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A Fundamental Role for Interleukin-21 in the Generation of T Follicular Helper Cells

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SUMMARY

T cell help to B cells is a fundamental property of adaptive immunity, yet only recently have many of the cellular and molecular mechanisms of T cell help emerged. T follicular helper (Tfh) cells are the CD4$^+$ T helper cells that provide cognate help to B cells for high-affinity antibody production in germinal centers (GC). Tfh cells produce interleukin-21 (IL-21), and we show that IL-21 was necessary for GC formation. However, the central role of IL-21 in GC formation reflected its effects on Tfh cell generation rather than on B cells. Expression of the inducible costimulator (ICOS) was necessary for optimal production of IL-21, indicative of interplay between these two Tfh cell-expressed molecules. Finally, we demonstrate that IL-21's costimulatory capacity for T helper cell differentiation operated at the level of the T cell receptor signalosome through Vav1, a signaling molecule that controls T cell helper function. This study reveals a previously unappreciated role for Tfh cells in the formation of the GC and isotype switching through a CD4$^+$ T cell-intrinsic requirement for IL-21.

INTRODUCTION

Germinal centers (GC) are specialized structures that develop within B cell follicles of secondary lymphoid tissues, such as lymph nodes, spleen, tonsils, and the Peyer's patches of mucosal-associated lymphoid tissues. The GC is the principle site for processes such as somatic hypermutation, class switch recombination, and selection of high-affinity B cells (Kelsoe, 1995; Liu et al., 1996; MacLennan, 1994). GC CD4$^+$ T cells provide direct help to antigen-specific naive B cells, which promotes the differentiation of antigen-selected high-affinity GC B cells into memory B cells or plasma cells, thereby ensuring long-term humoral immunity (Banchereau et al., 1994; Garside et al., 1998; Manz et al., 2005). Live imaging of the GC reveals that T-B cell interactions in the GC are weighted toward B cell competition for a small number of CD4$^+$ T cells (Allen et al., 2007; Hauser et al., 2007; Schwickert et al., 2007). Linked recognition of antigen and B cell competition for T cell help provides a stringent control that ensures self-tolerance during the production of high-affinity antibodies of varying specificities (Mitchison, 2004).

Despite the long-held appreciation of a fundamental role for CD4$^+$ T cells in the GC reaction, it is only recently that components of the cellular and molecular mechanisms for T cell help have emerged. T cell help for B cells has been considered the domain of Th2 and Th1 cells, which direct B cells to undergo Ig isotype switching to IgE and IgG2a, respectively. More recently, a subset of CD4$^+$ T helper cells termed follicular B-helper T (Tfh) cells was observed in the GC and displayed potent help for antibody responses (Breitfeld et al., 2000; Schaerli et al., 2000; Walker et al., 1999). At least six different follicular T cell subsets have been identified, on the basis of surface markers and/or anatomical location (Vinuesa et al., 2005b), including Tfh memory cells that reside in the lymph node and regulate the memory B cell response for long-term immunity (Fazilleau et al., 2007).

Expression of the chemokine receptor CXCR5 is transiently upregulated when T cells interact with peptide-MHC class II and costimulatory molecules on antigen-presenting cells, and continued CXCR5 expression after priming may reflect qualitative or quantitative aspects of this stimulation (Ansel et al., 1999; Sallusto et al., 1999). Characteristically, Tfh cells retain intense expression of CXCR5, which directs these cells toward CXCL13-rich areas within GC (Breitfeld et al., 2000; Schaerli et al., 2000; Walker et al., 1999). High amounts of costimulatory molecules, such as ICOS and CD40L on Tfh cells, reflect both the sustained multisignal pathways necessary for their generation as well as their function to provide cognate help to B cells (Bossaller et al., 2006; Coyle et al., 2000; Dong et al., 2001; Hutloff et al., 1999; Tafuri et al., 2001). Tfh cells also express a number of costimulatory molecules that have the capacity to restrain their interaction with B cells and APCs, including CTLA-4 and PD-1, which may reflect their discriminating role in the GC (Chtanova et al., 2004; Haynes et al., 2007).

CD4$^+$ T cell activation and differentiation is thought to require robust TCR signals, and studies demonstrating that genetic deficiencies in molecules integral to TCR signal strength exhibit greater defects in the CD4$^+$ (than CD8$^+$) T cell lineage support this notion (Acuto and Michel, 2003; Malissen et al., 2007). Tyrosine kinases are the most proximal mediators in TCR signaling. One such molecule, Vav1, is a guanine nucleotide exchange factor (GEF) for Rho-family GTPases, originally identified as an oncogene. Studies collectively demonstrate an important role for Vav1 in NF-ATc1 activity, downregulation of the cell-cycle inhibitor p27Kip1, interleukin-2 production, actin polymerization, and TCR clustering (Charvet et al., 2006; Tybulewicz et al., 2003). In accordance with these findings, Vav1 has a critical role in Th cell differentiation. Notably, a mutation in Vav1 perturbs PI3K-dependent pathways downstream of Vav1, affecting the TCR-induced proliferation of CD4$^+$ but not CD8$^+$ T cells (Prisco et al., 2005). Moreover, the defective GC reaction in *Vav1*$^{-/-}$ mice was found to be CD4$^+$ T cell intrinsic (Gulbranson-Judge

et al., 1999). However, a role for Vav1 in Tfh cell differentiation has not been demonstrated.

B helper cell function is not limited to cell-surface molecules, and Tfh cells also express a number of cytokines that facilitate antibody production, including interleukin-4 (IL-4) and IL-10 (Haynes et al., 2007). More recent studies have revealed that elevated expression of IL-21 and its receptor (IL-21R) distinguish Tfh cells from other Th subsets (Chtanova et al., 2004; Rasheed et al., 2006). IL-21 is a member of the common gamma-chain-signaling family of cytokines. The receptor for IL-21, comprising the alpha unit (IL-21Rα) and the common gamma chain, is expressed on various immune cells including T, B, NK, and dendritic cells. By contrast, IL-21 synthesis is restricted and largely limited to activated $CD4^+$ T cells and NK T cells (Coquet et al., 2007; Leonard and Spolski, 2005; Parrish-Novak et al., 2000). The almost exclusive production of this cytokine by $CD4^+$ T cells makes it a candidate for mediating T helper cell function. Analyses of the behavior of immune cells in response to IL-21 in vitro and studies of mice deficient in IL-21 or its receptor support this notion, revealing predominant actions on B cells and also $CD8^+$ T cells (Spolski and Leonard, 2007).

IL-21 is thought to deliver a costimulatory signal to lymphocytes (Parrish-Novak et al., 2000), and molecular studies are beginning to reveal pathways downstream of the IL-21R that might account for its costimulatory function. For instance, in T cells, Jak-STAT, MAPK, and PI3K pathways are all involved in IL-21 signaling through its receptor (Zeng et al., 2007). Consistent with its actions on lymphocyte populations, IL-21 contributes to development of autoimmune diseases in a number of animal models such as systemic lupus erythematosus (SLE or lupus), experimental autoimmune encephalomyelitis, and rheumatoid arthritis (Leonard and Spolski, 2005; Spolski and Leonard, 2007); moreover, studies describing the sanroque mouse strain that develops a lupus-like disease reveal excessive production of IL-21 (Vinuesa et al., 2005a). However, the mechanisms explaining IL-21's function in autoimmune disease pathogenesis remain unknown.

This study demonstrates that IL-21 was necessary for GC formation in response to T cell-dependent antigen. The GC requirement for IL-21 was found to reflect a $CD4^+$ T cell-intrinsic requirement for IL-21-driven Tfh cell generation. High expression of IL-21 and ICOS are features of the Tfh cell transcriptome, and here we show that ICOS was required for optimal production of IL-21. IL-21, in turn, costimulated T helper cell activation through its ability to initiate the activation of Vav1. In this manner, IL-21 modulated the expression of chemokine receptors that direct Tfh cells, expressing the IL-21R, into the GC. The IL-21-driven autocrine loop established by Tfh cells determines their phenotype and fate and distinguishes them from other Th cells, including Th17 cells.

