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Generation of T Follicular Helper Cells Is Mediated by Interleukin-21 but Independent of T Helper 1, 2, or 17 Cell Lineages

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SUMMARY

After activation, CD4$^+$ helper T (Th) cells differentiate into distinct effector subsets. Although chemokine (C-X-C motif) receptor 5-expressing T follicular helper (Tfh) cells are important in humoral immunity, their developmental regulation is unclear. Here we show that Tfh cells had a distinct gene expression profile and developed in vivo independently of the Th1 or Th2 cell lineages. Tfh cell generation was regulated by ICOS ligand (ICOSL) expressed on B cells and was dependent on interleukin-21 (IL-21), IL-6, and signal transducer and activator of transcription 3 (STAT3). However, unlike Th17 cells, differentiation of Tfh cells did not require transforming growth factor β (TGF-β) or Th17-specific orphan nuclear receptors RORα and RORγ in vivo. Finally, naive T cells activated in vitro in the presence of IL-21 but not TGF-β signaling preferentially acquired Tfh gene expression and promoted germinal-center reactions in vivo. This study thus demonstrates that Tfh is a distinct Th cell lineage.

INTRODUCTION

Naive CD4$^+$ helper T (Th) cells, upon encountering their cognate antigens presented on professional antigen-presenting cells (APCs), differentiate into effector cells that are characterized by their distinct cytokine production profiles and immune regulatory functions. In addition to Th1 and Th2 cells (Dong and Flavell, 2000), Th17 cells, a third subset of Th cells, have been recently identified; Th17 cells produce interleukin-17 (IL-17), IL-17F, and IL-22 and regulate inflammatory responses by tissue cells (Dong, 2008). Th17 cell differentiation, at least in mouse, is initiated by transforming growth factor β (TGF-β) and IL-6 (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006a), possibly via regulation of the chromatin remodeling of the *Il17-Il17f* locus (Akimzhanov et al., 2007). Whereas IL-6 is necessary for Th17 cell 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 cell differentiation (Korn et al., 2007; Nurieva et al., 2007a; Zhou et al., 2007). On the other hand, TGF-β signaling has also been clearly demonstrated to mediate Th17 cell differentiation in vivo (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006b). Th17 cell development is dependent on signal transducer and activator of transcription 3 (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 determine Th17 cell terminal differentiation (Ivanov et al., 2006; Yang et al., 2008b).

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 extent. For example, IFN-γ regulates IgG2a production whereasIL-4 is critical in IgE class switching. However, an additional Th cell subset called T follicular helper (Tfh) cells are recently found to be present in germinal centers and are characterized by their expression of chemokine (C-X-C motif) receptor 5 (CXCR5) (Vinuesa et al., 2005b). Although activated T cells may transiently express CXCR5, Tfh 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 Tfh cells, such as inducible costimulatory receptor (ICOS), IL-21 cytokine, and Bcl-6 transcription factor. ICOS was found essential for generation of Tfh cells in vivo (Akiba et al., 2005). In addition to Th17 cells, IL-21 is also expressed in Tfh cells and may serve as an important regulator of humoral responses by Tfh 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 Tfh cells and their relationship with other Th cell subsets is unclear, particularly considering that IL-21 is expressed by both Th17 and Tfh cells. A previous study revealed that human Tfh cells express genes distinct from those expressed by Th1 or Th2 cells (Chtanova et al., 2004). Here we show that mouse Tfh cells have a divergent gene expression profile from Th1, Th2, and Th17 cells and developed in vivo independently of these lineages. IL-21, IL-6, and STAT3 were critical in the generation of Tfh cells. Moreover, T cells activated in vitro in the presence of IL-21 but without TGF-β signaling preferentially acquired Tfh gene expression and functioned to promote humoral immunity in vivo. Thus, our data indicate that Tfh cells represent a distinct Th cell lineage and suggest a reciprocal relationship between the Tfh and Th17 cell lineages.

RESULTS

Distinct Gene Expression Profile of Tfh Cells

As a first step toward understanding Tfh cell regulation, we compared the gene expression profiles of Th1, Th2, and Th17 cells differentiated in vitro with in vivo-generated Tfh cells. Taking advantage of the coexpression of B and T lymphocyte attenuator (BTLA) by $CXCR5^+$ Tfh cells (Figure S1 available online), we FACS sorted $CD4^+CD44^{hi}CXCR5^+BTLA^+$ cells from splenocytes of C57BL/6 mice 7 days after immunization with keyhole limpet hemocyanin (KLH). These cells, as well as Th1, Th2, and Th17 cells, were restimulated with anti-CD3 for 4 hr and subjected to gene-profiling analysis in duplicates via Affimetrix gene chips. The microarray data were normalized with GC robust multiarray average (GCRMA) and the genes whose expression was changed across the Th1, Th2, Th17, and Tfh cells were then selected by 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 Tfh cells have a very distinct gene expression profile (Figure 1A, Figure S2A).

To confirm the above results, we performed real-time RT-PCR analysis on multiple subset-specific genes. The data indicate that Tfh cells did not express the typical markers for Th1 (IFN-γ and T-bet) or Th2 (IL-4 and GATA3) cells (Figure 1B), consistent with a previous report on human Tfh cells (Chtanova et al., 2004). Although Tfh 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 Tfh cells express mRNAs for CXCR5 as well as Bcl-6 (Figure 1B). In addition, Tfh 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 Tfh cells by IL-6 and IL-21. Moreover, consistent with human Tfh cells (Chtanova et al., 2004) and a recent report indicating that Tfh cells express programmed death-1 (PD-1) protein (Haynes et al., 2007), PD-1 mRNA was highly upregulated in Tfh cells compared to other Th cell subsets (data not shown).

To substantiate the above results, we also measured cytokine secretion of purified Tfh cells after they were activated ex vivo with anti-CD3 and anti-CD28 for 24 hr. High expressions of IL-21 but not IL-4, IL-10, IFN-γ, or IL-17 were observed in Tfh cells (Figure 1C). In addition, purified Tfh cells were activated with KLH and irradiated splenic APCs. Consistent with above results, Tfh cells preferentially produced IL-21, but not Th1, Th2, and Th17 cytokines (Figure S3). Furthermore, intracellular analysis on $CXCR5^+$ and $CXCR5^-$ cells after PMA and ionomycin restimulation also revealed that Tfh cells did not express IFN-γ or IL-17 (Figure 1D). These results indicate that Tfh cells are distinct from Th1, Th2, and Th17 cells in their gene expression and cytokine production.

Naive T Cell Differentiation into Tfh Cells Is Independent of Th1 and Th2 Cell Lineages

Previous analysis (Chtanova et al., 2004) and our current gene expression analysis on human and mouse Tfh cells, respectively, suggested that they are distinct from Th1 and Th2 cells. However, it was not clear whether generation of Tfh cells is distinct from Th1 or Th2 cell differentiation. To address this question, we transferred FACS-sorted naive $CD4^+$ 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. Seven days later, we examined splenic $CD45.1^+$ and $CD45.2^+$ $CD4^+$ T cells and found a substantial increase in the frequency of $CXCR5^+CD4^+$ T cells (Figure 2A). Interestingly, treatment of recipient mice with antibodies to IL-4 and IFN-γ did not reduce the proportion of $CXCR5^+CD4^+$ T cells (Figure 2A), suggesting that Tfh cell development is independent of Th1 or Th2 cell development. In addition, donor $CD44^{hi}CXCR5^+$ and $CD44^{hi}CXCR5^-$ OT-II cells from immunized mice were purified, and real-time RT-PCR analysis of Tfh-specific genes was performed. $CXCR5^+$ T cells were generated in the presence of blocking antibodies to IL-4, and IFN-γ exhibited gene expression patterns comparable to those from the control group (Figure 2A), suggesting that these $CXCR5^+$ cells were indeed Tfh cells.

