**Precocious Interleukin 21 expression in naïve mice defines a novel stage of T-follicular helper cell development**

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**Abstract**

T follicular helper cells (TFH) localized to germinal centers secrete Interleukin 21 (IL21), driving B cells to differentiate into memory cells and long-lived plasma cells. TFH are critical for humoral immunity to T-dependent antigens and contribute to several autoimmune diseases, yet their developmental origins remain obscure. Here, we describe activated, IL21-expressing CD4+ T cells present in neonatal mice and show that IL21 is their defining cytokine. Their generation is not dependent on B cells or CXCR5 but is dependent on IL6 and IL21 signaling. Their generation is not limited by stringent restriction of their diverse TCR repertoire and they are major targets of regulatory T cells (TREG). We propose that these “nascent” IL21-expressing TFH develop and persist because of hypersensitivity to weak tonic stimuli through which they are poised for rapid responses to foreign and potential autoantigenic challenges.

**Introduction**

Interleukin 21 (IL21) is a member of the c cytokine family that acts in a pleiotropic manner to modulate the differentiation and function of many lymphoid and myeloid cell types {Spolski, 2014 #53}. Through its broadly expressed receptor (IL21R), this potent cytokine acts in an autocrine and paracrine manner to support anti-tumor and anti-viral responses and numerous autoimmune disease processes {Liu, 2013 #56;Spolski, 2014 #53;Tangye, 2013 #43;Davis, 2015 #54}. While there is evidence that natural killer (NK) T cells and TH17 cells can produce IL21 ~~{Coquet, 2007 #33;Zhou, 2007 #525}~~, the major source is T follicular helper cells (TFH) {Crotty, 2011 #45} {Tangye, 2013 #43}. TFH are a specialized class of CD4+ helper T cells that localize preferentially to germinal centers (GC) within B cell follicles of the spleen, lymph nodes and Peyer’s patches. They are characterized by surface expression of ICOS, CXCR5, and PD1 and expression of the master transcriptional regulator, BCL6, which is critical for TFH development {Nurieva, 2009 #21e}. Through cognate engagements with B cells in in B cell follicles, TFH fully differentiate and drive the proliferation of antigen-stimulated B cells in GCs and their differentiation to class-switched IgG secreting plasmablasts, plasma cells and memory B cells{Barnett, 2014 #4}. Although IL21 is regarded as their signature cytokine, TFH can also express other cytokines, including IL4, IL10, IL17, and IFN, and can also assume alternative cytokine polarization states {O'Shea, 2010 #529;Tellier, 2013 #156}. Furthermore, while homing and positioning in B cell follicles is a defining feature of “GC TFH”, IL21-expressing TFH-like cells, are found in the circulation andin extrafollicular lymphoid sites (termed extrafollicular TFH (E TFH) {Lee, 2011 #44}) where they help extrafollicular B cells to differentiate to antibody-secreting plasmablasts. Given the importance of IL21 signaling and TFH in health and disease, there is need for a deeper understanding into how and when their biologies converge.

Currently, the ontogenic processes that give rise to TFH as opposed to other TH lineages are only partially understood. According to present-day concepts, this dynamic, multistep process starts when naïve CD4+ T cells in T cell zone of the spleen and lymph nodes are first activated and express ICOS through cognate TCR engagements with myeloid dendritic cells (DC) {Goenka, 2011 #8} expressing ICOSL{Choi, 2011 #15} and IL6 (xxx). In this “pre-TFH” stage, activation by DC initiates a series of transcriptional changes including the upregulation of the master TFH regulator, BCL6{Johnston, 2009 #22;Liu, 2012 #12}, as well as other transcription factors{Xiao, 2014 #2;Wang, 2014 #3;Liu, 2014 #42}, the repression of alternative TH fate determining factors{Chtanova, 2004 #27;Nurieva, 2008 #25}, and the expression of CXCR5 (XX). Programming is then continually reinforced and refined. When pre-TFH re-encounter cognate antigens presented by B cells at T/B border of the B cell follicles they increase expression of CXCR5 as they enter the follicles, and clonally expand and fully mature to PD1+ GC TFH.

Importantly, almost all the information we have on the development of TFH is based on studies of cells from immunized or infected adult animals or from the inducing the differentiation of naïve T cells *in vitro*. While studies have revealed important facets of TFH development and function, little attention has been paid to the processes through which TFH naturally develop in naïve mice and to the possible involvement of IL21. Knowledge of these natural ontogeny processes could prove to be important for understanding humoral immunodeficiencies, improving early vaccinations, and uncovering early events in the pathogenesis of autoimmune diseases.

To develop insights into these issues, we generated an IL21-Venus Fluorescent Protein (VFP) reporter mouse that made it possible to identify, phenotypically characterize and functionally assess cellular sources of IL21. We show that IL21 is the majorcytokine expressed within weeks of birth by the earliest CD4+ T cells to be activated in the spleens, circulation, and thymi of naïve mice. Using a variety of approaches, we show that these IL21-expressing cells arise naturally-and are the earliest precursors of mature TFH. These “nascent” TFH (nTFH) are clonally diverse but they develop and persist in naïve mice even with stringent restriction of their TCRs. Finally, we show that nTFH are a major target of suppression by regulatory CD4+ T cells (TREG) because their frequencies and maturation are increased strikingly in FOXP3-deficient mice. From these findings, we propose that nTFH develop spontaneously because they are hypersensitive to weak tonic stimuli, are constrained from their full autoreactive potential by TREG and persist already primed for rapid responses toforeign antigens and autoimmune stimuli.

**Results**

**Expression of an IL21-VFP IL21 knock-in reporter allele reliably detects IL21 expression by activated CD4+ T cells.**

We created an IL21-VFP reporter strain by inserting an internal ribosomal entry site (IRES)-VFP cassette into non-coding exon 5 of the mouse *Il21* locus by homologous recombination into C57BL6/N (B6)-derived embryonic stem cells and then excising the LoxP-flanked *NeoR* selection cassette by Cre-mediated deletion (**Supplementary Fig. 1a,b**). Heterozygous and homozygous B6.IL21-VFP mice were born in expected Mendelian ratios and developed normally.