RESULTS

IL-21 Is Necessary for GC Formation and B Cell Responses to T Cell-Dependent Antigen

IL-21 is known to play an important role in the generation of IgG1 antibody responses to T cell-dependent antigen (Ozaki et al., 2002). However, it is not known whether IL-21 imparts its effects on B cells or T cells to generate these responses. By using mice made genetically deficient in IL-21 ($Il21^{-/-}$), we analyzed the generation of GC after immunization with SRBC, which provides a strong polyvalent antigen stimulus for T cell-dependent IgG1 antibody responses.

GCs are detectable in histological sections of lymphoid tissue by the lectin PNA, which stains the surface of activated lymphocytes that express the nonsialylated core 1 *O*-glycans (Galβ1–3GalNAc) (Rose, 1981). $Il21^{-/-}$ mice exhibited a marked reduction in PNA^+ GC compared with WT mice 7 days after immunization with SRBC (Figure 1A). The fraction of PNA^+ $B220^+$ B cells was significantly reduced in the absence of IL-21 (Figures 1B and 1C), indicating a paucity of proliferating B cells in the GC (Figure 1D). In accordance with these findings, IL-21-deficient mice exhibited a marked defect in IgG1 production (Figure S1 available online), supporting previous observations in $Il21r^{-/-}$ mice (Ozaki et al., 2002). IL-21 deficiency had a similar effect on the T cell compartment, as shown by the fact that immunization failed to induce PNA^+ T cells in the GC of $Il21^{-/-}$ mice (Figures 1E and 1F).

IL-21 Is an Autocrine Growth Factor for Tfh Cells

As mentioned earlier, Tfh cells express ICOS and CXCR5, which correlates with their ability to provide cognate help to B cells (Rasheed et al., 2006). Tfh cells also express IL-21 and IL-21R, and this coexpression might reflect an autocrine loop (Chtanova et al., 2004). We analyzed the contribution of IL-21 to Tfh cell differentiation in $Il21^{-/-}$ mice after immunization with T cell-dependent antigen.

Analyses of the phenotype of GC $CD4^+$ T cells 7 days after immunization revealed a defect in the generation of Tfh cells, defined as $CXCR5^+ICOS^+CD4^+$ T cells, in the absence of IL-21 (Figures 2A and 2B). $Il21^{+/-}$ heterozygous mice gave more varied results, suggesting that the dose of IL-21 could be a factor in Tfh cell generation (Figures 2A and 2B). Immunization caused ICOS expression to increase on $CXCR5^-CD4^+$ T cells in the presence or absence of IL-21 (Figure 2A). By contrast, maximal expression of CXCR5 was not achieved on $CD4^+$ T cells in the absence of IL-21 (Figure 2C). Therefore, IL-21 was not necessary for ICOS expression per se, but was necessary for the maximal expression of $CXCR5^+$ that defines Tfh cells.

Tfh Cells Express the Receptor for IL-21

The dependence of Tfh cells on IL-21 suggested that they utilize IL-21 for their growth and/or survival. Therefore, we measured the expression of the IL-21R on $CD4^+$ T cells after immunization and observed prominent expression of IL-21R on $CXCR5^+$ Tfh cells but not on either $CXCR5^-$ memory ($CD44^{hi}$) $CD4^+$ T cells or naive ($CD44^{lo}$) $CD4^+$ T cells (Figure 2D). We also took advantage of the human system to assess IL-21R expression on different T cell subsets and observed that human Tfh cells from tonsils, marked by their high coexpression of PD-1 and CXCR5, exhibited an intense expression of IL-21R (Figure 2E). By contrast, we did not observe high expression of IL-21R on $CXCR5^-CD4^+$ or $CXCR5^{lo}CD4^+$ T cells. Tfh cells in human tonsillar tissue have been reported to be a terminally differentiated population with increased susceptibility to apoptosis (Rasheed et al., 2006), and we observed that many of the $IL\text{-}21R^+$ $CXCR5^+PD\text{-}1^+CCR7^-$ Tfh cells in human tonsillar tissue exhibited poor viability, staining with the vital dye 7AAD. These cells were excluded from our analyses in Figure 2E but are shown in Figure S2. Taken together, these findings are consistent with

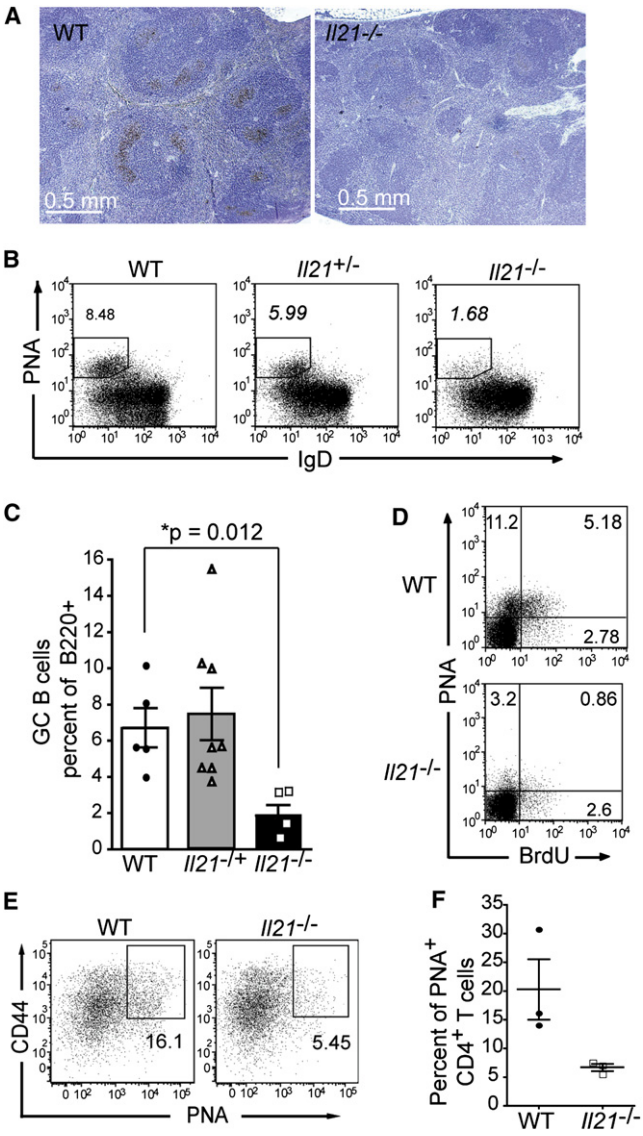

Figure 1. Analyses of Germinal-Center Formation in Wild-Type and *Il21*$^{-/-}$ Mice after T Cell-Dependent Immunization

(A) Representative light microscopy of hematoxylin-stained spleen sections, showing PNA$^+$ (brown) germinal centers on day 7 after SRBC immunization.

(B) Representative dot plot from flow cytometric analysis of IgD- PNA$^+$ germinal-center B cells, gated on total B220$^+$ cells.

(C) IgG1$^+$ germinal-center B cells as a percent of total B220$^+$ cells. Data are shown as values from individual mice ± SEM.

(D) Representative dot plot of flow cytometric analysis of BrdU$^+$ B cells in the spleen.

(E and F) Fraction of PNA$^+$ CD44^{hi} CD4$^+$ T cells shown as (E) representative dot plot and (F) a quantification of mean PNA$^+$ fluorescence intensity (mfi). Data are representative or combined individual values from two experiments where n = 5–8.

an autocrine activity of IL-21 mediating Tfh cell survival or activation in both mice and humans.

The GC Defect in IL-21-Deficient Mice Is CD4$^+$ T Cell Intrinsic

IL-21R is expressed intensely on both Tfh cells and B cells, and our data reveal a central role for IL-21 in the generation of Tfh cells and T cell-dependent antibody production. Previous compelling data support a role for IL-21 in B cell proliferation and antibody production from B cells (Ettinger et al., 2005; Good et al., 2006; Jin et al., 2004; Ozaki et al., 2002, 2004). However, it remained unclear whether the lack of responsiveness to IL-21 by T cells or B cells or both subsets explained the defect in GC formation and antibody production observed.

