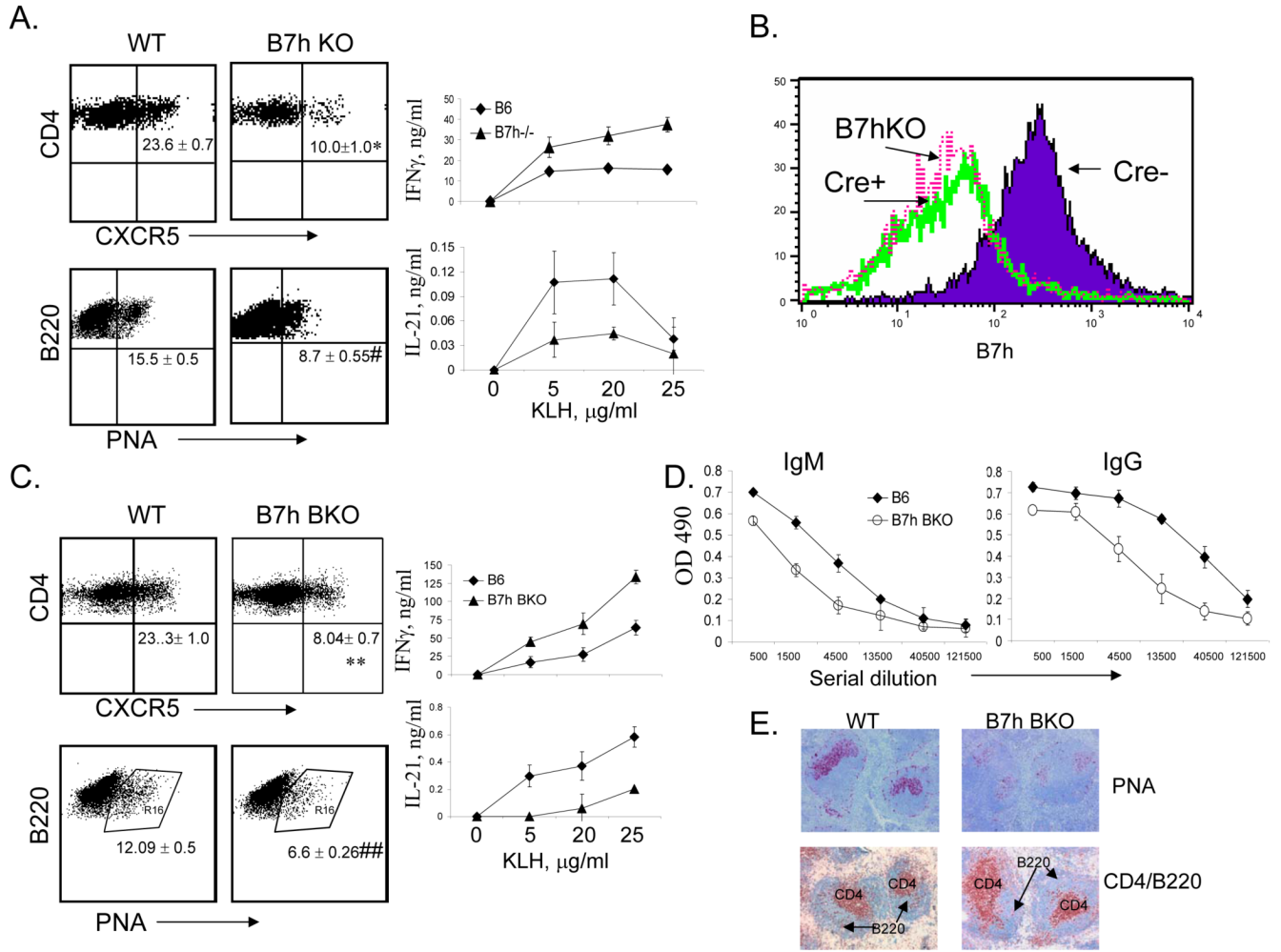
Figure 3. B7H expressed on B cells is required for generation of T$_{FH}$ cells
(A) B7h germline knockout mice and their controls (WT, 3 mice per group) were immunized with KLH in CFA. Seven days after the immunization, experimental mice were sacrificed and spleen cells from immunized mice were stimulated in 96-well plates as triplicates with the indicated concentration of KLH. Effector cytokines (IFN-γ and IL-21) were measured after 4 days of treatment. The germinal center B cells and T$_{FH}$ cells were analyzed. P values were calculated with the t-test by comparing the CXCR5+ cells and B220/PNA+ cells between wild-type and B7h KO mice and are indicated as followed: * $P < 0.005$; #, $P < 0.001$. Numbers in dot plot quadrants represent the percentages. (B). Splenic B220+ B cells from B7h germline KO (B7hKO), B7h B-cell conditional knockout (B7h BKO, cre+) and the cre- controls were analyzed for B7h expression. (C-E). Wild-type (WT) and B7h BKO (3 mice per group) were immunized with KLH in CFA. Seven days after the immunization, experimental mice were