To substantiate the above finding, we also immunized mice deficient in IL-4, IFN-γ, STAT6, or STAT4 and their appropriate controls with KLH. Seven days after immunization, we did not detect any defect in Tfh cells or PNA^+ germinal-center B cells in the IL-4-, IFN-γ-, STAT6-, or STAT4-deficient animals compared to wild-type controls (Figures 2B–2D, Figures S4A–S4C). To confirm the above results, we also measured cytokine secretion of purified $CXCR5^+$ cells from IL-4- and IFN-γ-deficient mice and their controls after they were activated ex vivo with anti-CD3 and anti-CD28 for 24 hr. Tfh cells from IL-4- or IFN-γ-deficient mice produced similar amounts of IL-21 but did not express IL-4, IL-10, IFN-γ, or IL-17 (Figure S4D), suggesting that they were Tfh cells. Thus, we conclude that $CXCR5^+CD4^+$ Tfh cells develop independently of the Th1 and Th2 cell lineages.

ICOSL Expressed on B Cells Regulates the Generation of Tfh 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,

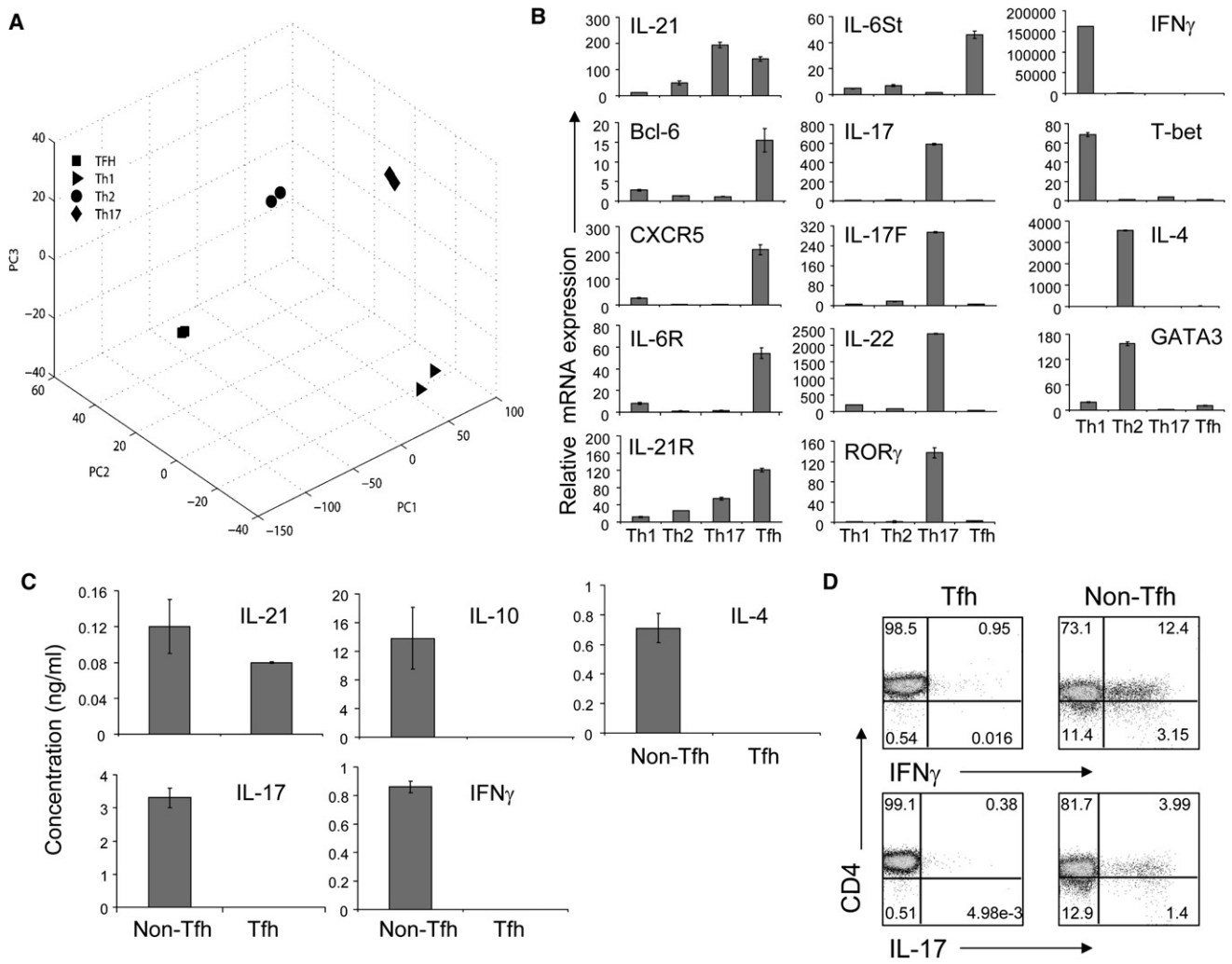

Figure 1. Tfh Cells Express Genes Distinct from those Expressed by Th1, Th2, and Th17 Cells

C57BL/6 mice were immunized with KLH in CFA. Seven days later, $CD4^+CD44^{hi}CXCR5^+$ $BTLA^+$ (Tfh) cells were sorted and restimulated with anti-CD3 for 4 hr 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 hr and were analyzed together with Tfh cells.

(A) A distinct gene expression profile of Tfh 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. The graph shows means ± standard deviation (SD).

(C and D) $CD4^+CD44^{hi}CXCR5^+$ $BTLA^+$ and $CD4^+CD44^{hi}CXCR5^-$ cells were restimulated with anti-CD3 plus anti-CD28 for 48 hr for cytokine measurement by ELISA (C) and with PMA and Ionomicin for 6 hr for detection of IL-17 and IFN-γ expression by intracellular cytokine staining (D).

In (C) the graph shows means ± SD. The data represent one of two independent experiments with similar results.

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 substantially reduced numbers of Tfh cells, indicating an essential role of ICOS in the differentiation of $CXCR5^+$ CD4 T cells (Akiba et al., 2005; Bossaller et al., 2006). Consistently, we also observed reduced percentages of $CXCR5^+CD4^+$ cells and decreased expression of IL-21 but not IFN-γ in our ICOSL germline-deficient ($Icosl^{-/-}$) animals (Nurieva et al., 2003b) after KLH immunization (Figure 3A). Tfh cells are regarded as regulators of the germinal-center reaction because they help to activated B cells that also express CXCR5. Because B cells constitutively express ICOSL, we asked whether the generation of Tfh cells may require B cell help via engagement of ICOS receptor on T cells. We thus bred mice carrying the ICOSL conditional flox (f) allele (Nurieva et al., 2003b) with CD19-cre mice (Rickert et al., 1995). In $Icosl^{f/f}$ mice carrying the CD19-cre ($Icosl^{f/f}Cd19Cre^+$), in comparison to those without cre expression, there was efficient deletion of the ICOSL gene in B cells, similar to those in ICOSL germline-deficient mice (Figure 3B). We then immunized $Icosl^{f/f}Cd19Cre^+$ mice with KLH and found that absence of ICOSL 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 $Icosl^{f/f}Cd19Cre^+$ mice, the numbers of $CD45.1^+$ Tfh cells were also greatly reduced

