**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) are critical for efficient humoral immunity and contribute to the development of autoimmune diseases. However, their origins remain obscure. Here, we show that Interleukin 21 (IL21) is the defining cytokine of a novel, activated CD4+ T cell population, referred to as natural (n) TFH, that develops shortly after birth in appreciable frequencies in thymi and peripheries of naïve mice. Molecular profiling, phenotypic characterizations, and capacity to fully mature after immunization establish that nTFH cells are early TFH precursors that precociously express IL21. Thymic development of nTFH requires AIRE while natural FOXP3+ CD4+ T cells play a key role in restraining their pathological, peripheral expansion. We propose that nTFH develop and are sustained by their intrinsic hypersensitivity to basal self-antigenic stimuli that poise them for rapid responses to foreign and autoantigenic challenges. Understanding this developmental pathway may suggest ways to enhance immunization or inhibit autoimmune diseases. (150 words)

**Key words**

T follicular helper cell; Interleukin 21; Regulatory T cells; T cell selection.

**HIGHLIGHTS** (85 character limit per 4 bullet points)

* TFH lineage commitment is established naturally in mice shortly after birth.
* IL21 is functionally expressed at the earliest stages of natural TFH cell development.
* AIRE is required for the positive selection of natural TFH cells.
* A primary function of FoxP3 is to prevent the pathological expansion of natural TFH cells.

**Significance statement**

IL21 was thought to be an end-stage product of TFH cells matured after immunization. We show that IL21 is a functional product a novel TFH population that develops naturally shortly after birth. These “natural” TFH cells provide insights into how CD4 T cell lineage commitments and immune capabilities are pre-determined.

**INTRODUCTION**

Interleukin 21 (IL21) acts through its broadly expressed receptor (IL21R) to support anti-tumor and anti-viral responses, promote autoimmune diseases and drive the development of lymphomas ([Davis et al., 2015](#_ENREF_14); [Ettinger et al., 2008](#_ENREF_17); [Spolski and Leonard, 2014](#_ENREF_52)). While there is evidence that natural killer (NK) T cells, TH17 cells and intestinal CCR9+ CD4 T cells can produce IL21, the major source is CD4 T follicular helper cells (TFH) ([Chtanova et al., 2004](#_ENREF_11); [Nurieva et al., 2008](#_ENREF_43); [Vogelzang et al., 2008](#_ENREF_59)). TFH are a specialized class of CD4 helper T cells that drive the proliferation of antigen-stimulated B cells in germinal centers (GCs) and their differentiation to class-switched IgG secreting plasmablasts, plasma cells and memory B cells ([Crotty, 2014](#_ENREF_13); [Tangye et al., 2013](#_ENREF_55); [Vinuesa et al., 2005](#_ENREF_58)). They are best characterized by surface expression of ICOS, CXCR5, and PD1 and expression of the master transcriptional regulator, BCL6 ([Johnston et al., 2009](#_ENREF_22); [Liu et al., 2012](#_ENREF_31); [Nurieva et al., 2009](#_ENREF_44); [Yu et al., 2009](#_ENREF_65)). Although IL21 is regarded as their signature cytokine, TFH can also express T-helper cytokines, including IL4, IL10, IL17 and IFN, and have the plasticity to further differentiate to memory and conventional helper T cells ([Choi et al., 2013](#_ENREF_10); [Lu et al., 2011](#_ENREF_32); [Luthje et al., 2012](#_ENREF_33)). Homing and positioning within B cell follicles are defining features of fully matured GC TFH. However, IL21-expressing TFH-like cells are also found in the circulation andin extrafollicular lymphoid sites, termed extrafollicular TFH (ETFH), where they help extrafollicular B cells differentiate to antibody-secreting plasmablasts and contribute to autoimmune diseases ([Lee et al., 2011](#_ENREF_25); [Odegard et al., 2008](#_ENREF_45)). Given the importance of TFH and their signature cytokine, IL21, and related cells in health and disease, there is need for deeper understandings of their ontologies.