sacrificed and analyzed as in A. P values were calculated with the t-test by comparing the CXCR5+ cells and B220/PNA+ cells between wild-type and B7h BKO mice and are indicated as followed: ** $P < 0.001$; ##, $P < 0.001$. (C). (D). Anti-KLH antibodies (IgM and IgG) were measured in the sera by ELISA. The sera from WT and B7h BKO mice were subject to a 3-fold serial dilution, and the concentrations of KLH-specific IgM and IgG were analyzed by ELISA and averaged for each group. (E). GC in the spleens of KLH-immunized WT and B7h BKO mice were identified by PNA staining (brown). T and B cells were identified by staining

with anti-CD4 (red) and anti-B220 (blue) Abs. The data represent at least three independent experiments with consistent results.
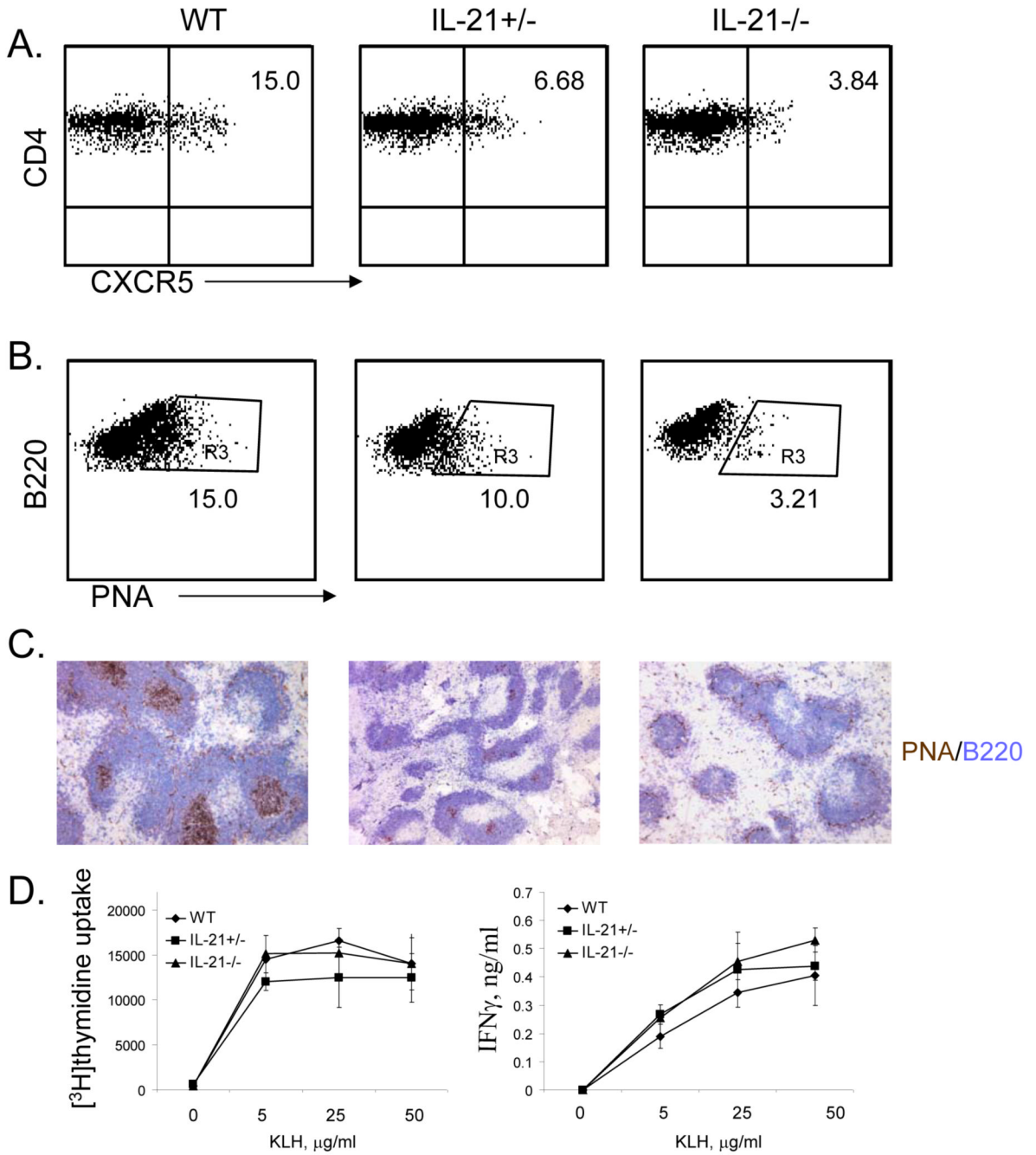
Figure 4. IL-21 is necessary for T_{FH} cell development

IL-21+/+, IL-21+/- and IL-21-/- mice (3 mice per group) were immunized subcutaneously with KLH emulsified in CFA. Seven days after the immunization, experimental mice were sacrificed and T_{FH} cells (A) and the germinal center B cells (B) were analyzed. Numbers