To determine the relative role of T cell responsiveness to IL-21 in GC formation and IgG1 production, we transferred 2 × 10^7 IL-21R-sufficient WT Ly 5.1 CD4$^+$ T cells (Figure 3A) into Ly 5.2 IL-21R-deficient recipients and immunized the recipient mice. The transferred CD4$^+$ T cells (Figure 3A) were found to fully differentiate into Tfh cells with high expression of IL-21R, CXCR5, and ICOS, which was presumably fuelled by the excess available IL-21 in the *Il21r*$^{-/-}$ mice (Figures 3B and 3C). However, the transfer of WT CD4$^+$ T cells into unimmunized *Il21r*$^{-/-}$ mice did not produce Tfh cells (data not shown), indicating that the effects of IL-21 required antigen for Tfh cell generation.

The IL-21-responsive WT CD4$^+$ T cells were particularly effective as shown by the fact that the transfer of just 10% of this splenic population could precipitate B cell activation (Figure 3D) and recover approximately 40% of the WT levels of IgG1-producing B cells (data not shown). Histological evaluation confirmed these findings by demonstrating PNA$^+$ cells (Figure 3E) and a small number of Ly5.1 CD4$^+$ T cells entering the B cell follicles of *Il21r*$^{-/-}$ hosts (Figure 3F). However, although the amount of IgG1 in the sera of recipient mice was double that of *Il21r*$^{-/-}$ sera, it remained lower than WT (Figure 3H). By contrast, the transfer of 2 × 10^7 IL-21R-sufficient WT B cells had no effect on IgG1 production. Similarly, the transfer of both CD4$^+$ T and B cells increased cell numbers in the host but did not increase IgG1 levels above that observed with CD4$^+$ T cells alone (Figures 3G and 3H).

These data demonstrated that the defect in GC formation and IgG1 production was CD4$^+$ T cell intrinsic but said little about the antigen specificity of the Tfh cells. Therefore, we tested two approaches. First, we used CD4$^+$ T cells primed to sheep red blood cells (SRBC), which afforded little improvement ($p > 0.5$) in the amount of IgG1 produced (Figure 3H). In our second approach, we transferred 4 × 10^4 OVA-specific TCR Tg CD4$^+$ T cells (OTII) into NP-OVA-immunized *Il21r*$^{-/-}$ mice, which generated Tfh cells (Figure 3I), and successfully recovered the NP-specific IgG1 production to WT amounts (Figure 3J). Thus, the defect in GC formation and IgG1 production in mice in which IL-21:IL-21R interactions were disrupted was due to the inability of CD4$^+$ T cells to respond to IL-21.

Tfh Cells Are Distinct from Th17 Cells

The newly described IL-17-producing Th17 subset has been reported to require IL-21 for their generation in vitro (Korn et al., 2007; Nurieva et al., 2007; Wei et al., 2007; Zhou et al., 2007). However, IL-21 is not required for the generation of Th17 cells in vivo where IL-6 is present (Frohlich et al., 2007; Sonderegger et al., 2008; Coquet et al., 2008). As mentioned earlier, Tfh cells can be distinguished from other T helper cell subsets, including Th17 cells, by their expression of CXCR5 (Acosta-Rodriguez et al., 2007), yet they share a number of attributes including high expression of IL-21, ICOS, CTLA-4, and Fas, as well as provision of help to B cells for antibody production (Annunziato

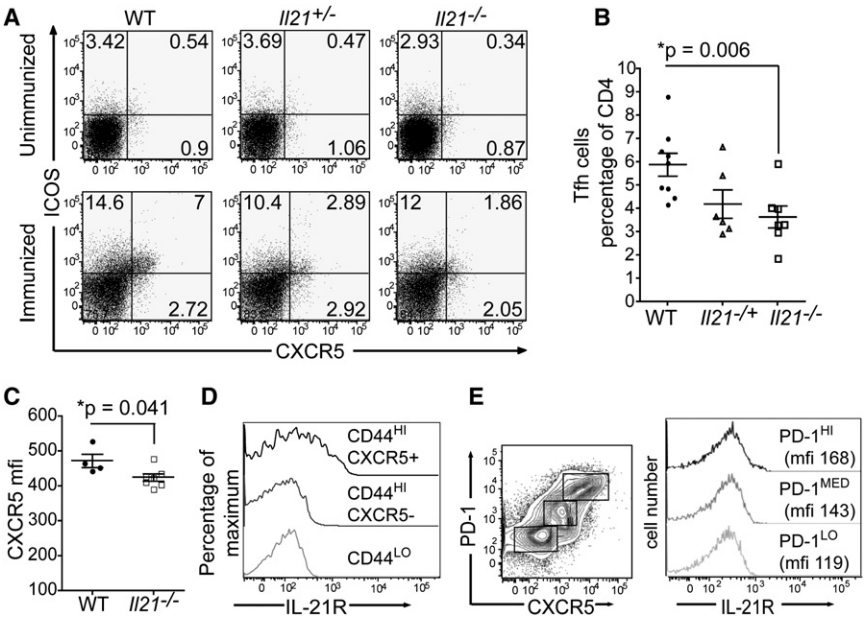

Figure 2. Analyses of the Effect of IL-21 Deficiency on Tfh Cell Generation after T Cell-Dependent Immunization

(A) Representative dot plot showing flow cytometric analysis of Tfh cells defined as ICOS$^+$ CXCR5$^+$ gated on total CD4$^+$ T cells before and 7 days after SRBC immunization.

(B) Tfh cells shown as a percent of total CD4$^+$ T cells after immunization.

(C) CXCR5 expression on Tfh cells shown as mfi. Data for (B) and (C) are shown as mean ± SEM including values from individual mice.

(D) Histogram overlay of IL-21R expression on representative CD4$^+$ T cell naive CD44^{lo}, CXCR5$^-$CD44^{hi} memory, and CXCR5$^+$CD44^{hi} Tfh cell subsets from immunized WT mice. Data are representative or combined individual values from three experiments where n = 6–9.

(E) Representative flow cytometric dot plot of 7AAD$^-$CD3$^+$CD4$^+$ T cell subsets from human tonsil sample showing PD-1 and CXCR5 expression and shown as a histogram overlay showing IL21R expression on PD-1^{hi}, PD-1^{med}, and PD-1^{lo} cells (n = 2).

et al., 2007). Furthermore, an expanded population of IL-17-producing Th17 cells has recently been demonstrated to drive spontaneous germinal-center formation in the BXD2 strain of mice that develop erosive arthritis (Hsu et al., 2008).

To investigate the connection between these two subsets, we analyzed Tfh cells for the presence of Th17 cell signature molecules. Analysis of Tfh cell gene transcription via Affymetrix microarrays failed to identify the gene transcripts associated with Th17 cells, such as RORγt, IL-17, or CCR6 (Figure S3). To directly address whether the Tfh cells that developed after T cell-dependent immunization were phenotypically distinct from Th17 cells, we performed intracellular immmunostaining for IL-17, which revealed that the IL-21$^+$CXCR5$^+$ Tfh cells generated by immunization of WT mice with SRBC (Figures 4A and 4B) and the CXCR5$^+$ OTII cells observed in NP-OVA-immunized hosts (Figure 4C) did not coexpress IL-17. Taken together, these data demonstrate that Tfh cells and IL-17-producing Th17 cells were distinct subsets in our models.