To determine if the VFP transgene reliably reports *Il21* expression, we first performed RT-qPCR analyses on FACS-purified splenic VFP+ and VFP- CD4+ T cells from adult mice stimulated with antibodies to CD3 and CD28 for 24 h. *Il21* and *VFP* transcripts were expressed at comparable levels in VFP+ cells (**Supplementary Fig. 1c**). To determine if the VFP transgene also reports *Il21* expression reliably *in vivo*, we immunized adult B6.IL21-VFP (VFP) mice with DNP-KLH in CFA and profiled spleen cell populations 11 days later by FACS. VFP expression was greatly elevated in ICOS+CD44+CD4+ T cells with the highest expression in PD1+CXCR5+ TFH (**Supplementary Fig. 1d,e**). VFP was detected exclusively on activated CD44hi CD4+ T cells, many also expressing the TFH marker ICOS and some expressing PD1. A minority expressed the NKT marker NK1.1 (**Supplementary Fig. 1d,e and data not shown**). In addition, RT-qPCR analysis of FACS-purified VFP- and VFP+ CD4+ T cells showed that *Il21* was among the T-helper cytokine gene transcripts that were significantly elevated in the VFPhi subset ofcells from immunized mice (**Supplementary Fig. 1g,h**). To examine the anatomic localization of VFP+ cells, we generated B6.*Sle1.Yaa* VFP mice that spontaneously develop GCs and stained their spleens with anti-GFP antibodies that cross-react with VFP. The results clearly showed that VFP+ cells localized to GCs of autoimmune disease-progressed, 14 wk old, B6.*Sle1.Yaa* mice (**Supplementary Fig. 1)** and VFP reporter mice immunized with DNP-KLH (data not shown). Taken together, these results demonstrated that the VFP reporter accurately marked cells expressing *Il21* and identified cells following immunization as activated CD4+ T cells closely resembling prototypic TFH.

**Early appearance of IL21-expressing CD4+ T cells in naïve mice.**

To begin to study the ontogeny of TFH in naïve mice, we analyzed the pattern of VFP expression by peripheral blood lymphocytes (PBL), spleen cells or thymocytes without immunization starting at 2 wks of age. Flow cytometric analysis of spleen cells from 2 and 4 wk old IL21-VFP mice revealed highly reproducible populations of VFP+CD4+ T cells at 0.5-1% and 2%, respectively (**Fig. 2a**). Their frequencies gradually increased to ~10% at 17 wks of age and were also detected in the circulation at reduced frequencies (**Fig. 2b**). Consistent with an activated state, all VFP+ cells expressed had elevated levels of CD44, with an appreciable fraction, ranging from 40 to 80%, also showing high levels of ICOS expression (**Fig. 1a,b and data not shown)**). 75 to 90% of VFP+ cells were CXCR5lo and none were PD1+, thus distinguishing them from previously described pre-TFH or fully mature TFH (**Fig. 1d**). Given their precocious presentation in the periphery, we next asked whether VFP+ cells were also found in the thymus. Analyses of thymi from 2 and 4 wk old IL21-VFP mice revealed a small (~0.2-0.5%) but reproducible population of VFP+ CD4+CD8- T cells that also expressed CD44 and ICOS (**Fig. 1e,f**). We did not detect VFP+ T cells in the CD4+CD8+ or CD4- CD8+ thymic populations (data not shown). Taken together, the results demonstrated that activated IL21-expressing CD4+ T cells are present in peripheral lymphoid compartments and in circulation of very young naïve mice and suggest that they originate from activated already IL21-expressing CD4 single-positive thymocytes.

**Early arising IL21-expressing CD4+ T cells are not dependent on B cells or expression of CXCR5 for their development.**

Studies of the requirements for B cells and the homing receptor, CXCR5, in directing the development of TFH after immunization are somewhat conflicting. Under most circumstances, B cells are critical for development of TFH {Akiba, 2005 #47;Johnston, 2009 #22} but only when antigen levels are limiting {Deenick, 2010 #17}. To explore these issues in regards to early-forming CD4+VFP+ T cells, we analyzed 4 wk old naïve B cell-deficient *Ighm*-/- mice bearing the IL21-VFP reporter. The frequencies of VFP+CD4+ T cells, which were primarily CD44+ICOS+, were comparable in the B cell-deficientand B cell-competent mice (**Fig. 2a,b**). Given the importance of CXCR5 for the proper homing of TFH cells to B cell follicles, we analyzed IL21-VFP reporter mice lacking CXCR5. These data showed that VFP+CD4+ T cells developed at normal frequencies in mice lacking CXCR5 (**Fig. 2c,d**). Taken together, these results indicated that neither B cells nor expression of CXCR5 was required for newly activated CD4+ T cells to express IL21 and that expression of CXCR5, which was found on a low proportion of VFP+ cells, was not important for their early development.

**IL21 is a major helper T cell cytokine expressed by CD4+ T cells of naïve mice**

Following activation, naïve CD4+ T cells can differentiate into a number of cytokine polarization states (XXX). Having documented the early appearance of activated CD4+ T cells expressing IL21, we sought to compare the frequencies of these cells with CD4+ T cells expressing other TH cytokines that might also develop in naïve mice. To examine this issue, we compared the frequencies of CD4+ T cells from naïve mice bearing other cytokine reporters to the VFP-IL21 reporter strain. Flow cytometry profiling of CD4+ T cells from blood and spleens of mice mice heterozygous for IL21-VFP, IL10-GFP, IL4-GFP and IFN-YFP showed that VFP+ cells were present at considerably higher frequencies compared to those expressing IFN, IL4 or IL10 reporters at both 6 and 13 wks of age (**Fig. 3a**). After establishing conditions for the spectral separation of GFP from VFP (**Fig. 3b**), we then investigated whether the IL21 expressing cells also expressed IL10, IL4 and IL17 by analyzing dual reporter mice carrying the IL21-VFP reporter and the IL10-GFP, IL4-GFP or IL17a-GFP reporters (**Figure 3c-e**). Studies of PBL or spleen cells from these mice at 7 to 15 wks of age showed that CD4+ T cells expressing IL21 alone were present at high frequencies while “double-expressors” were detected at only low frequencies. We conclude that IL21 is the major helper T cell cytokine expressed by activated CD4+ T cells in young mice.