in dot plot quadrants represent the percentages. Germinal centers were determined by immunohistochemical analysis (C). Spleen cells from immunized mice were stimulated in 96-well plates as triplicates with the indicated concentration of KLH peptide. Proliferation was assayed after 3 days of treatment by adding [^{3}H]thymidine to the culture for the last 8 h. IFN-γ was measured after 4 days of treatment. The experiments were repeated twice with consistent results.
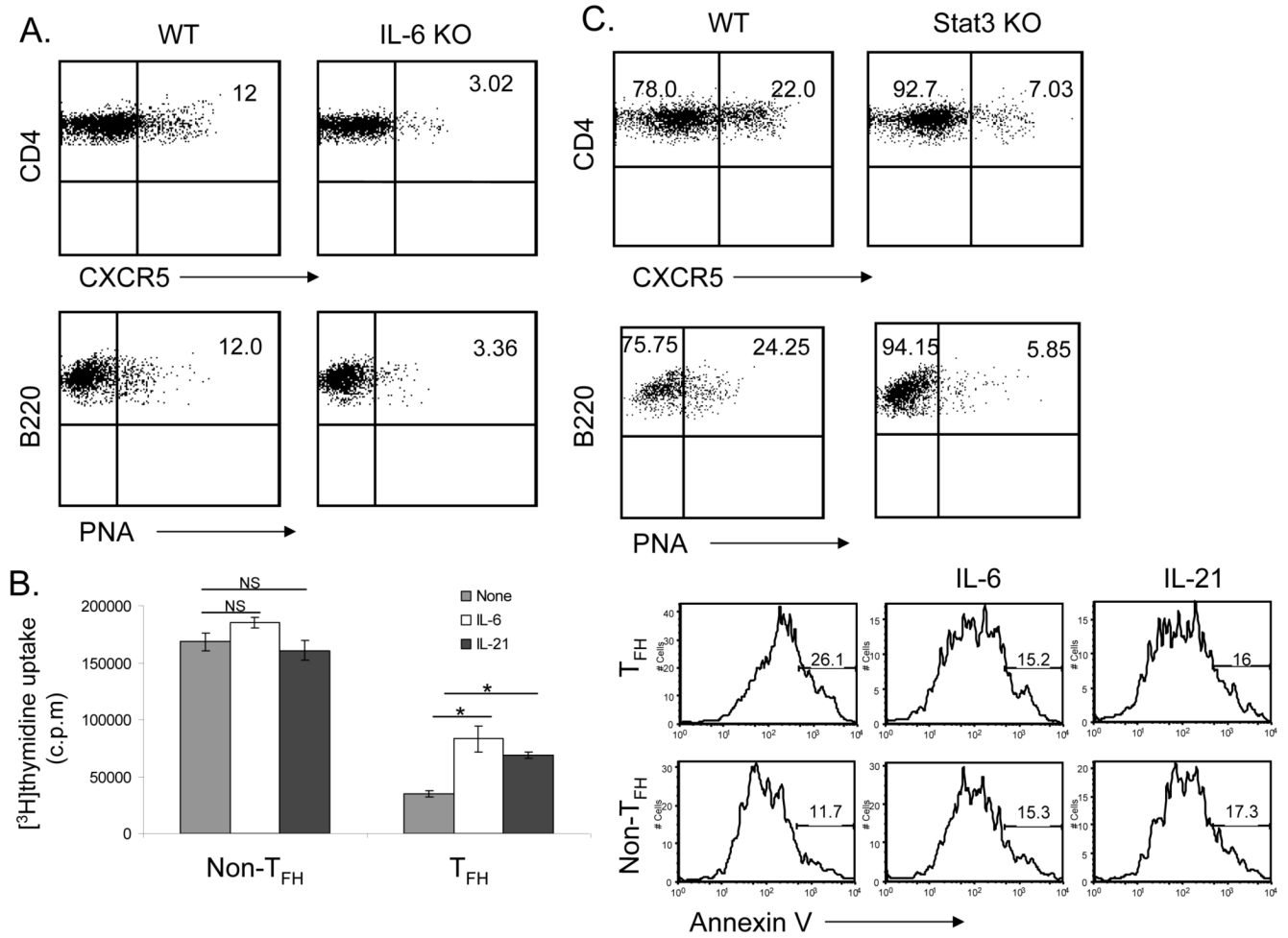
Figure 5. Generation of T$_{FH}$ cells requires IL-6 and STAT3

IL-6 KO (A) or STAT3 T-cell specific KO mice (C) and their appropriate controls (WT, 3 mice per group) were immunized subcutaneously with KLH emulsified in CFA. Seven days after the immunization, experimental mice were sacrificed and T$_{FH}$ cells and the germinal center B cells were analyzed. Numbers in dot plot quadrants represent the percentages. (B) C57BL/6 mice were immunized with KLH in CFA. 7 days later, CD4$^+$CD44^{hi}CXCR5$^+$ and CD4$^+$CD44^{hi}CXCR5$^-$ cells were sorted and restimulated with anti-CD3 and