ICOS Expression on CD4$^+$ T Cells Modulates IL-21 Production

Mice made genetically deficient in ICOS (*Icos*$^{-/-}$) do not generate Tfh cells and exhibit defective GC formation in response to T cell-dependent antigen (Akiba et al., 2005; Bossaller et al., 2006; Dong et al., 2001; Tafuri et al., 2001). Tfh cells are known to intensely express both ICOS and IL-21, but the relationship between these two molecules in Tfh cell generation remains unknown (Chtanova et al., 2004; Hutloff et al., 1999). We determined whether ICOS expression had an impact on IL-21 production by comparing IL-21 levels in splenocytes from *Icos*$^{-/-}$ and WT mice, after in vitro stimulation with CD3 monoclonal antibodies (mAb). Splenocytes from *Icos*$^{-/-}$ mice exhibited markedly reduced IL-21 mRNA expression when cells were analyzed directly ex vivo and after stimulation with CD3 mAb (Figure 5A) and when IL-21mRNA was normalized for background expression of IL-21mRNA present ex vivo in each genotype (Figure 5B).

These data revealed an integral role for ICOS in IL-21 production, but it remained unclear whether the splenocytes analyzed from *Icos*$^{-/-}$ mice harbored fewer activated IL-21$^+$ T helper cells or whether costimulation via ICOS:ICOSL interactions was necessary for IL-21 production. To differentiate between these two possibilities, we partitioned the naive and memory CD4$^+$ T cell subsets based on CD44 expression from *Icos*$^{-/-}$ and WT mice and measured IL-21 mRNA 4 hr after stimulation with CD3 mAb and CD28 mAb in vitro. The naive (CD44^{lo}) T cells from *Icos*$^{-/-}$ mice produced similar amounts of IL-21 mRNA to naive CD4$^+$ T cells from WT mice (Figure 5C). Similarly, the memory (CD44^{hi}) populations also exhibited no significant difference in their IL-21 mRNA levels after stimulation (Figure 5C). However, the provision of WT ICOSL-bearing B cells to the cultures boosted the IL-21 expression levels in WT CD4$^+$ T cells but had no effect on *Icos*$^{-/-}$ CD4$^+$ T cells (Figure 5D). In addition, ICOS-ICOSL interactions and CD28-B7 interactions were both found to be necessary for maximal IL-21 mRNA expression as blockade of these interactions in vitro reduced IL-21 mRNA levels (Figures 5E and 5F). Finally, we confirmed the decreased IL-21 from *Icos*$^{-/-}$ CD4$^+$ T cells and from cells with anti-ICOS blockade corresponded with a decreased production of IL-21 protein (Figure 5G). Taken together, these findings demonstrated that T helper cells utilize ICOS:ICOS-L interactions that quantitatively contribute to IL-21 production.

IL-21 Costimulates the TCR to Modulate the Tfh Cell Phenotype

Our findings suggested that IL-21 acts to drive the differentiation of Tfh cells, supporting the phenotypic changes in the expression of molecules that define this population of cells (Chtanova et al., 2005; Rasheed et al., 2006). IL-21 costimulates the TCR-induced proliferation of CD4$^+$ T cells (Parrish-Novak et al., 2000), indicating that IL-21 may mediate its effects on Tfh cell generation by potentiating signals through the TCR. Because most costimulatory signals are additive to the TCR, we argued

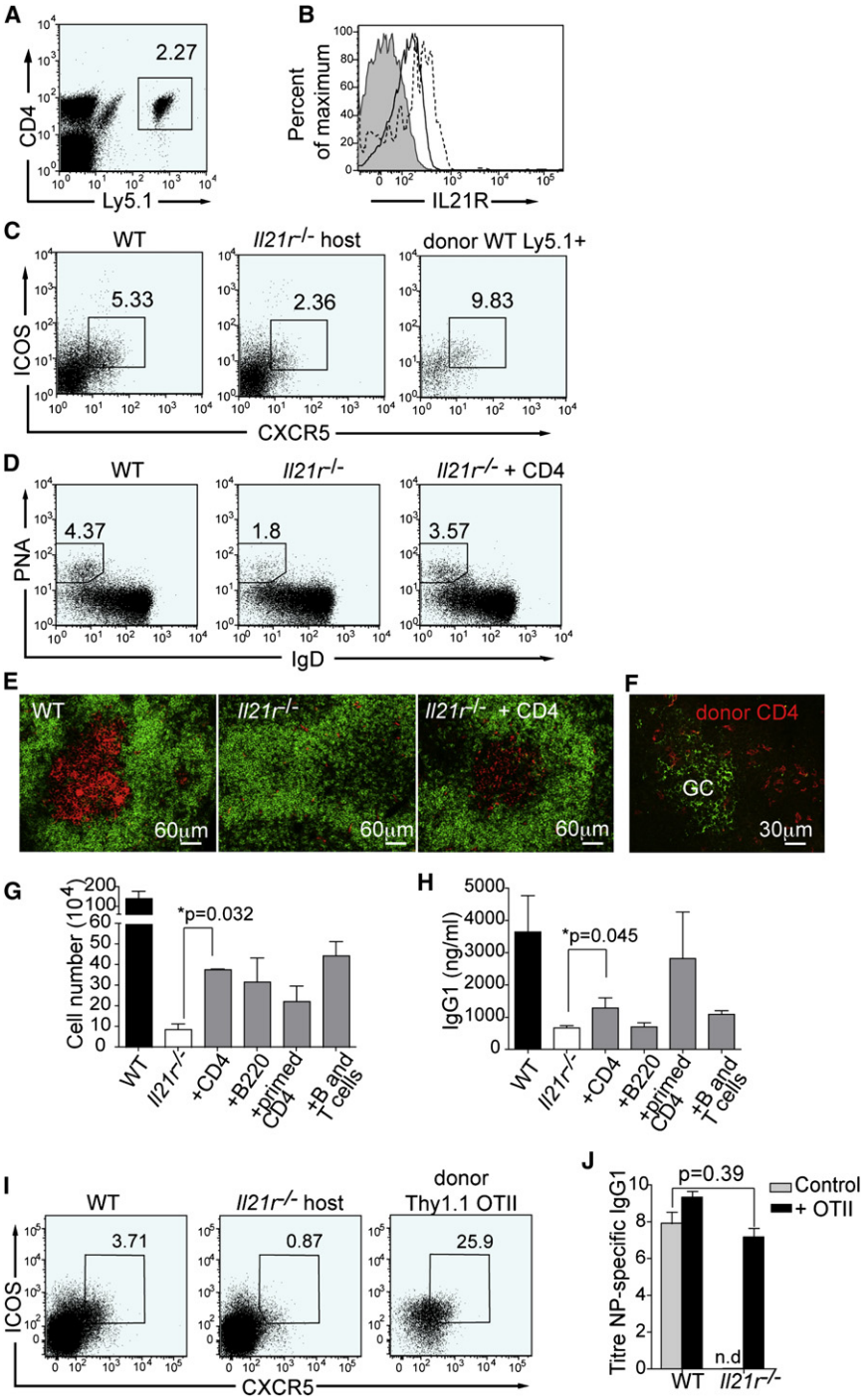

Figure 3. Contribution of IL-21 Responsiveness in CD4$^+$ T Cells to Germinal-Center Formation

(A) Representative dot plot showing donor WT Ly5.1$^+$ CD4$^+$ T cells as a percent of the total lymphocyte gate after immunization of host *Il21r*$^{-/-}$ with SRBC.

(B) Histogram overlay showing IL21R expression on CD4$^+$ T cells from host *Il21r*$^{-/-}$ (filled), WT (unbroken line), and donor WT Ly5.1$^+$ (dashed line) 7 days after SRBC immunization.

(C) Representative dot plots showing flow cytometric analysis of Tfh cells, gated on total CD4$^+$ T cells from WT, host *Il21r*$^{-/-}$, and donor WT Ly5.1$^+$ 7 days after SRBC immunization.

(D) Representative dot plots showing flow cytometric analysis of IgD- PNA$^+$ germinal-center B cells gated on total B220$^+$ cells on day 7 of SRBC immunization.

(E and F) Representative confocal images of spleen sections on day 7 of SRBC immunization showing (E) PNA expression (red) within IgD$^+$ (green) B cell follicles and (F) GL7$^+$ (green) germinal centers with infiltrating donor ly5.1$^+$ (red) CD4$^+$ T cells.