**Analyses of cytokines that may affect the frequencies of early-forming IL21-VFP cells**

To determine whether cytokines previously reported to support or repress the generation of mature TFH also affect the development of early-forming IL21-expressing T cells, we crossed the IL21-VFP reporter onto mice homozygous for knockout alleles of *Il6*, *Il21r, Il10*, *Il12b*, and *Ifnar1* and evaluated the frequencies of VFP+CD4+ T cells. 4-6 wk old IL21-VFP mice deficient in expression of IL21R, IL6 or IFNAR1 had significantly lower frequencies of VFP+ cells than control mice with the overall frequencies of ICOS+CD4+ T cells being less affected (**Fig. 4 a,b,c**). In contrast mice deficient in IL12b (IL12p4) did not show changes in the frequencies of total ICOS+ cells and VFP+CD4+ T cellseither arising spontaneously (**Fig. 4d**) or after immunization (data not shown). Moreover, a deficiency in IL10 resulted in increased frequencies of VFP+ and ICOS+ CD4+ T cells (**Fig. 4e**).We conclude that various cytokines operate at the earliest stages to support (IL6, IL21, IFN1) or retard (IL10) the activation and development of IL21-expressing CD4+ T cells.

**RNAseq profiling of early-forming IL21-expressing CD4+ T cells describes a nascent TFH precursor state**

To define early forming IL21-expressing CD4+ T cellsat the transcriptional level, we performed RNAseq on FACS-purified splenic CD4+ T cells from naïve 4 wk old IL21-VFP reporter mice based on the following criteria: naïve ICOSlo/- VFP- (N); activated ICOShi VFP- cells not expressing IL21 (ACT); and activated IL21-expressing cells ICOShi VFP+ cells (ACT IL21) (**Fig. 5a**). Confirming the fidelity of the IL21 reporter and the sorting strategy, *Il21* and *VFP* were expressed coordinately and almost exclusively in the ACT IL21 population, whereas *Icos* and *Cd44* were transcriptionally elevated well above naive T cells and to equivalent levels between the ACT and ACT IL21 populations (**Fig. 5b**).

Analyses of the gene expression patterns for these three CD4+ T cell subsets identified a total of 330 most discriminating genes: **[rest belongs in Figure legend?]** 111 for Naive; 100 for ACT; and 119 for ACT IL21+ cells (**Fig. 5c,d, Supplementary Table 1**). Functional enrichment analysis showed that ACT IL21 cells were enriched in pathways of T cell development, differentiation, activation, and TCR signaling. In contrast, ACT cells (which include all subsets of activated CD4+ T cells not expressing IL21) lacked developmental and differentiation enrichments (**Fig. 5b, Supplementary Table 1**).

To interpret these cell population categorizations, we first compared our gene expression data to the publically available ImmGen microarray-based datasets that are based on analysis of purified CD4+ T cell subpopulations derived primarily from naïve B6 mice{Heng, 2008 #49;Shay, 2013 #48}. Pearson’s correlation followed by principle component analysis (PCA)-based clustering of our sorted populations with the ImmGen CD4+ T cell categorizations showed that ACT IL21 cells clustered closely to ImmGen’s effector memory CD4+ T cell categories, while the ACT cells clustered most closely to NKT cell populations (**Supplementary Fig. 2**). However, since the ImmGen database does not include information on TFH as a distinct T cell subset, we then compared our data with published microarray-based gene expression datasets of activated CD4+ T cells of differing differentiation states isolated from mice after immunization with a T-dependent antigen CD44+ CD4+ T cells: non TFH (CD44+BCL6-CXCR5-); intermediate TFH (CD44+BCL6loCXCR5+); and TFH (CD44+ BCL6hi CXCR5+){Liu, 2012 #12}. Two-way hierarchical clustering showed a strong correlation of our ACT IL21 to the TFH population, whereas our N and ACT cells clustered with the Liu et al intermediate non-TFH and intermediate TFH populations, respectively **Fig. 5e; Supplementary Table 3)**. Thus, even despite different methods and criteria for population identification, ACT IL21 cells were consistent with a TFH lineage.

To gain a more detailed understanding of the N, ACT and ACT IL21 populations, we examined selected genes that have been implicated in various CD4+ T cell lineages. In agreement with the clustering described above, the ACT population exhibited expression signatures distinct from the ACT IL21 population that were consistent with a mixture of TH1, TH2, TREG and NKT cells with minimal evidence for TH17 cells (**Fig. 5f**). The categorization of ACT IL21cells to a TFH lineage was evidenced by the fact that they showed conspicuous elevations in prototypic TFH cell markers – *Btla*, *Cd4*, *Cd28*, *Cd200*, *Slamf6*, *Gpm6b, Sostdc1* and *Cxcr5*. Moreover, the ACT IL21 subset showed a unique upregulation of the canonical TFH transcription factor, *Bcl6,* another TFH-promoting transcription factor, *Ascl2* (expressed at only low levels){Liu, 2014 #42}, and *E2f2*, *Fosb*, *Pou2af1* and *Tox2,* which have not been considered to be TFH-specific. The increased expression of *Maf* and *Egr2* and the downregulation of the *Prdm1* provided additional support for a TFH lineage association for ACT IL21 cells. However, reduced expression of the recently described transcriptional repressors of TFH, *Foxo1* and *FoxP1{Wang, 2014 #3;Weber, 2015 #39;Stone, 2015 #50}*, was not a characteristic of ACT IL21 cells and the expression of *Itch2*, which encodes a ubiquitin ligase that degrades FOXP1{Xiao, 2014 #2}, was expressed at equivalent levels in the three subpopulations. Finally, and consistent with the cytokine reporter studies described above (**Fig. 3**), ACT IL21+ cells were quite restricted in their expression of cytokine genes. In addition to *Il21*, only the TH1 associated cytokine, *Ifng,* was appreciably expressed, but at reduced levels compared with the ACT population. ACT IL21+ cells also uniquelyexpressed *Tgfb3*, *Tnfsf8,* and *Angptl2*. Overall, these results strongly suggest that early IL21 expression marks a nascent stage of TFH development (nTFH) that can be distinguished transcriptionally from alternative TH lineages that also arise in naïve mice within weeks of birth.

**nTFH cells display a diverse TCR repertoire but stringent restriction of the TCR repertoire does not alter their generation**

Given the early activation of nTFH cells, we considered the possibility that they might show a biased TCR repertoire. We therefore utilized the RNAseq data to evaluate TCR usage by the three subpopulations of CD4+ T cells. The results showed that the *Trav* and *Trbv* repertoires were quite similar for naïve and ACT IL21 nTFH cells, while ACT cells showed more bias including substantially increased usage of *Trav11* and *Trav11b* (**Fig. 6a**). The results show that despite being activated, the TCR repertoire of nTFH is as diverse as that of naïve CD4+ T cells.