anti-CD28 with or without IL-6 or IL-21 for 48 hours. Proliferation was assayed by adding [^{3}H]-thymidine to the culture for the last 8 h. P values were calculated with the t-test by comparing the CXCR5 + cells proliferation in the absence and in the presence of IL6 (*p<0.005) or in the absence and in the presence of IL21 (*p<0.005). CD4$^+$CD44^{hi}CXCR5$^+$ and CD4$^+$CD44^{hi}CXCR5$^-$ cells were restimulated with anti-CD3 and anti-CD28 for 24 hours and stained with Annexin V-FITC. The experiments were performed two times with consistent results.
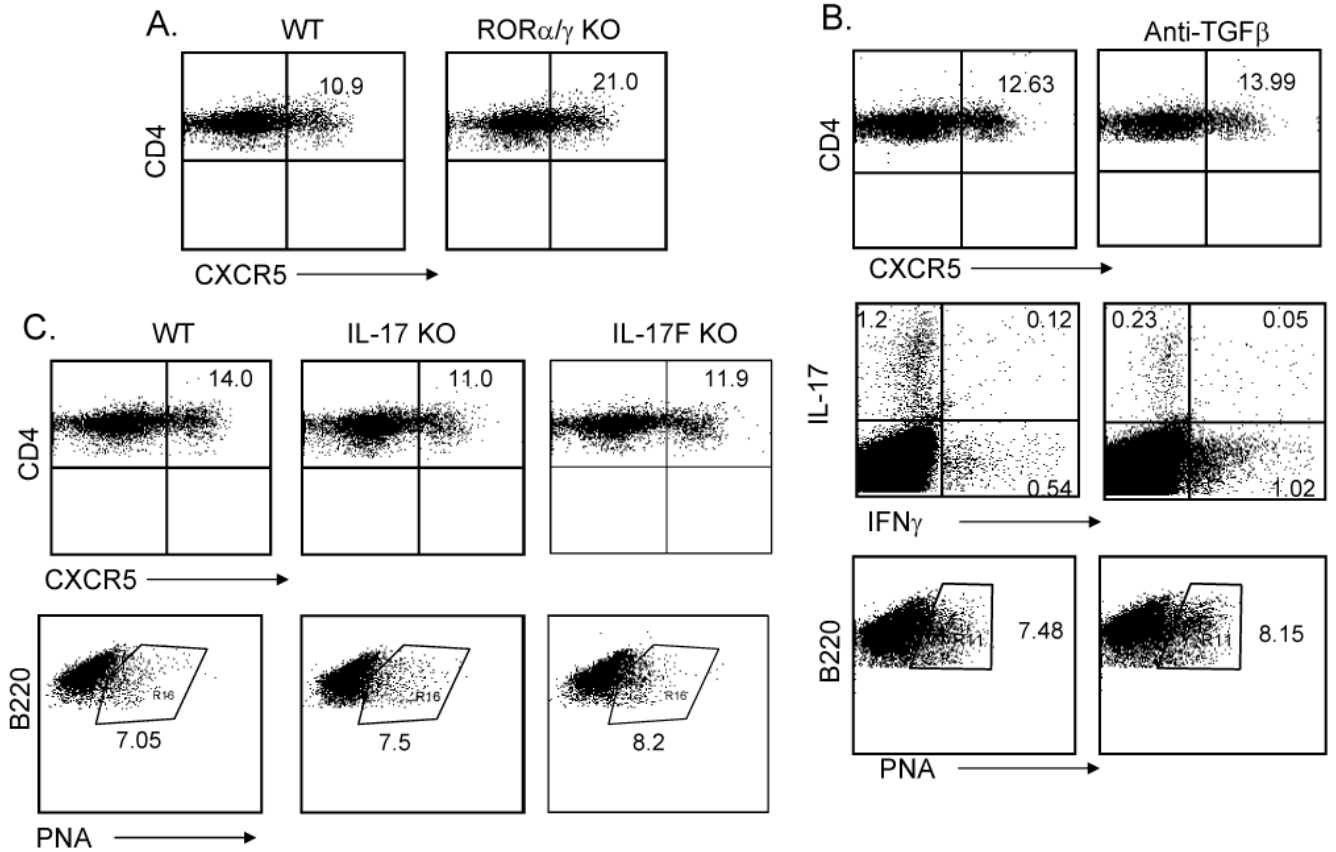
Figure 6. Generation of T$_{FH}$ cells is independent of TH17 lineage
Rag1-/- reconstituted with WT and RORαsg/sg/RORγ KO bone marrow cells (A) or IL-17 KO and IL-17F KO mice and their controls (C) (3 mice per group) were immunized subcutaneously with KLH in CFA. Seven days after the immunization, experimental mice were sacrificed and the T$_{FH}$ cells were determined. Numbers in dot plot quadrants represent the percentages. (B). C57BL/6 mice were immunized with KLH emulsified in CFA with 100 μg of isotype control antibodies or anti-TGFβ blocking Abs (3 mice per group). 