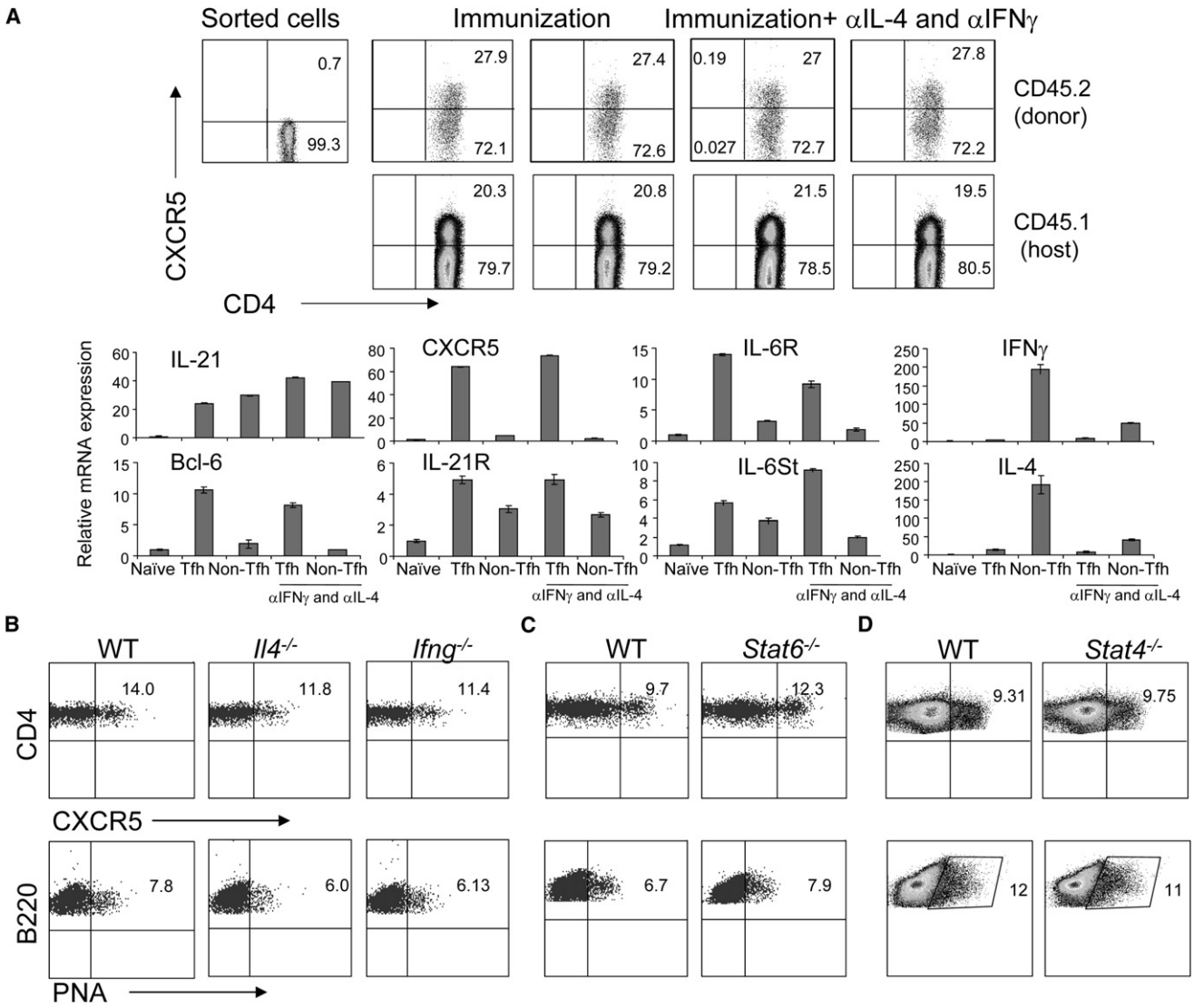

Figure 2. Generation of Tfh Cells Is Independent of Th1 and Th2 Cell Lineages

(A) $CD4^+$ T cells from OT-II mice (CD45.2) were transferred into C57BL/6 (CD45.1) mice, which were subsequently divided into two groups (three mice per group). Mice were immunized subcutaneously with Ova protein emulsified in CFA and treated with a 300 μg of control rat Ig or anti-IFN-γ and 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 CXCR5 mAb, followed by APC-labeled streptavidin. Numbers in dot-plot quadrants represent the percentages. $CD44^{hi}CXCR5^+$ and $CD44^{hi}CXCR5^-$ cells from immunized mice were purified, and real-time RT-PCR analysis of Tfh-specific genes were performed. The graph shows means ± SD.

(B and C) *Il4*$^{-/-}$, *Ifng*$^{-/-}$ (B), *Stat6*$^{-/-}$ (C), and *Stat4*$^{-/-}$ (D) and their appropriate controls (WT, three 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 B220 mAb. The Tfh cells were analyzed by staining with PerCP-labeled CD4 mAb and biotinylated CXCR5 mAb, followed by APC-labeled streptavidin. Numbers in dot-plot quadrants represent the percentages. The experiments were repeated three times with consistent results.

after Ova-peptide immunization (data not shown). In addition to CXCR5 expression, we found that the expression of IL-21 was also greatly reduced in *Icosl*$^{f/f}$*Cd19Cre*$^+$ mice, whereas IFN-γ expression was elevated in these mice (Figure 3C). Moreover, PNA^+ germinal-center B cells were greatly reduced in these animals (Figures 3C and 3E). KLH-specific IgG production was also reduced (Figure 3D). Overall, these data indicate that B cell expression of ICOSL is necessary for IL-21 production and for the generation of Tfh cells and appropriate antibody responses, indicating that Tfh cell differentiation is regulated by B cells.

IL-21 and IL-6 Are Required for Generation of Tfh 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 cell differentiation (Nurieva et al., 2007a). In addition, Tfh T cells produced greater amount of IL-21 compared to Th1 and Th2 cell 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). Because IL-21 is also expressed in Tfh cells, we assessed whether IL-21 is important for Tfh cell generation. *Il21*$^{+/+}$,