The diversity lack of TCR bias among nTFH argues against conventional antigen-driven processes and was more akin to their activation through more promiscuous, low affinity TCR engagements. Ovalbumin-specific OT2 TCR transgenic (TG) T cells are selected and maintained homeostatically by their weak TCR reactivity to self-antigens (XXX). If nTFH arise from naïve T cells through similar weak engagements, we reasoned that limitation of the T cell repertoire to OT2 T cells might still permit nTFH development. To test this possibility, we generated TCR-deficient IL21-VFP mice expressing the OT2 transgene as their only TCR and compared them with TCR-unrestricted IL21-VFP wild-type controls. Flow cytometric comparisons of cells from these two cohorts groups at 4 and 14 wks showed that while the overall frequencies of ICOS+ T cells were markedly reduced in *Tcra*-/- OT2 TG mice, appreciable populations of nTFH still developed (**Fig. 6c)**. Therefore, stringent restriction of the TCR repertoire did not compromise the generation of nTFH.

**nTFH cells are stable, persist robustly and differentiate efficiently to mature TFH and memory cells after immunization.**

Our data clearly indicate that nTFH cells are in a state of differentiation that is distinct from that of pre and mature TFH. To directly test if nTFH cells are progenitors of more mature TFH, we performed adoptive transfer experiments in which spleen cells from 4 wk old IL21-VFP or B cell-deficient *Ighm-/-* Il21-VFP mice were enriched for CD4+ T cells, sort-purified for nTFH cells (VFP+CXCR5-/lo PD1-/lo) and injected intravenously (i.v) into 3 sex-matched T cell-deficient B6.*Tcra-/-* recipients. PBL from the recipients were analyzed by over a period of 9 wks (**Fig. 7a**). Two wks after transfer, 50% to 70% of the transferred cells retained the VFP+ nTFH phenotype and increased numerically at later timepoints (**Fig. 7 b,d**).

We then asked how the persisting nTFH would respond to immunization. The B6.*Tcra-/-* recipients described above were immunized with DNP-KLH in CFA and their splenic T cells were characterized 11 days later (**Fig. 7b**). Immunization resulted in an increase in CD4+ VFP+ cells that included all stages of TFH differentiation (nTFH, pre-TFH and full TFH), but with a dramatic phenotypic shift towards full TFH (**Fig. 7b,e**). These results showed that nTFH were fully capable of responding and differentiating into phenotypically mature TFH after immunization.

To determine if the VFP+ cells that developed and persisted after immunization could respond to a secondary immunization, we transferred splenocytes from the immunized recipients into a second group of B6.*Tcra-/-* recipients and monitored the transferred cells (**Fig. 7b**). The VFP+ cells persisted for at least 8 wks and, consistent with an effector memory phenotype, they were uniformly CD44hi CD62Llo (**Fig. 7f**). We then immunized the secondary recipients with DNP-KLH in CFA (**Fig. 7b**). Two of the 3 recipients showed progressive increases in the frequencies of VFP+ TFH (**Fig. 7g**). Taken together, the results show that nTFH are maintained homeostatically, able to respond and efficiently differentiate into full TFH and IL21 expressing effector memory after antigen stimulation and rechallenge.

**Natural TREG potently constrain post-thymic expansion and differentiation of nTFH**

The spontaneous development of nTFH suggested parallels with the ontogeny of natural CD4+ TREG. Analysis of FOXP3-GFP+ IL21-VFP dual reporter mice was performed to compare the frequencies and patterns of FOXP3 and IL21 expression in CD4+ T cells in blood of naïve 4 wk old mice. After filtering for spectral overlap, average frequency of naïve GFP+ TREG was ~3.7%, while the frequency of GFP+ cells in an activatedICOS+CD44+ state was ~1.5% and similar to that of VFP+ TFH (~1.2 (**Fig. 8a**). Since TREG and nTFH are prominent CD4+ T populations in young mice, we hypothesized that a primary function of TREG is to constrain nTFH. To test this possibility, we first analyzed FOXP3-deficient (scurfy) mice that lack TREG and develop a profound autoimmune disease associated with increased frequencies of mature TFH cells within 3-4 wks of age{Ramsdell, 2014 #40}. We generated *Foxp3*-/- VFP reporter mice and analyzed their male progeny at 2 wks of age before disease onset and at 4 wks of age when they display overt signs of disease. While substantial increases in the frequencies of total splenic VFP+ cells all stages of TFH differentiation (CXCR5-PD1- nTFH; CXCR5+PD1- pre-TFH; CXCR5+PD1+ full TFH) were found, the increase was most striking in the nTFH population(**Fig. 8 b,c**). Knowing that nTFH are also present in the neonatal thymus, we also evaluated the frequencies of VFP+ CD4 single positive thymic T cells of FOXP3-deficient and WT mice. Unlike that observed in the periphery, The frequencies of VFP+ cells did not differ in FOXP3-deficient and WT mice (**Fig. 8d**). Overall the results suggest that an important early function of TREG is to limit the post-thymic expansion of nTFH thereby limiting their further pathogenic differentiation and resulting autoimmune disease.

**Discussion**

Differentiation into the various TFH as opposed to alternative TH lineages is a dynamic, multiphase process that is only partially understood and subject to continual revision (XROTTY REF). Canonical models based largely on analyses performed after immunization or infection of adult mice place IL21 as a late stage product of IL21 TFH differentiation (XXXX). By taking advantage of a novel IL21-VFP reporter mouse to investigate the ontogeny of TFH based on IL21 expression in naïve neonatal and adolescent mice, our results provide new insights into the earliest lineages decisions made by CD4+ T cells when they are first activated by endogenous stimuli.

First, we found that is IL21 is the preferred T-helper cytokine expressed by newly activated thymic and peripheral CD4+ T cells that develop spontaneously within weeks of birth. Second, we have shown that precocious expression of IL21 is a property of nascent precursors of TFH. We interpret these findings to indicate that commitment to the TFH lineage is made shortly after the earliest stages of activation. Third, while upregulated ICOS has been thought to be required to promote the differentiation TFH to sufficient maturity for IL21 expression (XXX), we have found appreciable IL21 in CD44hi ICOSlo CD4+ T cells, suggesting that the activated CD44hi state is sufficient for nTFH development. These observations are congruent with recent studies arguing that the requirement for ICOS in promoting TFH differentiation is not absolute and is dependent on situations in which cognate antigens are limiting (CRAFT).

Fourth, we find that nTFH do not require B cells or CXCR5 for their development, arguing that the expression of IL21 develops at the earliest stages of activation by DC. However, even at this early phase, we found that they are influenced by the same cytokine signals that are known to promote (IL6, IL21 and IFN1) or retard (IL10) TFH development and function in adult mice (XXX). This indicates that such cytokines, importantly including IL21 acting in an autocrine manner, are expressed and biologically influential during the earliest stages of TFH ontogeny.