(G) Total IgG1$^+$ IgD$^-$ PNA$^+$ germinal-center B cell numbers in spleen after SRBC immunization.

(H) Serum IgG1 levels measured by ELISA on day 7 of immunization with SRBC.

(I) Representative dot plots showing flow cytometric analysis of Tfh cells, gated on total CD4$^+$ T cells from WT, host *Il21r*$^{-/-}$, and donor Thy1.1$^+$ OTII CD4$^+$ T cells on day 7 of NP-OVA immunization.

(J) Serum levels of NP-specific IgG1 measured by ELISA 7 days after NP-OVA immunization (n.d. = none detected). Data are shown as the mean ± SEM. Data are representative of three experiments (n = 4–9).

that crosslinking the TCR or providing ample CD28 mAb might overcome the need for IL-21. Therefore, we examined the effect of IL-21 on the expression of the Tfh cell signature molecules ICOS, CXCR5, as well as CCR7 and CTLA-4 on CD4$^+$ T cells from *Il21*$^{-/-}$ and WT mice after activation with CD3 mAb and increasing concentrations of CD28 mAb in vitro.

IL-21-deficient CD4$^+$ T cells stimulated with CD3 mAb and low-dose CD28 mAb failed to upregulate either ICOS or CTLA-4 in vitro (Figures 6A and 6B). However, the expression of CTLA-4 on *Il21*$^{-/-}$ CD4$^+$ and WT CD4$^+$ T cells could not be distinguished at the highest dose of CD28 mAb nor in the presence of cross-linked CD3 mAb (Figure 6A). Similarly, the activation-induced downregulation of CCR7 was impaired in IL-21-deficient CD4$^+$ T cells but this defect was overcome by increasing the strength of signal through the TCR (Figure 6A). By contrast, the levels of ICOS remained relatively reduced on *Il21*$^{-/-}$ CD4$^+$ T cells (Figure 6A), but ICOS levels were boosted after the addition of IL-21 to CD3 mAb-stimulated *Il21*$^{-/-}$ CD4$^+$ T cells, confirming that IL-21 upregulated ICOS expression (Figure 6B). However, purified CD4$^+$ T cells from both groups failed to upregulate CXCR5 under these in vitro conditions (data not shown).

A specific requirement for IL-21 in CD4$^+$ T cell activation or survival was also evident when we performed comparative analyses of the proliferation of *Il21*$^{-/-}$ and WT CD4$^+$ T cells in response to CD3 and CD28 mAbs. A considerably reduced

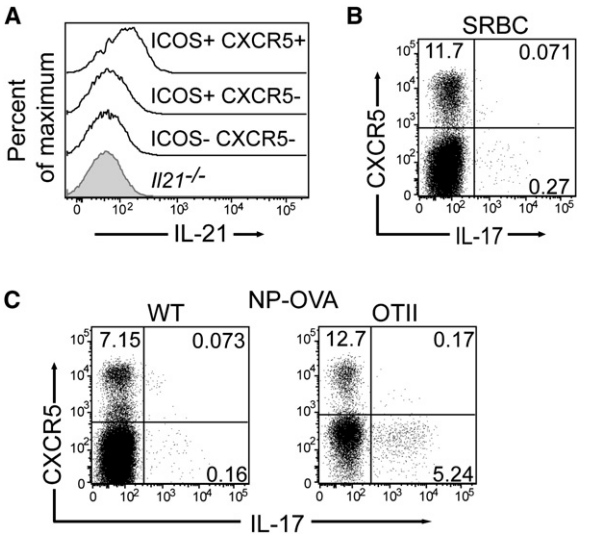

Figure 4. IL-21-Producing Tfh Cells Do Not Express IL-17

(A) Histogram overlay of IL-21 expression on representative *Il21*$^{-/-}$ and CD4^{+} subsets from mesenteric lymph node: ICOS^{-}CXCR5^{-}, CXCR5^{-}ICOS^{+}, and CXCR5^{+}ICOS^{+} Tfh cells.

(B) Representative dot plot showing intracellular immunostaining for IL-17 in CXCR5^{-}CD4^{+} T cells on day 7 of immunization with SRBC.

(C) Representative dot plots showing IL-17 in CXCR5^{-}CD4^{+} T cells from WT host and OTII donor cells on day 7 of immunization with NP-OVA. These data are representative of two experiments (n = 4–9).

fraction of *Il21*$^{-/-}$ CD4^{+} T cells were observed in the divided populations and this was found to be a direct effect of IL-21 deficiency because the addition of exogenous rmIL-21 recovered the proliferation of *Il21*$^{-/-}$ cells (Figure S4). Collectively, these findings indicated that the defect in activation of CD4^{+} T cells in the absence of IL-21 could be partially explained by decreased costimulation of the TCR.

Tfh Cells Are Uniquely Dependent upon IL-21

We next determined whether increasing signals through the TCR might restore the defect in Tfh cell generation in *Il21*$^{-/-}$ mice in vivo. To this end, we administered a single dose of CD28 mAb to *Il21*$^{-/-}$ and WT mice 1 day prior to immunization with SRBC and monitored Tfh cell generation by FACs. The CD28 antibody potentiated the generation of Tfh cells in WT mice but had little or no effect on Tfh cells in the *Il21*$^{-/-}$ mice (Figure 7A). However, although Tfh cells did not respond to CD28 mAb in *Il21*$^{-/-}$ mice, CD25^{hi}CD4^{+} Treg cells did expand, confirming that IL-21-deficient CD4^{+} T cells were responsive to signals delivered through CD28 (Figure S5) but that Tfh cells were uniquely and absolutely dependent upon IL-21.

IL-21 Acts at the Level of the TCR Signalosome to Impact T Helper Cell Fate

Because IL-21 potentiated the TCR-mediated activation of CD4^{+} T cells, we determined whether signaling through the TCR was affected by IL-21. Vav1 is activated by tyrosine phosphorylation after TCR stimulation and is important for PI3K-dependent pathways that lie downstream of the IL-21R. We focused our attention on Vav1 for two reasons; first, Vav1 is an integral component

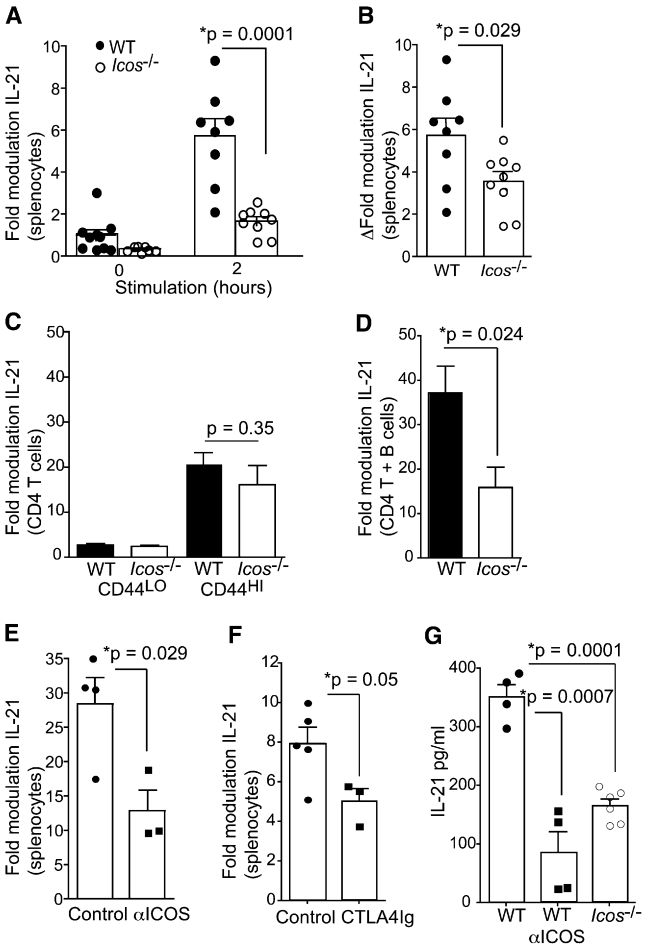

Figure 5. The Contribution of ICOS Expression to IL-21 Production by CD4^{+} T Cells

(A) IL-21 mRNA levels from WT and *Icos*$^{-/-}$ splenocytes ex vivo (0 hr) and after 2 hr stimulation with soluble anti-CD3 measured by real-time PCR. IL-21 mRNA expression is presented as fold modulation compared to WT ex vivo levels.