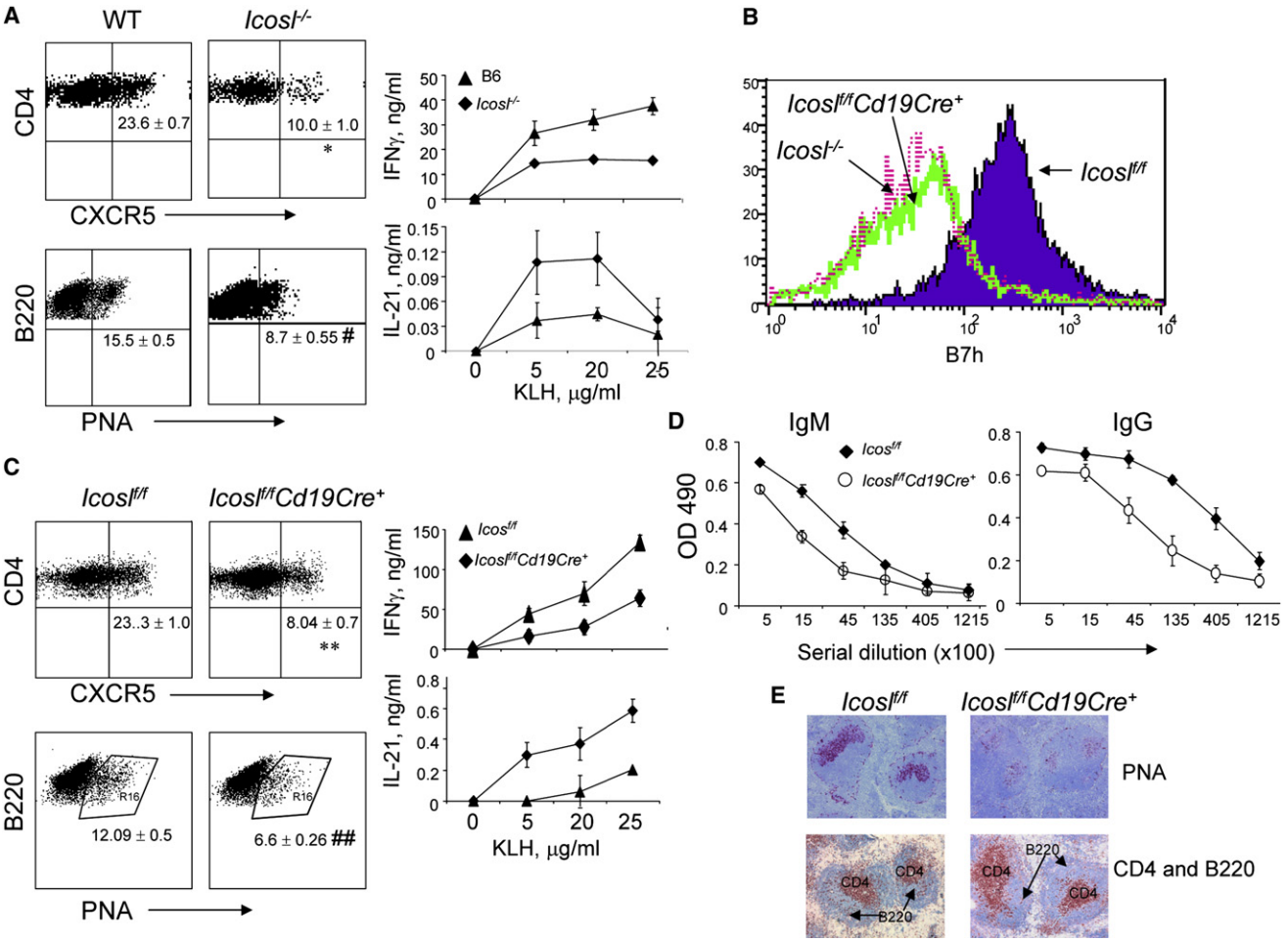

Figure 3. B7H Expressed on B cells Is Required for Generation of Tfh Cells

(A) ICOSL germline-deficient mice (*Icosl*$^{-/-}$) and their controls (WT, three 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 graph shows means ± SD. The germinal-center B cells and Tfh cells were analyzed. p values were calculated with the t test by comparison of the CXCR5$^+$ cells and B220$^+$PNA$^+$ cells between wild-type and *Icosl*$^{-/-}$ mice and are indicated as follows: *, $p < 0.005$; #, $p < 0.001$. Numbers in dot-plot quadrants represent the percentages.

(B) Splenic B220$^+$ B cells from ICOSL germline-deficient mice (*Icosl*$^{-/-}$), B cell-specific ICOSL-deficient mice (*Icosl*$^{f/f}$*Cd19Cre*$^+$), and their controls (*Icosl*$^{f/f}$) were analyzed for ICOSL expression.

(C–E) *Icosl*$^{f/f}$ *and Icosl*$^{f/f}$*Cd19Cre*$^+$ mice (three mice per group) were immunized with KLH in CFA. Seven days after the immunization, experimental mice were sacrificed and analyzed as in (A). The graph shows means ± SD. In (C), p values were calculated with the t test by comparison of the CXCR5$^+$ cells and B220$^+$PNA$^+$ cells between *Icosl*$^{f/f}$ and *Icosl*$^{f/f}$*Cd19Cre*$^+$ mice and are indicated as follows: **, $p < 0.001$; ##, $p < 0.001$. (D) KLH-specific antibodies (IgM and IgG) were measured in the sera by ELISA. The sera from *Icosl*$^{f/f}$ and *Icosl*$^{f/f}$*Cd19Cre*$^+$ mice were subjected 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 *Icosl*$^{f/f}$ *and Icosl*$^{f/f}$*Cd19Cre*$^+$ mice were identified by PNA staining (brown). T and B cells were identified by staining with anti-CD4 (red) and anti-B220 (blue). The data represent at least three independent experiments with consistent results.

Il21$^{+/-}$, and *Il21*$^{-/-}$ mice were immunized with KLH, and splenic Tfh cells were analyzed in these mice. *Il21*$^{+/-}$ mice exhibited a reduced number of Tfh cells, which was further reduced in *Il21*$^{-/-}$ mice (Figure 4A). In addition, PNA$^+$ germinal-center B cells were also greatly reduced in *Il21*$^{-/-}$ mice (Figures 4B and 4C). In contrast, CD4 T cells from *Il21*$^{+/-}$ and *Il21*$^{-/-}$ mice showed normal proliferation and IFN-γ expression after restimulation with KLH ex vivo (Figure 5D). Thus, these results indicate that IL-21 is necessary for Tfh cell development.

Because IL-6 induces IL-21 expression, we also tested Tfh cell generation in mice lacking IL-6. It has been previously shown 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, *Il6*$^{-/-}$ mice exhibited greatly reduced numbers of Tfh cells and germinal-center B cells (Figure 5A), indicating that IL-6 is also necessary for Tfh cell generation.

Because Tfh cells expressed high amounts of IL-21R, IL-6R, and IL-6st (Figure 1B), we also examined whether IL-6 and IL-21 signaling regulates Tfh 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