Lastly, we have shown that precocious IL21 expression identifies a population of CD4+ T cells with a remarkable capacity to persist in an activated, IL21-expressing state after adoptive transfer. These cells potently respond and differentiate after immunization with a foreign T-dependent antigen into fully mature IL21-expressing TFH and persist in an activated memory state. The CD4+ T cells that have lost IL21 expression may be TFH cells that have become effector memory cells, as it has already been shown that IL21 expression can be turned off in these memory cells until rechallenge{Luthje, 2012 #5}.

Our RNAseq analysis sheds new light on the earliest molecular processes through which naïve T cells differentiate towards TFH in contrast to alternative TH lineages. A variety of cell purification procedures based on cell surface markers and/or transcriptional factor reporters have been applied to characterize the gene expression patterns of fully differentiated TFH elicited after immunization (XXx). Our approach differed in that we used IL21 reporter expression as the primary cellular phenotype and focused on activated CD4+ T cells that naturally develop in young naïve mice. Our results supported early lineage commitments towards TFH, TH1, TH2, but not TH17 cells, but with TFH being a preferred fate choice. The gene expression signature of nTFH clearly established their TFH lineage. Among the transcription factorsthat have beenpreviously shown to promote TFH development (*Bcl6*, *Acsl2*, *Maf* and *Irf4*), only *Bcl6,* and to a lesser extent *Maf*, provided best specificity for nTFH. We also found that transcription factors including *E2f2, Fosb, Pou2af1, Tox2*, *Egr2*, and *Nfatc1,* which have not been previously regarded to demarcate TFH, were also substantially upregulated in nTFH. However, the observation that the transcription factors, *Foxo1* and *Foxp1,* which were thought previously to act early on to negatively impact TFH development{Stone, 2015 #50;Weber, 2015 #39;Wang, 2014 #3}, were highly expressed by nTFH suggests that they may act to suppress later stages of TFH differentiation. Overall, we propose that the commitment to a TFH lineage is determined at the earliest stages of naïve T cell activation by subsets of transcriptional factors, especially those closely adjoined to the BCL6 regulatory axis. This primary transcriptional state then can be modulated by any combination of cell extrinsic stimuli and intrinsic transcriptional controllers through which nTFH clonally expand and differentiate to mature TFH, E TFH and GC TFH.

The nature of antigens that cause naïve CD4+ T cells to become activated and differentiate into nTFH remains to be determined. While controversial (XXX), there is growing support for models in which ultimate TH lineage fates are a swayed by the nature of TCR-pMHC engagements (CROTTY??Jenkins cell){Keck, 2014 #51}, with antigen abundance rather than high TCR-pMHC affinity thought to promote to TFH (XXX). We have shown that nTFH develop from the naïve CD4+ T cell pool in the absenceof overt immunization with as diverse TCR usage. We have also shown that limitation of the T cell repertoire to singularity in *Tcra-/-* OT2 transgenic mice has minimal impact on nTFH development. These behaviors are reminiscent of the homeostatic behavior of naïve T cells, which are supported by high abundance but low affinity tonic TCR/self-pMHC ligand signals (Sprent XXXand othersGERMAIN). We therefore propose that nTFH may be manifestation of this same homeostatic behavior, with naïve T cells that are particularly sensitive to MHCII-presented self-antigens being activated and differentiating selectively into nTFH. Furthermore, it is further possible that this intrinsic hypersensitivity is established in the thymus. Recent studies have provided evidence linking self-pMHC ligands first encountered by developing T cells during thymic selection to their response potential in the periphery (XXXJaimeson and Allen). This “instructive” model (XXX) proposes that naïve T cells selected in the thymus for higher self-reactivity are advantaged in comparison to weakly self-reactive T cells by enhanced basal TCR signals in response to tonic self-antigen stimuli that is continually reinforced in the periphery. In this way, thymically-acquired self-reactivity has been proposed to poise naïve T cells for more efficient immune responses to foreign antigens {Mandl, 2013 #522} (XXX). Our findings that nTFH are present not only in the circulation and spleens but also in thymi of naïve mice by 2 weeks after birth, albeit at low frequencies, suggests that they are first activated in the thymus. Newly selected CD4+ T cells may thus differentiate into activated, IL21-expressing nTFH rather than remaining in the naïve state because their TCRs are unusually self-reactive.

This suggested ontogeny for nTFH has parallels with that of CD4+ TREG. nTFH and TREG are numerically similar in early post-natal life, clonally diverse, and intrinsically self-reactive. Both develop through thymic selection and emerge shortly after birth into the periphery already activated and poised for effector or regulatory functions, respectively. In agreement with recent reports {Wing, 2014 #52;Iwamoto, 2014 #41}, we have shown that fully matured IL21-expressing TFH are increased considerably in the absence of TREG. Our findings that the most striking increases in the absence of TREG was in the neonatal frequencies of nTFH rather than more differentiated TFH suggests that TREG target the nTFH stage of TFH development. A primary function of TREG may thus be to constrain nTFH that arise in parallel through the same thymic selection processes but with opposing functions.

Overall, our identification nTFH may be shed light on the origins of TFH and related IL21-expressing populations that confer humoral immunity after infection or promote autoimmune diseases. nTFH maintained peripherally in an activated state by tonic TCR/self-pMHC stimulation may be poised for rapid responses to foreign antigens by selecting among those with high affinity, permitting their rapid antigen-driven clonal selection by B cells and resulting in full differentiation to TFH in GCs. In contrast, self-reactivity, rather than high affinity and clonal selection by foreign antigens, is thought to be a general property of autoimmune disease-promoting CD4+ T cells {Wing, 2014 #52}(NEW REF??MARK DAVIS). Genetic and environmental factors that drive the polyclonal expansion and further differentiation of this already self-reactive nTFH population may underlie autoimmune diseases, such as SLE, in which IL21-producing TFH and related TFH-like cells are important pathogenic drivers.