(B) IL-21 mRNA from (A) presented as delta change from ex vivo levels from each genotype.

(C) IL-21 mRNA from either naive (CD44^{lo}) CD4^{+} T cells or memory (CD44^{hi}) CD4^{+} T cells as indicated, and cultured with CD3 mAb and CD28 mAb (2 μg/ml).

(D) IL-21 mRNA expression in memory (CD44^{hi}) CD4^{+} T cells cultured with CD3 mAb and WT B cells.

Data in (C) and (D) are presented as fold modulation compared to ex vivo WT naive cell expression.

(E and F) IL-21 mRNA from splenocytes stimulated with soluble CD3 mAb in the presence or absence of (E) ICOS mAb (5 μg/ml) or (F) CTLA4-Ig (5 μg/ml), presented as fold modulation from ex vivo splenocytes. Data are shown as the mean ± SEM and individual values from 2–5 experiments where n = 4–11.

(G) IL-21 production measured by ELISA in supernatants from duplicate cultures of splenocytes stimulated for 4 days with CD3 mAb. Data are shown as the mean ± SEM as well as individual points from cultures derived from individual mice (n = 2), representative of two experiments.

of the TCR signalosome and, second, Vav1-deficient CD4^{+} T cells are defective in their T cell helper function (Gulbranson-Judge et al., 1999; Villalba et al., 2001). Crosslinking of the TCR with CD3 mAb led to rapid phosphorylation of Vav1 in CD4^{+} T cells purified from both *Il21r*$^{-/-}$ and WT mice (Figure 7B).

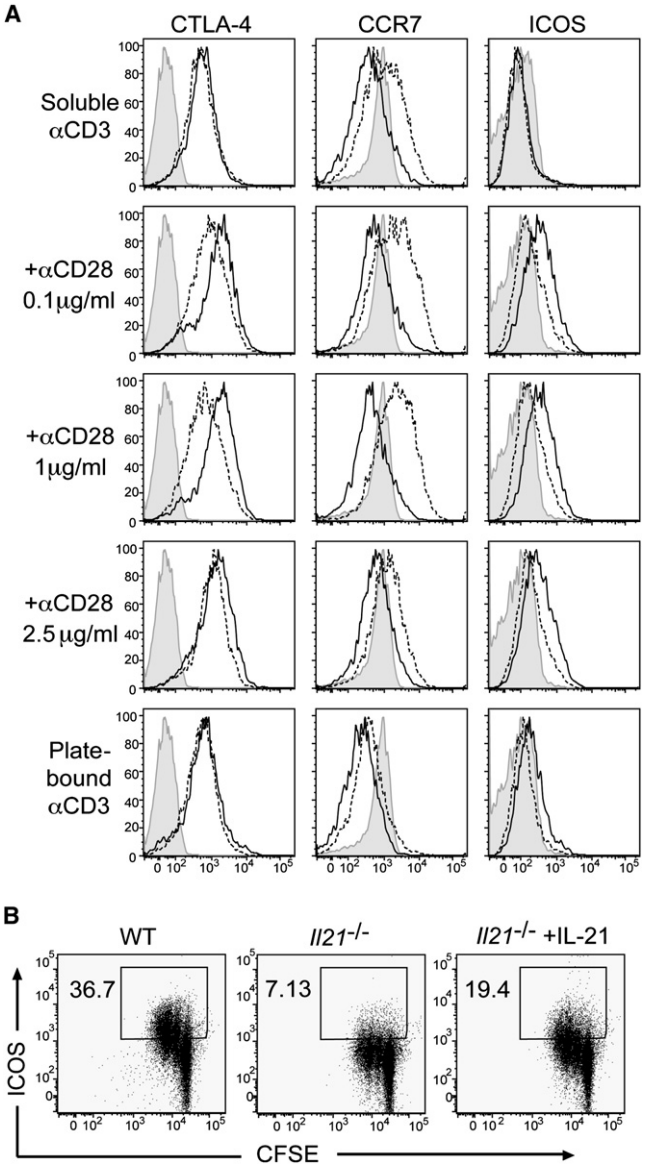

Figure 6. IL-21's Modulation of Tfh Cell Signature Molecules Reflects Its Costimulatory Capacity

(A) Representative histogram overlay showing flow cytometric analysis of $CD4^+$ T cells stimulated for 48 hr with soluble CD3 mAb (2.0 μg/ml) in the presence of increasing doses of CD28 mAb or plate-bound CD3 mAb. Overlays show modulation of CTLA-4, CCR7, and ICOS from WT ex vivo (filled) and cultured WT (unbroken line) and $Il21^{-/-}$ (dashed line).

(B) Representative dot plot showing ICOS expression on CFSE-stained $CD4^+$ T cells from WT or $Il21^{-/-}$ after 48 hr culture with CD3 mAb (2 μg/ml) and CD28 mAb (0.1 μg/ml) in the presence or absence of exogenous IL-21 (50 ng/ml). Data are representative of three experiments where n = 7.

The addition of IL-21 to WT cells greatly potentiated the TCR-induced phosphorylation of Vav1 with no such effect on $IL21r^{-/-}$ cells (Figure 7B). The lack of Vav1 phosphorylation observed by adding rmIL-21 to $IL21r^{-/-}$ $CD4^+$ T cells confirmed the specificity of the effect (Figure 7B). Furthermore, IL-21 was found to phosphorylate Vav1 in the absence of TCR ligation in WT $CD4^+$ T cells (Figure 7C). In contrast, experiments performed in parallel demonstrated that IL-21 did not phosphorylate Zap70, indicating that IL-21 signaling intersects with the TCR downstream of Zap70 (data not shown and Figure S6). These findings support the notion that IL-21 costimulates TCR signals to promote T helper cell activation and differentiation.

DISCUSSION

This study demonstrates that IL-21 is critically important for the generation of Tfh cells and that responsiveness of Tfh cells to IL-21 drives the formation of the GC reaction. Unexpectedly, IL-21 responsiveness was required on $CD4^+$ T cells but not B cells for the generation of the GC and production of IgG1. Previous studies indicate that IL-21 is a switch factor for IgG1 (Pene et al., 2004). In contrast, our data indicate that IL-21 responsiveness by B cells was not necessary for the switch to IgG1, but it clearly impacted on the amount of antibody produced. Our findings support a quantitative effect of IL-21 on IgG1 production in which GC B cells require IL-21 for their expansion rather than for switching to the IgG1 isotype. These findings are consistent with the notion that the induction of isotype switch recombination is imprinted prior to the GC reaction (McHeyzer-Williams and McHeyzer-Williams, 2005). Whether B cell responsiveness to IL-21 influences the affinity maturation of the antibody produced remains an important unanswered question. Our data also reveal a previously unappreciated role for Tfh cells in the establishment of the GC, and the high expression of CXCL13 reported in Tfh cells suggests that such a capacity exists (Kim et al., 2004; Rasheed et al., 2006).