7 days later, experimental mice were sacrificed and splenic T$_{FH}$ cells and germinal center B cells were analyzed. Splenocytes were restimulated with KLH for overnight and the production of IL-17 and IFNγ was analyzed in CD4^{+} gate by intracellular cytokine staining. The results represent one of three individuals with similar results.
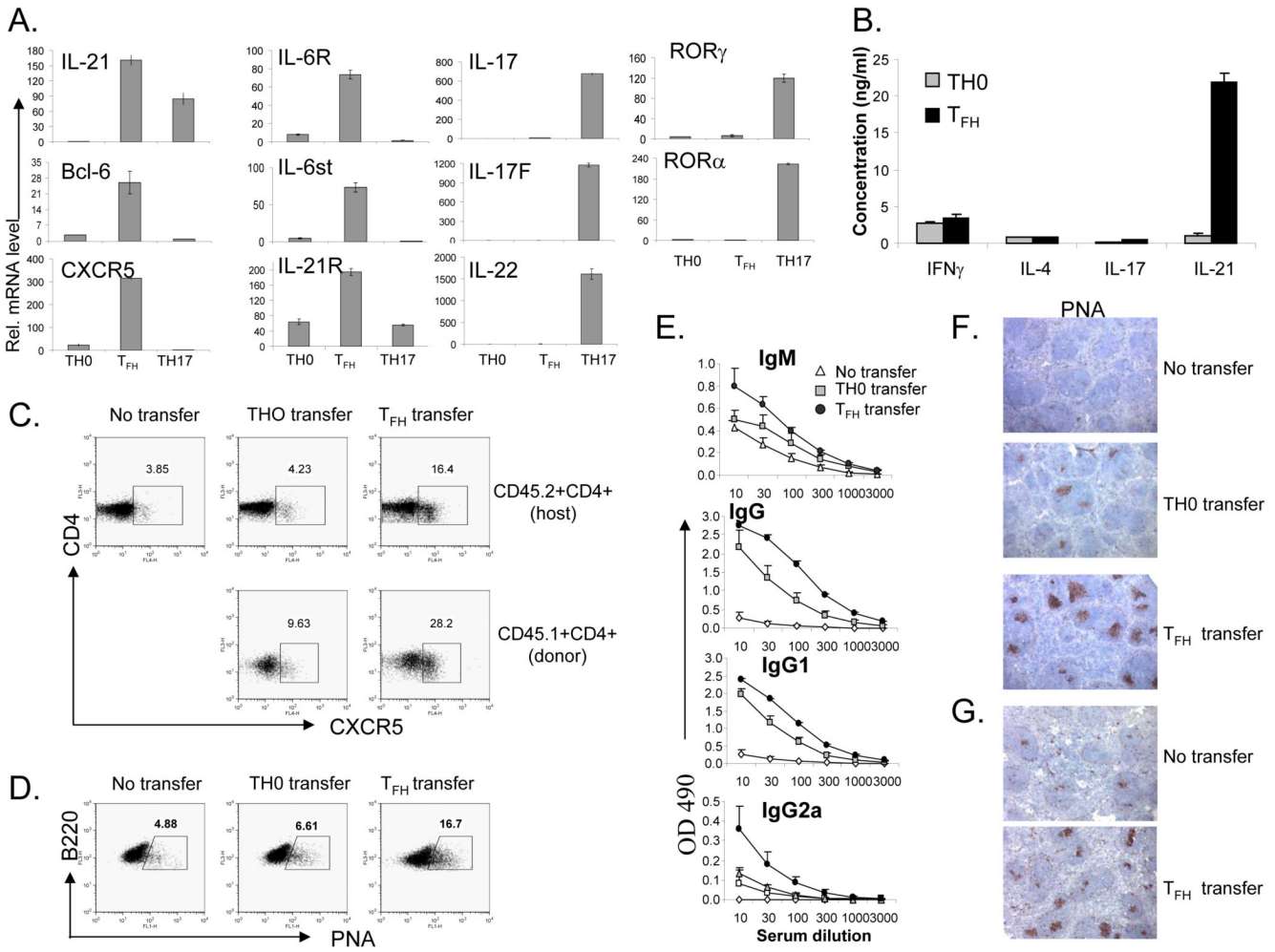
Figure 7. IL-21, in the absence of IL-4, IFNγ and TGF-b signaling, generates T$_{FH}$ cells
A-F, FACS-sorted CD62^{hi}CD44^{lo}CD25^{neg}CD4$^+$ T cells from CD45.1$^+$ OT-II mice were cultured with irradiated splenic APC plus OVA$_{323-339}$ peptide under TH0, T$_{FH}$ (IL-21 plus antibodies to IL-4, IFNγ and TGFβ) or TH17 condition for 5 days. After 5 days, CD4 T cells were restimulated with anti-CD3 for 4 hours for real-time PCR analysis (A) or for 24 hours for cytokine measurement by ELISA (B). (C-F). Five days after in vitro differentiation, cells were adoptively transferred into CD45.2$^+$ congenic mice (n=3-4) before the recipient mice were subcutaneously immunized with OVA in IFA. A group of mice that did not receive T cells was used as a control (No transfer). Seven days after immunzation, lymphoid cells from the draining lymph nodes of recipient mice were isolated and T$_{FH}$ cells and germinal center B cells were analyzed (D). Numbers in the boxes represent the percentages. (E). The sera from the recipient mice were subject to a 3-fold serial dilution, and the concentrations of OVA-specific IgM and IgG were analyzed by ELISA and averaged for each group. (F). Germinal center in the spleens of the recipient mice were identified by PNA staining (brown). The results are a representative of multiple mice of two independent experiments with similar results. (G). CXCR5$^+$CD44^{hi} cells were sorted from CD45.1 mice on day 7 after immunization with KLH and transferred to CD45.2 (n=3) recipient mice following immunization with KLH in CFA. A group of mice that did not receive T cells was used as a control (No transfer). Seven days after immunization, germinal center in the spleens of the recipient mice were identified by PNA staining (brown).