**METHODS**

***Generation of IL21-VFP reporter mice***

The IL21-VFP mice were generated by Ozgene Pty. Ltd. (Bentley WA) using a C57BL/6 targeting strategy as part of a contract with NIAID, NIH. In the targeting locus, IRES-Venus was inserted in exon 5 of the IL21 gene downstream of the stop codon. The PGK-neo selection cassette was inserted downstream of IRES-Venus. The cassette was flanked by loxP sites so that it could be deleted by Cre recombinase. Mice with an appropriately targeted vector were crossed with a Cre-expressing C57BL/6 mouse and the deletion of the PGK-neo cassette was confirmed in their progeny. Genotypic confirmations and further breeding experiments performed using a three primer PCR assay using the following primers: *a,* 5’-AATGCATTTCTTTCACTTCCATGTT-3’; *b,* 5’-TTAGTTAATGGGCGAAAGGATCTTA-3’; *c*, 5’-AACGAGAAGCGCGATCACAT-3’. The wild type band (primers a & b) has an expected length of 293bp and the VFP band (primers c and b) is 501 bp. PCR was performed using 40 cycles of 95C for 30 seconds, 60C for 1 min and 70C for 1 min. VFP-IL21+ mice were born in the expected Mendelian ratios. The mice used in the study were bred and used at the Jackson Laboratory following protocol 01022 approved by the institutional ACUC.

***Mice***

All mice were bred and maintained in a specific pathogen-free mouse colony

at The Jackson Laboratory. All experiments were performed under protocol 01022 approved by the Institutional Animal Care and Use Committee.

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***Activation in vitro and immunization in vivo***

***NEED GILJUNS PROTOCOL***

Mice were immunized intraperitoneally with 200µL of TNP-KLH in emulsified complete Freunds adjuvant at a concentration of 100 µg/100 ul and analyzed 7-11 days after immunization.

***Flow cytometry and sorting***

Flow cytometry was performed using established procedures (XXX). Antibodies are listed in **Supplementary Table 1**. Most analytical studies were performed on a four-laser/13- color BD LSRII analytical cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star). Cell sorting as was performed using a FACSAria (BD Bioscience). FlowJo version 8 and 9 were used for FACS analysis.

***Adoptive Transfer***

Splenocytes from ten B6.IL21-VFP mice were pooled. CD4+ T cells were isolated via negative cell depletion using streptavidin microbeads (Milltenyi) coupled with CD11b, CD11c, B220 and CD8 antibodies (**Supplemental Table 1**, BD Bioscience) and the autoMACS pro separator (Milltenyi) according to the manufacturer’s protocols. Cells were then sorted for CD4+ IL21-VFP+ CXCR5- and PD1- and tail vein injected into recipient mice (BETTER TO PUT THIS IN Fig LEGEND🡪insert injection amounts).

***Quantitative RT-PCR analysis***

Total RNA was extracted with the RNeasy Micro kit (Qiagen) followed by cDNA synthesis using the QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer’s protocol. The INSERT PRODUCT NAME was used for RT-PCR (primers listed in **Supplementary Table 2**). Samples were run in triplicate and mean expression was normalized to 18sRNA using the Ct method.

***RNAseq***

Total RNA from FACS-purified VFP-ICOS-, VFP-ICOS+, and VFP+ICOS+ CD4+ cell pools were extracted with Qiagen RNeasy Mini Kits (Qiagen, Hilden, Germany) in 2 replicates. Poly-A-enriched mRNA was reverse transcribed and amplified using the Nugen Ovation Kit (NuGEN, San Carlos, CA, USA). Paired-end cDNA was sequenced with an Illumina MiSeq at 106 base pair length (Illumina, San Diego, CA, USA). Reads were checked with FASTX-Toolkit (http://hannonlab.cshl.edu/fastx\_toolkit), trimmed with Trimmomatic (Bolger et al., 2014), and aligned to the GRCm38.73 assembly transcriptome with Bowtie (Langmead et al., 2009). Transcript expression levels were estimated in transcripts per million (TPM) using RSEM (Li and Dewey, 2011). Signature genes for the three samples were defined as those that were expressed the highest in their signature sample (mean value), had a TPM more than 50 and were differentially expressed between their signature sample and the other two samples (*P* < 0.05, pairwise t-test). EBSeq was utilized to identify genes that were expressed significantly higher or lower in at least one of the three samples with posterior probability larger than 0.95 (Leng et al., 2013). All analyses were done with R (www.r-project.org) except when specified otherwise. To estimate the expression of VFP relative to native mouse genes, the VFP sequence reads were aligned and combined with the mouse transcriptome file.

***Comparison of RNAseq profiles with ImmGen and antigen-induced TFH* microarray datasets**

For Immgen, we compared transcriptional profiles of our purified samples with those of 214 cell types in six major immune system cell types: B cells, αβ T cells, γδ T cells, NKT cells, myeloid cells, and stromal cells (http://www.immgen.org/). Genes that were expressed differently in at least one of the three samples (N, ACT, ACT IL21) as identified by EBSeq with probability larger than 0.95 were taken in the comparison. Pearson’s correlation on z-transformed gene expression in each sample identified the top-correlated ImmGen samples with each of our three samples (*VFP\_N\_ICOS\_N*: T\_8Nve\_Sp\_OT1, T\_4Nve\_Sp, T\_4Nve\_PP, T\_4Nve\_LN; *VFP\_N\_ICOS\_P*: NKT\_44NK1\_1\_Th1, NKT\_4\_Sp1, NKT\_4\_Sp, NKT\_4\_Lv; *VFP\_P\_ICOS\_P*: T\_4Mem44h62l\_Sp, T\_4Mem44h62l\_LN, T\_4Mem\_Sp, T\_4Mem\_LN). Principal component analysis was applied to cluster the 12 ImmGen datasets with our samples. The first three principal components explained 73% of the total variance. Principal Component 1 was correlated with the different data sources: microarrays from ImmGen and RNAseq of our study. Principal Components 2 and 3 were used for sample clustering.

For direct comparison with TFH induced after immunization, microarray datasets of CXCR5-BCL6-, CXCR5+BCL6lo, and CXCR5+BCL6hi CD4+ T cells isolated after immunization with TNP-KLH {Liu, 2012 #12} were compared to the transcriptional profiles of the 1211 differentially expressed genes in our N, ACT and ACT IL21 populations. The first principal component of the six combined samples was subtracted from each transcript to account for platform and study differences, and hierarchical clustering was performed on the residual transcript levels.

***T cell receptor repertoire analysis***

We identified all transcripts with Ensembl TR\_V\_genes and used TPM estimates to quantify the differential usage of T cell receptor usage in our samples. TCR\_J and TCR\_D segments were unidentifiable in our protocol because their sequences are too short for appropriate expression estimates. Transcripts with maximum expression less than 5 TPM were filtered out, retaining a total of 68 *Tcrav* and *Tcrbv* genes.