One important issue pertaining to Tfh cells is their relationship to other T helper cell subsets, such as Th1, Th2, Th17, and T regulatory (Treg) cells (King et al., 2008). IL-21 is required for the differentiation of Th2 cells (Frohlich et al., 2007; Wurster et al., 2002), and studies indicate an autocrine role for IL-21 in Th17 generation in vitro (Korn et al., 2007; Nurieva et al., 2007; Zhou et al., 2007). The differentiation of T cells toward the Th17 lineage is influenced by several cytokines and is characterized by expression of the transcription factor RORγt (Ivanov et al., 2006). Th17 cells are dependent upon the transcription factor RORγt for their generation and the cytokine IL-23 for their function in vivo (Zhou et al., 2007). The differentiation of Th17 cells in vitro can be achieved in the presence of both IL-6 and TGF-β, and studies indicate that IL-21 can substitute for IL-6 in vitro (Korn et al., 2007; Nurieva et al., 2007; Zhou et al., 2007). Th17 cells express IL-21 in response to IL-6 but are unlikely to be the predominant source of IL-21, as indicated by the fact that $Ror\gamma t^{-/-}$ mice express normal levels of IL-21 (Zhou et al., 2007). In this study, we showed that Tfh cells express IL-21 and high levels of the IL-21R but do not produce IL-17. In addition, our analysis of human Tfh cell gene transcription via Affymetrix microarrays failed to identify gene transcripts associated with Th17 cells, such as RORγt, IL-17, or CCR6. Taken together with the observation that Th17 cells are generated in the absence of IL-21:IL-21R interactions (Frohlich et al., 2007; Sonderegger et al., 2008; Coquet et al., 2008), these data support the notion that Tfh and Th17 cells are indeed distinct subsets.

In contrast, separating Tfh and Th2 cell subsets on the basis of phenotype and function proves more difficult. For instance, IL-21 is required for the homing and differentiation of Th2 cells in response to infection (Frohlich et al., 2007). Similarly, mice made

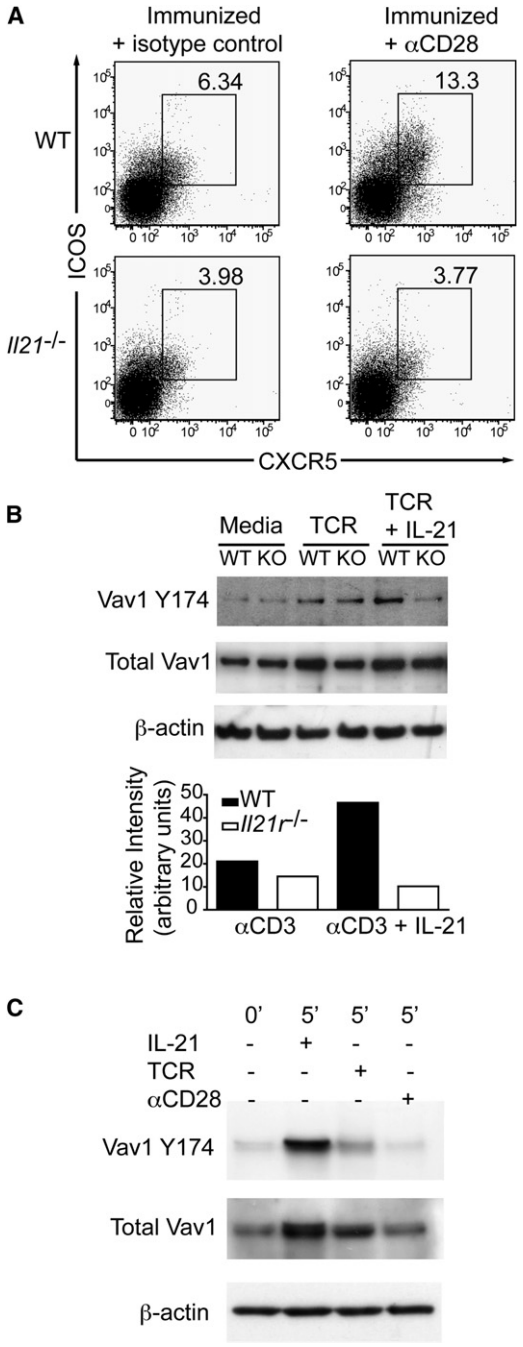

Figure 7. IL-21 Delivers a Costimulatory Signal for Tfh Cell Differentiation

(A) Representative dot plot of $CD4^+$ T cells showing the percent of Tfh cells in immunized WT and *Il21*$^{-/-}$ mice after administration of CD28 mAb, from three experiments (n = 5–11).

(B and C) Immunoblots probed for tyrosine phosphorylation of Vav1, total Vav1, and β-actin after addition of exogenous IL-21, crosslinked CD3 mAb, or CD28 mAb as indicated in (B) purified WT and *Il21r*$^{-/-}$ $CD4^+$ T cells after 10 min of anti-CD3 monoclonal antibody stimulation in the presence or absence of exogenous IL-21 and (C) purified WT $CD4^+$ T cells and Densitometry is shown of relative tyrosine phosphorylation of Vav1 compared to unstimulated control samples. Data are representative blots from three experiments.

genetically deficient in Tfh cell signature molecules such as Bcl6, ICOS, IL-21R, or c-Maf all have defective Th2 cell responses. Studies that have distinguished Th2 cells from Tfh cells demonstrate that IL-4-deficient mice can generate T cell-dependent Ab responses (Kopf et al., 1995; Shimoda et al., 1996), indicating that B cell help still occurs in the absence of IL-4 (and presumably typical Th2 cells). However, the observation that fully polarized Th2 cells do not express CXCR5 segregates Th2 cells from their GC-residing counterparts (Moser and Ebert, 2003). In light of these studies, although Tfh cells and Th2 cells may represent distinct subsets, Tfh cells could arise from an IL-21-dependent pre-Th2 cell pool for their discriminating role in the GC.

Our data indicate that IL-21 exerts its effects by costimulation of the TCR. IL-21-driven costimulation was required for the high expression levels of CXCR5 that guides Tfh cells into the CXCL13-rich GC. It is likely that after the initial activation by antigen-loaded antigen-presenting cells in the T cell zone, T cells destined to become Tfh cells must receive additional signal(s) provided by cells located in or close to the B cell follicles (Ebert et al., 2004). Our data showed that B cells could costimulate $CD4^+$ T cells for optimal production of IL-21 through ICOS:ICOSL interactions. The level of expression of ICOS has been previously suggested to define a qualitative aspect of T helper cell function based upon cytokine production (Lohning et al., 2003), and in this context, the highest production of IL-21 is also associated with the highest expression of ICOS in Tfh cells. However, although IL-21 was an absolute requirement for ICOS expression in vitro, it was not necessary for ICOS expression on $CXCR5^-CD4^+$ T helper cells in vivo. This discrepancy possibly reflects that our in vitro culture conditions incompletely mimic T cell activation events in vivo and indicates that factors in the lymphoid microenvironment driving ICOS expression could compensate for the lack of IL-21. Thus, IL-21 acted as a soluble costimulator, but Tfh cells were further dependent on IL-21 for their growth or survival because increased costimulation per se (via CD28) could not recover Tfh cells in vivo.

Engagement of lymphocyte antigen receptors sets in motion the tyrosine phosphorylation of numerous proteins that are essential for cellular activation and differentiation. Vav1 has been shown to be necessary for T cell help during the GC response (Gulbranson-Judge et al., 1999), and this finding is supported by diminished IL-4 production and Th2 cell responses in *Vav1*$^{-/-}$ T cells (Kim et al., 1999; Tanaka et al., 2005). Our data demonstrated that IL-21, alone or in conjunction with CD3 mAb, induced the tyrosine phosphorylation of Vav1. These findings are a good indication that IL-21 increases the strength of the TCR signal, and, because stronger TCR signals are known to favor Th2 cell differentiation, they are also likely to favor Tfh cell differentiation. Our data propose an intriguing relationship between costimulation of the TCR by IL-21 and activation of Vav1 during the generation of Tfh cells that is likely to involve P13K (Figure S6). Moreover, the functional coupling of Vav1 and IL-21 reveals a molecular mechanism explaining a fundamental role of IL-21 in Th cell differentiation.

In this study, we uncover important information on the biology of IL-21 and IL-21R for Tfh cell generation. Tfh cells intensely express ICOS and IL-21, and elevated expression of these molecules are found in models of spontaneous autoimmunity. This study demonstrates that ICOS is required for optimal IL-21

production, which is produced in abundance by Tfh cells (Bryant et al., 2007; Chtanova et al., 2004). B cells have been thought to be the predominant recipient of IL-21's effects, driving class switch and antibody production. This study challenges that notion by revealing an IL-21-driven autocrine loop in Tfh cells that controls isotype switching and GC formation. Together, these findings reveal a fundamental, but previously unknown, aspect of T helper cell differentiation and the central role of IL-21 and IL-21R in Tfh cell biology.