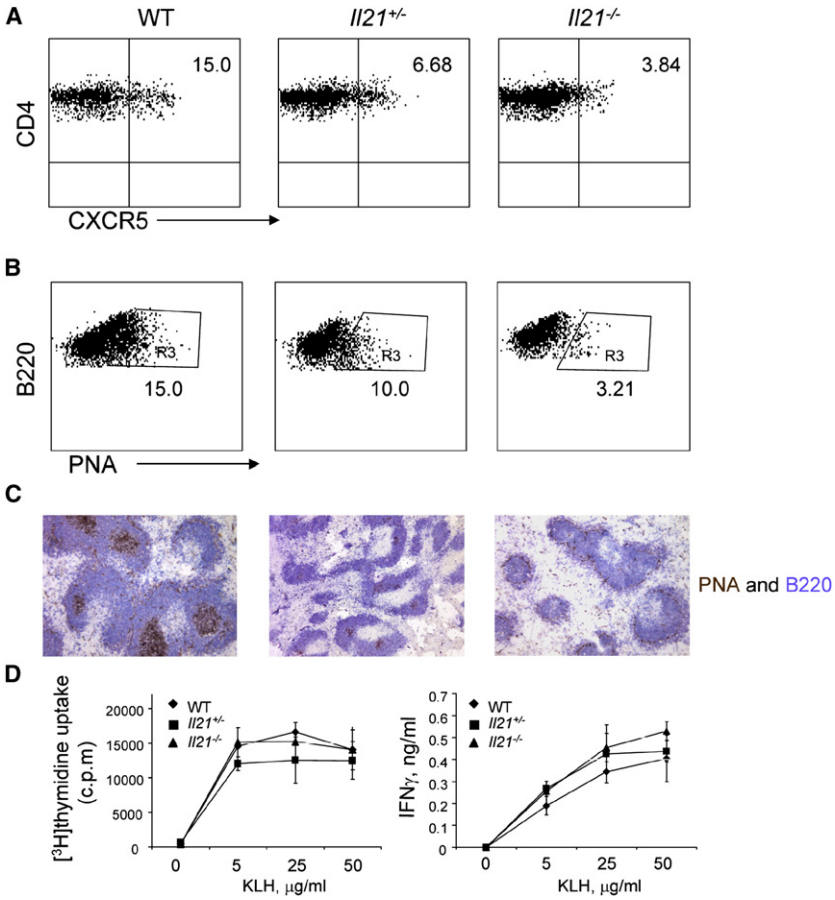

Figure 4. IL-21 Is Necessary for Tfh Cell Development

Il21$^{+/+}$, *Il21*$^{+/-}$, and *Il21*$^{-/-}$ mice (three mice per group) were immunized subcutaneously with KLH emulsified in CFA. Seven days after the immunization, experimental mice were sacrificed and Tfh 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 (D). Proliferation was assayed after 3 days of treatment by adding [^{3}H]thymidine to the culture for the last 8 hr. IFN-γ was measured after 4 days of treatment. The graph shows means ± SD. The experiments were repeated twice with consistent results.

and anti-CD28 in the absence or presence of IL-21 or IL-6. Compared to non-Tfh cells, CXCR5$^+$ T cells showed a substantial 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 ($p < 0.005$) (Figure 5B), suggesting that IL-6 and IL-21 preferentially regulate Tfh cells. Furthermore, we stained restimulated CXCR5$^+$ and CXCR5$^-$ cells with Annexin V. We found that a substantial portion of CXCR5$^+$ cells underwent apoptosis compared to CXCR5$^-$ (Figure 5B). However, IL-6 or IL-21 treatment reduced apoptosis of Tfh cells.

Because both IL-6 and IL-21 signal through STAT3, we analyzed Tfh cell generation in *Stat3*$^{f/f}$ 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$^+$ Tfh 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 (Figure S5). Overall, these data indicate that Tfh cell generation is dependent on IL-21, IL-6, and STAT3.

Tfh Cell Generation Is Independent of Th17 Cell Differentiation or Function

The above results indicate that the IL-6-IL-21 axis and STAT3 transcription factor are necessary for Tfh cell generation, which is similar to the regulation of Th17 cell differentiation. To test whether the development of Tfh cells is dependent on or independent of the Th17 cell lineage, we utilized *Rag1*$^{-/-}$ mice reconstituted with bone-marrow cells from wild-type, *Rora*$^{st/st}$, or *Rora*$^{st/st}$*Rorc*$^{-/-}$ mice. We recently reported that the latter mice were completely impaired in Th17 cell 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 Tfh cells can be generated in the absence of Th17 cell development. Moreover, PNA$^+$ germinal-center B cells were not affected in these animals (Figure S6B).

Because Th17 cell differentiation requires also TGF-β in addition to IL-6 or IL-21, we examined whether TGF-β signaling is required for Tfh cell generation. C57BL/6 mice were immunized with KLH in the absence or presence of TGF-β blocking antibody as previously described (Veldhoen et al., 2006b). Whereas IL-17 expression was substantially decreased in anti-TGF-β-treated mice, CXCR5 expression was not (Figure 6B), indicating that TGF-β signaling is not essential for Tfh cell generation. Moreover, PNA expression on B cells was not affected either (Figure 6B).

To understand whether Tfh cell generation requires Th17 cell function, we also immunized mice deficient in IL-17 or IL-17F. Lack of IL-17 or IL-17F did not substantially reduce the number of Tfh cells in spleen or the number of germinal-center B cells (Figure 6C, Figure S6B). IL-17 or IL-17F is thus not essential in the generation of Tfh cells in vivo.

IL-21 in the Absence of TGF-β Initiates Tfh Cell Differentiation

Our results thus far suggest that although IL-6 and IL-21 are required for both Tfh and Th17 cell differentiation, these two subsets appear to have distinct genetic programs and differ in their dependency on TGF-β signaling. We next assessed whether IL-21 is sufficient to drive Tfh cell development in vitro in the absence of TGF-β signaling. Naive OT-II cells were activated by Ova peptide and splenic APCs in the absence (neutral condition)