***Immunohistochemisty***

Formalin fixed paraffin embedded sections of spleen and lymph nodes of IL21-VFP mice were treated with Proteinase K (DAKO) for 5’ @ RT then stained with a rabbit polyclonal anti-GFP antibody (Abcam; ab6556) followed by biotinylated goat anti-rabbit IgG (Vector Labs) and ABC Elite Reagent (Vector Labs).  Detection with DAB was followed by hematoxylin counterstain.

***Statistical analyses***

The non-parametric Mann-Whitney U test was used for two sample group comparisons of flow cytometry and RT-qPCR data.

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**Figure Legends**

**Figure 1: IL21-expressing CD4+ T cells are found in naïve mice at early ages.** **(a)** Flow cytometric profiling of VFP expression by splenic CD4+ T cells from 2 and 4 wk old naïve B6.IL21-VFP mice. Example (left) with frequencies of each gated population indicated and combined results (right) with each symbol indicating data from an individual mouse. Samples from B6 mice lacking the IL21-VFP reporter are shown as negative controls. **(b)** Percentagesof IL21-VFP+ CD4+ PBL and splenocytes for groups of mice analyzed at varying ages. **(c)** Patterns of CD44, ICOS, CXCR5, PD1 expression by VFP+ splenic CD4+ T cellsfrom a 4 wk old naïve B6.IL21-VFP mouse. **(d)** Analysis of CD4+ single positive thymocytes from 2 and 4 wk old naïve B6.IL21-VFP and control B6 mice examining frequencies of VFP+ cells and patterns of CD44 and ICOS co-expression. Data are representative of XXX experiments. need to do comparative stats!! Especially to make the point that the frequencies of VFP increase over time for peripheral but not thymus.

**Figure 2: IL21-expressing CD4+ T cells develop in the absence of B cells and CXCR5.**

**(a)** Flow cytometry results comparing the frequencies of (SPLEEN OF PBL? VFP-expressingCD4+ T cells from *Ighm-/-* IL21-VFP and IL21-VFP wild-type (WT) controls and co-expression of CD44, ICOS, CXCR5 and PD1. **(b)** Similar analyses comparing the frequencies of blood VFP+ CD4+ T cells from *Cxcr5-/-* IL21-VFP and wild-type controls. NS, not significantly different by the non-parametric Mann Whitney U test. Data are representative of XXX experiments.

**Figure 3: Cytokine reporter-based comparisons of helper T cell cytokine expression by CD4+ T cells from naïve mice**

**(a)** CD4+ T cells fromblood ofgroups of6 and 13 wk oldB6-backgroundIL21-VFP (n=?), IL4-GFP (n=?), IL10-GFP (n=?) and IFNγ-YFP (n=?) reporter mice were analyzed for reporter protein expression by flow cytometry after (need to do comparative stats!!). **(b)** Spectral separation of GFP and VFP. B6.IL21-VFP and B6.IL4-GFP reporter mice were immunized with DNP-KLH in CFA and CD4+ T cells were FACS-purified 11 days later. Sorted VFP+ and GFP+ samples were analyzed by separately or when mixed together by flow cytometry. **(c)** Analysis ofVFP and GFP expression by CD4+ T cells from blood or spleens of B6.IL21-VFP IL4-GFP dual reporter mice of differing ages after filtering for spectral overlap. **(d)** Analysis ofVFP and GFP expression by CD4+ T cells from B6.IL21-VFP IL4-GFP dual reporter mice in at 7 wk and 15 wk of age. **(e)** Analysis ofVFP and GFP expression byCD4+ T cells from B6.IL21-VFP IL17a-GFP dual reporter mice at 7 and 15 wks of age. NEED STAT COMPARISONS IF STATING DIFFERENCES and # OF EXPERIMENTS.

**Figure 4:** **IL21, IL6, IFN1 and IL10 signaling influence the development of IL21 expressing CD4+ T cells in naïve mice.**

Flow cytometry results comparing the frequencies of blood CD4+ T cells expressing ICOS and VFP in: (**a**) groups of 5 wk old B6.*Il21r-/-* IL21-VFP and B6.IL21-VFP WT mice; (**b**)6 wk old *Il6-/-* IL21-VFP and WT mice; (**c**) groups of 4 wk old *Ifnar-/-* IL21-VFP and WT mice; **(d)** 4 wk old *Il12b-/-* IL21-VFP and WT mice; and (**e**)6 wk old *Il10-/-* IL21-VFP and WT mice. \*, p≤0.05; \*\*, p≤0.01; NS, not significantly different. Results are representative of XXX experiments.

**Figure 5: RNAseq-based transcriptomic analysis of isolated populations of splenic CD4+ T cells from IL21-VFP reporter mice.**

**(a)** Splenocytes from naïve 4 wk old VFP mice were pooled into 2 biological replicates. Each sample was then enriched for CD4+ T cells and FACS-sorted. Representative plot showing the criteria used for sorting: N, naïve (ICOS-/lo VFP-); ACT, activated (ICOS+ VFP-); and ACT IL21+ (ICOS+ VFP+). **(b)** Gene expression confirmations of gating parameters showing equivalent expression of VFP and *Il21* by the ACT IL21+ population and equivalently high levels of *Icos*and *Cd44* expression in ICOS+ VFP- and ACT IL21+ populations. **(c)** Heat map of 213 most discriminating genes and functional enrichments on GO terms and KEGG with FDR < 0.05. See **Supplementary Table 1** for more details.(**d**) Scatterplot of the Log2 difference between the two ACT samples and the N sample with genes most discriminating the N, ACT, ACT IL21+ populations colored and reference genes in grey. **(e)** Comparisons between microarray-based profiling datasets of Liu at al of non TFH (CD44+BCL6-CXCR5-), intermediate TFH (CD44+BCL6loCXCR5+) and TFH (CD44+ BCL6hi CXCR5+)to RNAseq profiles of N, ACT, and ACT IL21+ populations, respectively. **(f)** Categorizations of N, ACT, ACT IL21+ to TH categories based on genes of interest. Mean TPM are shown. See **Supplementary Table 2** for underlying data and statistical information.