EXPERIMENTAL PROCEDURES

Animal Procedures

The *Il21*$^{-/-}$ mice in this study were created through an NIH initiative with Lexicon and Deltagen, on a mixed C57BL/6 and 129 background and bred to B6 N5. *Il21r*$^{-/-}$ mice were obtained from W. Leonard (NIH) via M. Smyth (Melbourne) at B6 N6 and backcrossed to N7 for experimental use. Thy1.1^{+} OTII mice were bred in-house. Ly5.1 congenic mice were purchased from the Animal Research Center in Perth, Australia. Animals were housed under specific pathogen-free conditions and handled in accordance with the Australian code of practice for the care and use of animals for scientific purposes. Age-matched littermate mice used for experimental purposes were between 7 and 14 weeks of age. Mice were given 2 × 10^{8} sheep red blood cells (IMVS, Australia) i.p. injection or 100 μg of NP$_{17}$-OVA absorbed to alum, and spleens were analyzed at day 7. 100 μg of anti-CD28 (Ebioscience, San Diego, CA) in PBS was given 1 day prior to immunization. For transfer experiments, 2 × 10^{7} B220^{+} or CD4^{+} Ly5.1 congenic cells (day 6 post SRBC immunization for primed CD4^{+} T cells), or 4 × 10^{4} OTII-purified CD4^{+} T cells were given i.v. at the time of immunization.

Flow Cytometry, Enzyme-Linked Immunosorbance Assay

Single-cell suspensions were stained in PBS 0.5% BSA-containing antibodies. To reduce nonspecific binding, cells were pretreated with anti-CD16 (2.4G2 made in-house). The following anti-mouse antibodies were purchased from Ebioscience: CD44, CD4, CTLA-4, IgD, and Thy1.1. Antibodies purchased from BD Biosciences include ICOS, CXCR5, CD4, B220, Ly5.1, GL7, CCR7, IgG1, and BrdU. Monoclonal antibody 9N1.1 was used to stain human IL21R (Chtanova et al., 2005). PNA was purchased from SIGMA. To detect IL-17, splenocytes were cultured for 6 hr with PMA (50 ng/ml) and ionomycin (750 ng/ml), and then intracellular IL-17 was stained according to the manufacturer's instructions (BD Biosciences). Cells were acquired with Canto cytometer (BD Biosciences) and analyzed with Flowjo (Treestar). IL-21 was assayed from the supernatant by ELISA, according to the manufacturer's instructions (R&D Systems). Serum IgG1 was captured with anti-mouse Ig(H^{+}L) (Southern Biotech) or NP$_{30}$OVA (Molecular Solutions) and detected with AP conjugated anti-mouse IgG1 (BD Biosciences). The titer of NP-specific IgG1 was calculated as Log$_{2}$ of the last dilution factor where the OD was 3× that of background.

Cell Purification, Culture, and Transfer

Lymphocyte populations were purified with biotin MACSbeads kits and the negative population was collected with an Automacs (Miltenyi) giving cell purity of >95%. Naive (CD44^{-}CD4^{+}) and activated (CD4^{+}CD44^{hi}) T cells and B cells (B220^{+}) used for mRNA analysis were sorted with a FACS Aria (BD Biosciences). Sorted CD4^{+} lymph node T cells were cultured in RPMI supplemented with 10% fetal calf serum, 1 mM sodium pyruvate, 1 mM HEPES, non-essential amino acids, and 5 × 10^{5} M 2-mercaptoethanol (GIBCO, Invitrogen). Some wells contained plate-bound or soluble anti-CD3 at 2.5 μg/ml (145.2C11) and soluble anti-CD28 antibody (37-51, Ebioscience). For analysis of proliferation by CFSE, cells were stained for 10 min with 0.5 μM CFSE (Invitrogen) in PBS 0.1% BSA at 37°C and stimulated as described above for 48 hr before flow cytometry analysis.

Immunohistochemistry and Microscopy

GC formation was assessed by staining 5 μm sections of OCT embedded (Tissue-Tek), acetone-fixed spleen with biotinylated primary antibodies overnight, followed by incubation with ST-HRP or ST-Cy3 (Jackson Immunoresearch) for 1 hr. Slides were analyzed with a Leica laser scanning confocal microscope (Leica Microsystems). Each image represents a single 0.14 μm Z optical section. The images were processed with the Leica confocal software and Adobe Photoshop, version 7.

SDS-PAGE and Immunoblotting

Purified CD4^{+} cells were stimulated as described previously (Charvet et al., 2006) with or without recombinant mouse IL-21 (Peprotech) at 50 ng/ml. Cell lysates were prepared in standard lysis buffer with protease inhibitors (Sigma Aldrich) and fractionated by SDS polyacrylamide-gel electrophoresis. Membranes were probed with anti-Vav1, anti-phospho-Vav1 Tyr174 (Santa Cruz), and anti-βactin (Sigma Aldrich). Antibody binding was detected with goat anti-rabbit or anti-mouse IgG-HRP (DAKO) and developed with ECL (Perkin-Elmer). Densitometry was performed to calculate the relative intensity of phosphorylated Vav1 compared to B-actin loading, normalized to background phosphorylation in unstimulated controls according to the following equation: ((test-βactin)/(media-βactin))*100.

RNA Analysis

For RNA extraction, 6 × 10^{5} pooled splenocytes or sorted cells from 2–3 mice per genotype were stimulated for 3 hr in triplicate as above. RNA was isolated with TRIzol reagent (Invitrogen) and cDNA was prepared with Superscript II reverse transcriptase (Invitrogen) and oligo-dT primers (Promega). We determined the relative abundance of cDNAs in triplicate by qRT-PCR analysis with the ABI Prism 7700 Sequence Detection System (Applied Biosystems). Real-time PCR primers for IL-21 and GAPDH were obtained from Applied Biosystems. For each assay, standard curves were generated to identify positive signals on the linear part of the curve. Values for IL-21 were normalized to GAPDH expression in each sample. Fold modulation of mRNA was calculated by employing a comparative CT method.

Microarray

Lymphocyte mRNA for microarray analysis was generated as described previously (Liu et al., 2006). In brief, cell subsets were prepared as follows; Tfh cells (CD57^{+}CXCR5^{+}CD4^{+} T cells) were isolated from tonsil by FACS; Th1 cells were generated from FACS-sorted cord blood CD4^{+} T cells polarized under Th1 cell conditions; Th2 cells were generated from FACS-sorted cord blood CD4^{+} T cells polarized under Th2 cell conditions; TCM (CCR7^{+}CD4^{+}CD45RO^{+} T cells) were FACS-sorted from PBMC; TEM (CCR7^{-}CD4^{+}CD45RO^{+} T cells) were isolated from PBMC; total T cells (CD3^{+}CD4^{+}) were isolated from PBMC by FACS; CD19 B cells (CD19^{+}) were isolated by FACS from PBMC. Each genechip was normalized to its respective median across all chips and the expression signatures computed by taking the mean of all replicates by GeneSpring (Agilent).

Baf3 Cell Transfection

Baf3 cells for retroviral transfection of *Il21r* was described previously (Yu et al., 2006). The primers used for cloning the human and murine *Il21r* can be provided on request.

Data Analysis and Statistics

Data were analyzed with Prism software (Graphpad software) to calculate unpaired, two-way Student's t test, including an F test to compare variances.

ACCESSION NUMBERS

All human microarray data can be found online at the Garvan public database (http://linkage.garvan.unsw.edu.au/public/microarrays/Arthritis_Inflammation/human/Tcells/index.html) or online in the GEO database (accession number GSE3982).

SUPPLEMENTAL DATA

Supplemental Data include six figures and can be found with this article online at http://www.immunity.com/cgi/content/full/29/1/127/DC1/.

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