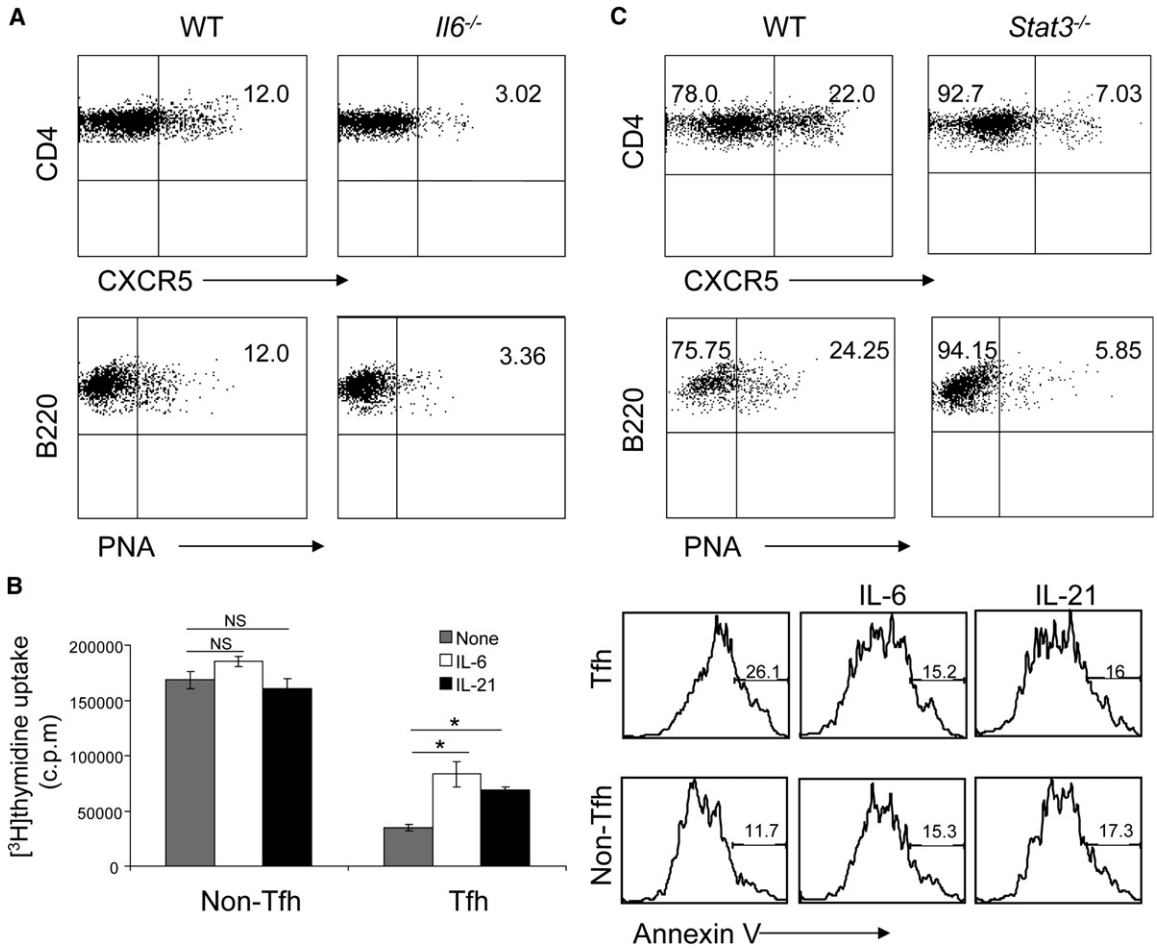

Figure 5. Generation of Tfh Cells Requires IL-6 and STAT3

Il6$^{-/-}$ (A) or T cell-specific *Stat3*$^{-/-}$ mice (C) and their appropriate controls (WT, three mice per group) were immunized subcutaneously with KLH emulsified in CFA. Seven days after the immunization, experimental mice were sacrificed and Tfh 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. Seven 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 hr. Proliferation was assayed by adding [^{3}H]-thymidine to the culture for the last 8 hr. The graph shows means ± SD. p values were calculated with the t test by comparison of the CXCR5$^+$ cells proliferation in the absence and in the presence of IL-6 (*, $p < 0.005$) or in the absence and in the presence of IL-21 (*, $p < 0.005$). CD4$^+$CD44^{hi}CXCR5$^+$ and CD4$^+$CD44^{hi}CXCR5$^-$ cells were restimulated with anti-CD3 and anti-CD28 for 24 hr and stained with Annexin V-FITC. The experiments were performed two times with consistent results.

or presence of IL-21, TGF-β, and antibodies to IL-4 and IFN-γ (Th17 cell condition) or IL-21 plus antibodies to IL-4, IFN-γ, and TGF-β. Five days later, the activated T cells were extensively washed and restimulated with anti-CD3 for 4 hr, and their gene expression was assessed by real-time RT-PCR. As expected, cells cultured under Th17 condition highly expressed TH17-specific genes, including genes encoding IL-17, IL-17F, IL-22, RORα, and RORγt (Figure 7A). In contrast, T cells treated with IL-21 in the absence of TGF-β signaling upregulated genes that are specifically expressed in Tfh cells, including those encoding CXCR5, Bcl-6, IL-6R, and IL-6st (Figure 7A). They also upregulated IL-21R expression but did not express Th17 genes (Figure 7A). The gene encoding PD-1 was also highly expressed in these cells (data not shown). To confirm this result, we measured cell supernatants 24 hr after restimulation for cytokine secretion by ELISA. IL-21 in the absence of TGF-β signaling did not support IL-17 expression, but the resulting cells expressed high levels of IL-21 (Figure 7B), suggesting that IL-21 expression is independent of TGF-β signaling.

To test the function of these Tfh-like cells in vivo, we transferred OT-II cells (CD45.1$^+$) activated under neutral condition or with IL-21 plus blocking antibodies to IFN-γ, IL-4, and TGF-β 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$^+$ Tfh cells (Figure 7C). Interestingly, host T cells in these mice also had approximately four times the Tfh cells of those receiving T cells activated under neutral condition (Figure 7C). The transferred cells also promoted Ova-specific antibody production and germinal-center reactions (Figures 7D–7F). As a control for the above experiment, we sorted CD45.1$^+$ CXC5$^+$CD44^{hi} cells from KLH-immunized

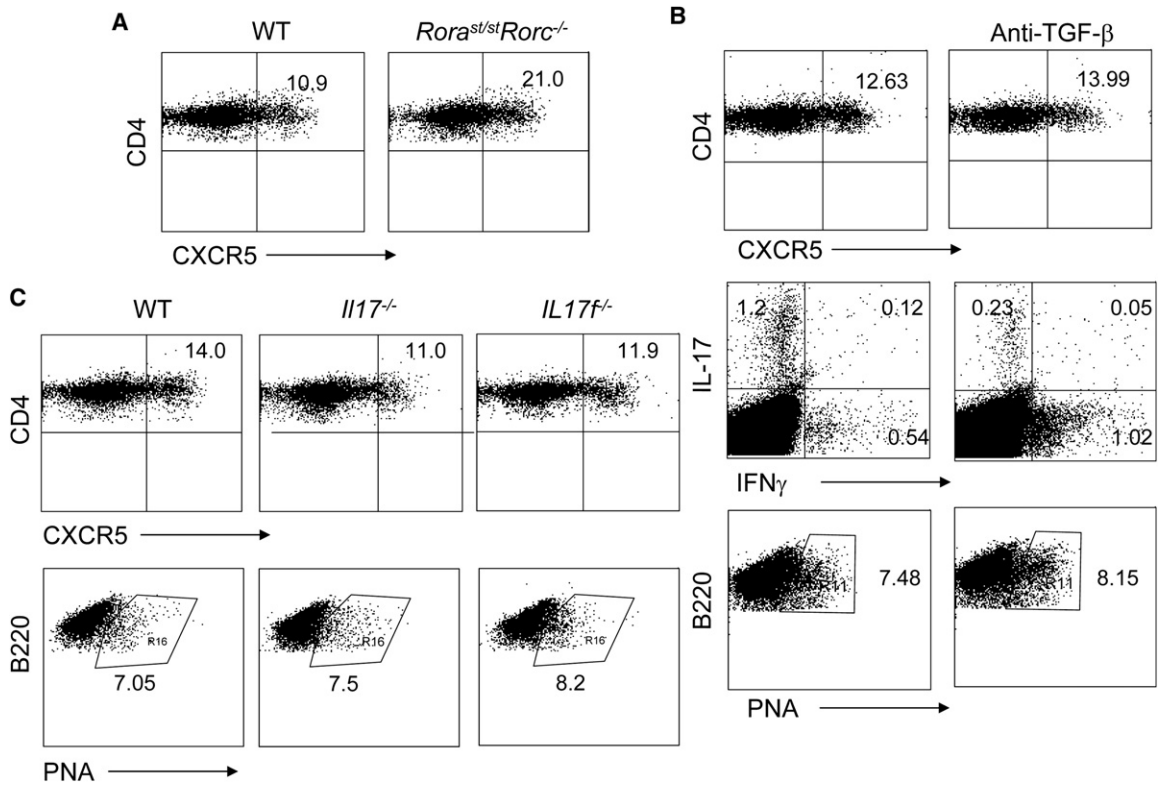

Figure 6. Generation of Tfh Cells Is Independent of TH17 Cell Lineage

Rag1$^{-/-}$ reconstituted with WT and *Rora*$^{st/st}$/*Rorc*$^{-/-}$ bone-marrow cells (A) or *Il17*$^{-/-}$ and *Il17f*$^{-/-}$ mice and their controls (C) (three mice per group) were immunized subcutaneously with KLH in CFA. Seven days after the immunization, experimental mice were sacrificed and the Tfh cells (A and C) and germinal-center B cells (C) 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 TGF-β blocking Abs (three mice per group). Seven days later, experimental mice were sacrificed and splenic Tfh cells and germinal-center B cells were analyzed. Splenocytes were restimulated with KLH overnight, and the production of IL-17 and IFN-γ was analyzed in CD4^{+} gate by intracellular cytokine staining. The results represent one of three individuals with similar results.

mice and transferred them into recipient mice (CD45.2^{+}), followed by immunization with KLH. Similar to in vitro-generated Tfh cells, in vivo-generated CXCR5^{+} cells also promoted a substantial increase in PNA^{+} germinal-center B cells (Figure 7G). These results indicate that IL-21, in the absence of Th1, Th2, and Th17 cell differentiation, drives Tfh cell development.