**Figure 6: nTFH display a diverse TCR repertoire but restricting its specificity does not alter nTFH development.**

**(a)** Analysis of *Trav* and *Trbv* gene utilization of naïve (ICOS- VFP-), activated (ICOS+, VFP-) and nTFH (ICOS+ VFP+). Mean TPM of read counts for each *Tcrav* and *Tcrbv* gene is shown. Pearson correlation coefficients: N vs. ACT IL21+, *r* = 0.866; N vs. ACT, *r* = -0.00361; ACT vs. ACT IL21+, 0.0760. **(b)** Flow cytometry of CD4+ T cells from 4 and 15 wk old B6.IL21-VFP *Tcrα*-/- OT2 Tg and non-transgenic B6.IL21-VFP WT mice. Representative profiles of VFP and ICOS staining (left) and combined results (left) are shown. \*, p≤0.05; \*\*, p≤0.01; NS, not significantly different. Results are representative of XXX experiments.

**LIZ MAYBE BETTER TO SHOW THE SCATTERGRAMS FOR 4 WK OLD MICE – BETTER CONNECTED PERHAPS WITH OUR POINTS?**

**Figure 7: nTFH cells persist after transfer and differentiate into full TFH after immunization.**

**(a)** CD4+ T cells were enriched by negative cell depletion of pooled spleen cells from 4 wk old IL21-VFP or *Ighm*-/- IL21-VFP mice and further purified by FACS for VFP+, CXCR5-/lo and PD1-/lo expression. X to X million cells of the gated populations were injected intravenously into 3 B6.*Tcrα*-/- mice. (**b**) The frequencies of VFP+ CD4+ T cells in the adoptively transferred recipients were monitored serially for 9 wks by flow cytometric analysis of PBL, and the mice were immunized with DNP-KLH in CFA. 11 days later spleen cells from the immunized mice were analyzed frequencies of VFP+ CD4+ T cells and the remaining cells were injected i.v. into new *Tcrα*-/- mice. Frequencies of blood VFP+ CD4+ T cells from the secondary recipients were monitored serially for 10 wks. The secondary recipients were then immunized with DNP-KLH in CFA, and splenic VFP+ CD4+ T cells were analyzed 11 days later mice. Lower panel, % VFP+ nTFH of total viable lymphocytes; upper panel, % VFP+ CXCR5+ PD1+ full TFH of total viable lymphocytes. **(c-g)** Representative profiles of VFP-expressing CD4+ T cells: (**c**) PBL?? from a negative control *Tcrα*-/- mouse that received no donor cells; (**d**) PBL from a recipient 4 wks after adoptive transfer; (**e**) spleen cells from a recipient 11 days after immunization; (**f**) PBL from a secondary recipient 3.5 wks after adoptive transfer of immunized cells; and (**g**) spleen cells from a secondary recipient 11 days after immunization.

**Figure 8: TREG develop with and potently restrain nTFH.**

**(a)** Flow cytometry of IL21-VFP and FOXP3-GFP dual reporter expression by CD4+ T cells from blood of 4 wk old mice after filtering for spectral overlaps. #of mice tested per group. (**b**) Upper, flow cytometry examples of expression of VFP and co-expression of CXCR5 and PD1 of the VFP+ gated splenic CD4+ T cells from 2 and 4 wk old IL21-VFP *Foxp3-/-* and IL21-VFP WT mice; lower, frequencies of VFP+ CD4+ T cells in cohorts. (**c**) Frequencies from **b** of VFP+ CD4+ T cells further distinguished as nTFH (ICOShi CXCR5-/lo PD1-), pre-TFH (ICOS-/lo CXCR5hi PD1-), full TFH (ICOShi CXCR5hi PD1+). (**d**) Examples and group comparisons of VFP expression by CD4+ single positive thymocytes from 2 wk old IL21-VFP *Foxp3-/-* and IL21-VFP WT mice. \*, p≤0.05; \*\*, p≤0.01; NS, not significantly different. Results are representative of XXX experiments.

**Supplementary Material**

**Supplementary Table 1**

Expression values, ANOVA p-values, and fold changes of the 330 most discriminating genes for N (111), ACT (100), and ACT IL21+ cells (119).

**Supplementary Table 2**

Expression values, ANOVA p-values, and fold changes of selected Tfh-related genes as shown in Figure 5e.

**Supplementary Table 3**

Antibodies used for flow cytometry

**Supplementary Table 4**

Oligonucleotide primers used for RT-qPCR

**Supplemental Figure 1: An IL21-VFP IL21 knock-in allele reliably reports IL21 expression by activated CD4+ T cells and TFH.**

**(a)** IL21-VFP reporter construct design. An IRES-VFP cassette was inserted into non-coding exon 5 of *Il21* along with a *NeoR* selection cassette flanked by *loxP* sites. The *NeoR* cassette was excised by cre-mediated deletion resulting in mice transmitting the bicistronic IL21-IRES-VFP reporter. **(b)** PCR confirmation of germline transmitting mice. **(c)** Cytokine and VFP transcription ofCD4+ T cells fromIL21-VFP mice after stimulation *in vitro.* Splenocytes from IL21-VFP mice were cultured with antibodies to CD3 and CD28. VFP- or VFP+ CD4+ T cells were then FACS purified and RT-qPCR was performed on their RNAs. Data shown are the mean fold changes +/- SD of cells from 3 B6.IL21-VFP mice  Ct normalization to 18S RNA. \*, p≤0.05; \*\*, p≤0.01 by the non-parametric Mann Whitney U test. **(d)** VFP is expressed in immunized mice almost exclusively by CD44hi ICOShi PD1hi TFH.IL21-VFP mice were immunized with DNP-KLH in CFA and their spleen cells as well as gating control cells from an unimmunized B6 mouse were analyzed by flow cytometry 11 days later. Data shown are representative of 3-5 mice in 2??? experiments. (**e**) VFP+ CD4+ T cells from immunized mice show elevated transcriptional expression of a number of T-helper cytokines, including *Il21*. CD4-gated spleen cells from 3-XXX immunized mice described in **d** were FACS-sorted for VFP- or VFPhi expression, and analyzed by RT-qPCR as in **c**. **(f)** VFP expressing cells localize to a splenic germinal center (circled) of a XXX wk old B6.*Sle1* *Yaa* IL21-VFP mouse.

**Supplementary Figure 2**

Integrate with the ImmGen dataset. (a) Top 20 most correlated ImmGen samples with each of N, ACT, and ACT IL21 samples. (b) PCA clustering of N, ACT, ACTIL21 and all ImmGen samples in Naïve T, NKT, T memory categories.

**Supplementary Figure 3**

Heatmap of 811 genes for the hierarchical clustering of CD44+BCL6-CXCR5-, CD44+BCL6loCXCR5+, CD44+ BCL6hi CXCR5+ samples from Liu et al.and N, ACT, ACT IL21+ samples. The gene expression values were corrected for the batch effect as described in the methods.