DISCUSSION

Although strongly implicated in antibody production and germinal-center reactions, the ontogeny of Tfh cells has been unclear. In the current study, we find that Tfh cells are distinct from Th1, Th2, or Th17 cells in their gene expression and developmental regulation. Generation of Tfh cells requires IL-21, IL-6, and STAT3.

Although implicated in humoral immunity and antibody-mediated autoimmunity, the regulation of Tfh cell development is unclear. ICOS-ICOSL 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 to be important in the generation of Tfh cells in mouse (Akiba et al., 2005). Interestingly, ICOS deficiency in human patients also causes a severe reduction of Tfh cells (Bossaller et al., 2006). Conversely, impaired negative regulation of ICOS by Roquin E3 ubiquitin ligase led to increased numbers of CXCR5^{+} Tfh cells and IL-21 hyperproduction (Vinuesa et al., 2005a; Yu et al., 2007). However, the mechanism by which ICOS and ICOSL regulate Tfh cell development has not been clear. In our current study, we find that the ICOS-B7h interaction is necessary for IL-21 expression by T cells. ICOS may thus regulate Tfh 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., 2003a, 2007b). ICOS may act through NFATc1 to regulate IL-21 expression. Given that IL-21 also regulates Th17 cell 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 ICOSL-deficient mouse, we show that ICOSL expression on B cells is required for the generation of Tfh cells and IL-21 expression. This result not only substantiates the importance of ICOS-ICOSL interaction in Tfh cell development but also suggests an important function of B cells in vivo as APCs in the generation or maintenance of Tfh cells. Consistent with our findings, Ebert

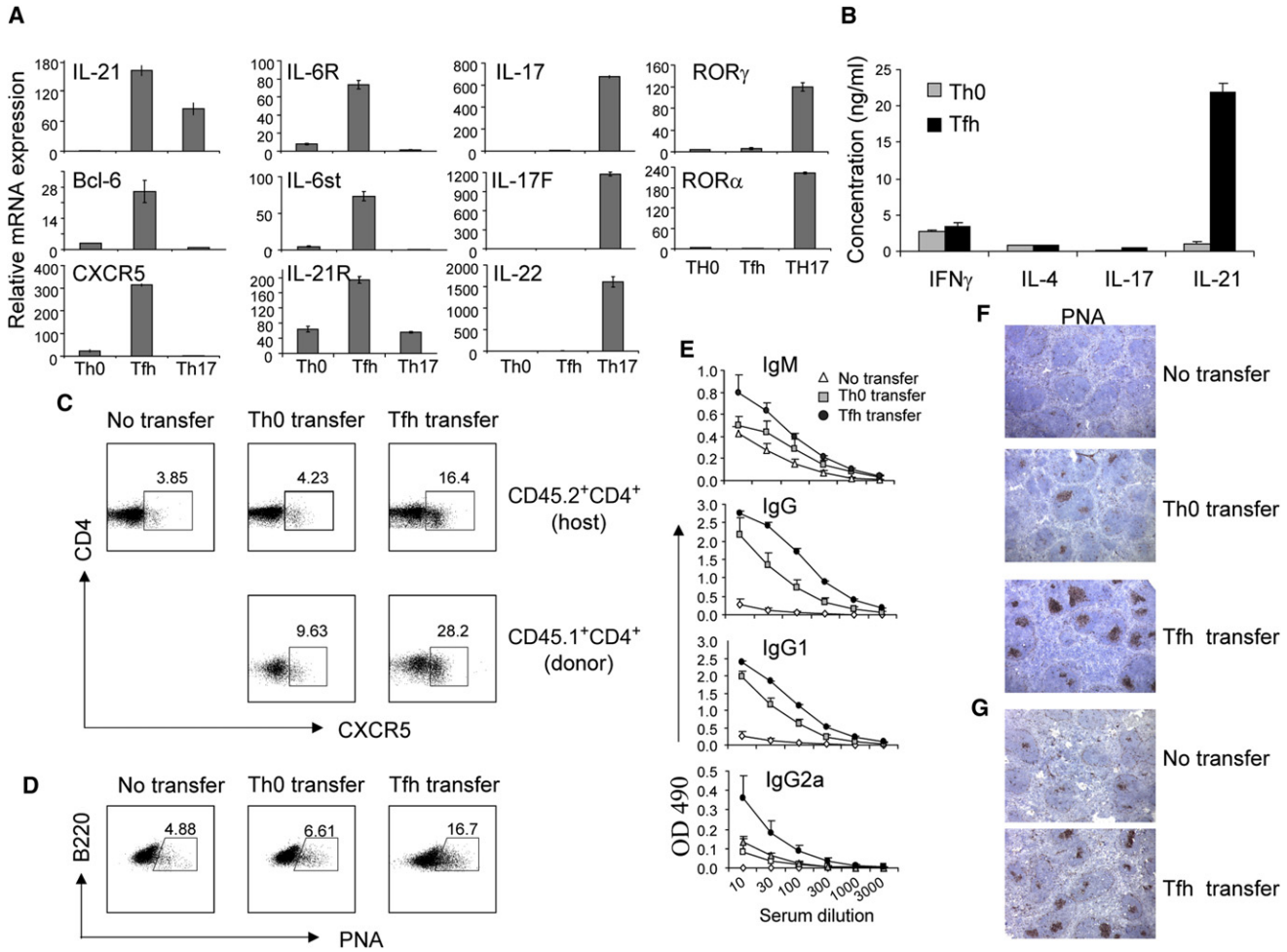

Figure 7. IL-21, in the Absence of IL-4, IFN-γ, and TGF-β Signaling, Generates Tfh Cells

(A–F) FACS-sorted $CD62^{hi}CD44^{lo}CD25^{-}CD4^{+}$ T cells from CD45.1^{+} OT-II mice were cultured with irradiated splenic APC plus $OVA_{323-339}$ peptide under Th0, Tfh (IL-21 plus antibodies to IL-4, IFN-γ, and TGF-β), or TH17 cell condition for 5 days. After 5 days, $CD4^{+}$ T cells were restimulated with anti-CD3 for 4 hr for real-time PCR analysis (A) or for 24 hr for cytokine measurement by ELISA (B). In (A) and (B), the graphs show means ± SD. (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 Tfh cells and germinal-center B cells were analyzed (D). Numbers in the boxes represent the percentages. (E) The sera from the recipient mice were subjected to a 3-fold serial dilution, and the concentrations of OVA-specific IgM and IgG were analyzed by ELISA and averaged for each group. The graph shows means ± SD. (F) Germinal centers 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 after 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 centers in the spleens of the recipient mice were identified by PNA staining (brown).

et al. previously showed that human B cells regulate Tfh cell phenotypes (Ebert et al., 2004).

Previous gene expression analysis has revealed that human Tfh cells are distinct from Th1 and Th2 cells (Chtanova et al., 2004); (Kim et al., 2004). Our current study also reveals that Tfh cells do not produce Th1 or Th2 cell cytokines. Interestingly, although human Tfh cells appear to express IL-10 and CXCL13, a ligand for CXCR5 (Chtanova et al., 2004; Ebert et al., 2004; Kim et al., 2001, 2004), mouse Tfh cells do not. What accounts for this species difference would need further investigation. Moreover, we found that naive T cells differentiate into Tfh cells in vivo, which is independent of IFN-γ, IL-4, and STAT6. From these data, we conclude that Tfh cells develop independent of Th1 and Th2 cell lineages.

On the other hand, Tfh 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, Tfh cells differ from Th17 cells in the following aspects. First, they are distinct in their gene expression profiles. Second, Tfh cells do not produce IL-17, IL-17F, or IL-22. Most importantly, Tfh cell development does not require RORα or RORγt. Thus, we believe that Tfh cells develop independently of the Th17 cell lineage. Th17 cell development in mouse is not only mediated by the IL-6-IL-21 axis, but also by TGF-β. We show here that IL-21 can be induced

in T cells independently of TGF-β signaling. T cells activated in the presence of IL-21 but in the absence of IL-4, IFN-γ, and TGF-β signaling produced IL-21 but not IL-4, IFN-γ, IL-17, IL-17F, or IL-22. Furthermore, these cells acquired expression of CXCR5, Bcl-6, IL-6R, and IL-6st, genes that were expressed by in vivo generated Tfh cells, suggesting that Tfh cells may be generated in vitro under the above condition. Moreover, these Tfh-like cells generated in vitro preferentially expressed CXCR5 and functioned to promote humoral immunity, similar to in vivo-generated Tfh cells. Furthermore, TGF-β signaling, although required for IL-17 expression in vivo, is not essential for Tfh cells, indicating a reciprocal relationship of Tfh and Th17 cells. Interestingly, Bcl-6 has recently been shown as a repressor of TGF-β-SMAD signaling (Wang et al., 2008), suggesting that lack of TGF-β signaling may favor Tfh cell development. These results further indicate that Tfh cell 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 Tfh cells also enhanced the Tfh cell generation in recipient mice, suggesting that IL-21 may function in a paracrine fashion to regulate Tfh cell development.

In summary, our current study has extensively characterized the developmental regulation of Tfh cells. Our data indicate that Tfh 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 cell lineages (Figure S7). In mice defective in Tfh cells, there existed still-detectable amounts of antigen-specific antibodies, suggesting that other Th cell subsets may independently regulate the humoral immunity. This knowledge may help us to find ways to treat antibody-mediated autoimmune diseases.

EXPERIMENTAL PROCEDURES

Mice

IL-4-, IFN-γ-, 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. *Rora*$^{st/st}$, *Rora*$^{st/st}$ *Rorc*$^{-/-}$, and wild-type bone-marrow chimeras were generated as described (Yang et al., 2008b). *Stat3*$^{f/f}$ mice (Takeda et al., 1999) were bred with CD4-Cre mice provided by C. Wilson (Lee et al., 2001). IL-21-deficient mice on 129xC57BL/6 F1 mixed background were obtained from the National Institutes of Health (NIH) Mutant Mouse Regional Resource Centers (MMRRC) (Nurieva et al., 2007a). *Icosl*$^{f/f}$*Cd19Cre*$^{+}$ mice were created by breeding ICOSL flox mice (Nurieva et al., 2003b) with CD19-cre mice (Rickert et al., 1995). IL-17- and IL-17F-deficient mice were recently generated in our lab (Yang et al., 2008a). Mice were housed in the specific pathogen-free (SPF) animal facility at M.D. Anderson Cancer Center, and the animal experiments were performed at the age of 6–10 weeks with protocols approved by Institutional Animal Care and Use Committee.

T Cell Differentiation

Differentiation of OT-II cells in Figures 1A and 1B was performed as previously described (Chung et al., 2006). In Figure 7A, the cytokine stimuli for Th17 cell differentiation were as follows: 100 ng/ml IL-21, 5 ng/ml TGF-β, 10 μg/ml anti-IL-4, and 10 μg/ml anti-IFN-γ, and for generation of Tfh cells were 50 ng/ml IL-21, 10 μg/ml anti-IFN-γ, 10 μg/ml anti-IL-4, and 20 μg/ml TGF-β (1D11) neutralizing Abs. IL-4, IL-6, IL-12, and TGF-β were purchased from Peprotech. IL-21, IL-23, and TGF-β (1D11) neutralizing Abs were purchased from R&D. For characterization of the in vitro-differentiated CD4^{+} T cells under these conditions, these cells were restimulated with plate-bound anti-CD3 (5 μg/ml) for 4 hr for real-time PCR analysis, or for 24 hr for cytokine measurement by ELISA.

Keyhole Limpet Hemocyanin 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-TGF-β (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 Tfh cell induction was determined by staining with PerCP-labeled anti-CD4 mAb (PharMingen) and biotinylated anti-CXCR5 mAb (PharMingen), followed by APC-labeled streptavidin (Jackson ImmunoResearch Laboratories). In some experiments, antigen-specific IgM antibodies and IgG antibodies in sera from immunized mice were measured with ELISA. In brief, 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 anti-mouse IgM or rat anti-mouse IgG antibodies (Southern Biotechnology Associates). For analysis of the role of ICOSL in regulation of T cell responses in vivo (Figures 3A and 3C), 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 6B, spleen cells from immunized mice were restimulated with 50 μg KLH for 24 hr. In the final 5 hr, Golgi-stop (BD Bioscience) was added, and IL-17- and IFN-γ-producing cells were analyzed with 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 with Affymetrix Mouse 430 2.0 chips. The total RNA samples were labeled according to the manufacturer's instruction by the 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 with GCRMA (Wu et al., 2004). We then selected the genes whose expression was changed across the Th1, Th2, Th17, and Tfh cells by 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 nondifferentially expressed probe sets (m_0) was estimated as 2 × 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 resampling 500 times; 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 with the given ANOVA F statistic value (FDR estimated for various F statistic value is shown in Figure S2B). Then, the expression 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) was 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) (three groups; three mice per group). Two 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-IFN-γ 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 Figures 7C–7E, FACS-sorted naive (CD4^{+}CD62L^{hi}CD44^{-}CD25^{-}) T cells from CD45.1^{+} OT-II mice were activated under TH0 or Tfh 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 Figure 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) (three mice per group). The 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, followed by APC-labeled streptavidin, or stained with FITC-labeled PNA and PerCP-labeled anti-B220.

Quantitative Real-Time PCR

Total RNA was prepared from T cells with TriZol reagent (Invitrogen). cDNA were synthesized with Superscript reverse transcriptase and oligo(dT) primers (Invitrogen), and gene expression was examined with a Bio-Rad iCycler Optical System with iQ SYBR green real-time PCR kit (Bio-Rad Laboratories). The data were normalized to β-actin reference. The following primer pairs for Bcl-6, IL-6R, IL-6st, and CXCR5 was used: Bcl6 forward: 5′-CACACCCGTCCATCATTGAA-3′, reverse: 5′-TGTCCTCACGGTGCCTTTTT-3′; IL6R forward: 5′-GGTGGCCCAGTACCAATGC-3′, reverse: 5′-GGACCTGGACCACGTGCT-3′; CXCR5 forward: 5′-ACTCCTTACCACAGTGCACCTT-3′, reverse: 5′-GGAAACGGGAGGTGAACCA-3′; IL-6st forward: 5′-ATTTGTGTGCTGAAGGAGGC-3′, reverse: 5′-AAAGGACAGGATGTTGCAGG-3′. The primers for IL-21, IL-17, IL-17F, IL-4, IFN-γ, 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 followed by biotinylated anti-rat secondary Ab and avidin-peroxidase complex reagent. Novared was used as substrate. For double staining, biotin-labeled B220 was applied and followed by 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.

ACCESSION NUMBERS

The microarray data have been deposited into NCBI GEO with the accession number GSE11924.

SUPPLEMENTAL DATA

Supplemental Data include seven figures and can be found with this article online at http://www.immunity.com/cgi/content/full/29/1/138/DC1/.

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