Much of the current understanding of the ontogeny and behavior of TFH is based on lessons from immunized or infected adults animals or induced differentiation of adult T cells *in vitro*. Even then the differentiation process of TFH is not fully understood, nor can it be characterized by a single event or factor (XXX). It is further complicated by the plasticity of these cells, which can also give rise to memory cells and conventional effector helper T cells ([Luthje et al., 2012](#_ENREF_33)). According to the current understanding of TFH differentiation, a pre-TFH ICOShiCXCR5+ stage in which IL21 is not expressed is established by the costimulatory and cytokine milieu in which naive T cells are first activated by antigens presented on DC. Signaling through upregulation of ICOS, IL6R, CXCR5 and IL12R (in humans) is thought to be vey important for TFH differentiation ([Crotty, 2014](#_ENREF_13)). These extrinsic signals intertwine a transcriptional network governed largely by the upregulation of the master transcriptionalregulator, BCL6, and associated early acting TFH-determining factors, including ASCL2, TCF1 and LEF1 ([Choi et al., 2015](#_ENREF_8); [Liu et al., 2014](#_ENREF_30)), that prevents alternative TH fates primarily by repressing BLIMP1, FOXO1 and FOXP1 ([Johnston et al., 2009](#_ENREF_22); [Stone et al., 2015](#_ENREF_53); [Wang et al., 2014](#_ENREF_60)). TFH programming is then reinforced and modulated by these factors as pre-TFH re-encounter cognate antigens presented by B cells at the T/B border of B cell follicles. They can then divert to E TFH or increase expression of CXCR5 as they enter the follicles, clonally expand and fully mature to CXCR5hiPD1+ GC TFH expressing higher levels of IL21.

It is well established that functionally capable natural (n) FoxP3+ TREG and CD1-dependent NKT cells develop spontaneously through activation by endogenous stimuli early in life ([Chen et al., 1997](#_ENREF_7); [Mendiratta et al., 1997](#_ENREF_39)). The possibility that natural TFH develop in a similar manner has not been explored. To develop insights into this issue, we generated an IL21-Venus Fluorescent Protein (VFP) reporter mouse making 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 a subset of activated CD4 T cells in the spleens, circulation, and thymi of naïve mice. Using a variety of approaches, we show that these IL21-expressing cells arise intrathymically in an AIRE-dependent manner as the earliest activated precursors of mature TFH. These “natural” TFH (nTFH) are clonally diverse but develop and persist even with stringent restriction of their TCRs. We further show that the thymic development of nTFH is strictly dependent on AIRE, while nTFH are a major target of suppression by regulatory CD4 T cells (TREG) as their frequencies and maturation are increased strikingly in FOXP3-deficient mice.

**RESULTS**

**Expression of a 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 (**Figure S1A and S1B**). Heterozygous and homozygous B6.IL21-VFP (IL21-VFP) mice were born in expected Mendelian ratios and developed normally.

To determine if the VFP transgene reliably reports *Il21* transcription, we performed RT-qPCR analyses on FACS-purified splenic VFP+ and VFP- CD4+ T cells from adult mice that had been stimulated for 24 h with antibodies to CD3 and CD28 *in vitro*. *Il21* and *VFP* transcripts were expressed at comparable levels in VFP+ CD4+ T cells but not in VFP- T cells (**Figure S1C**). To determine if expression of the IL21-VFP reporter correlates with secreted IL21, supernatants collected after culture of anti-CD3/CD28 stimulated purified VFP+ and VFP- CD4+ T cells were analyzed by ELISA. IL21 was only found in supernatants of VFP+ cells accompanied by higher levels of IL2 and IL10, while IL17 and IFN were prevalent in supernatants of VFP­- CD4+ T cells (**Figure S1D**). To determine if the IL21-VFP transgene is expressed preferentially by TFH, we immunized IL21-VFP mice with DNP-KLH and profiled their spleen populations 11 days later by FACS. Consistent with the placement of VFP within the TFH lineage, VFP expression was greatly elevated in ICOS+CD44+ CD4+ T cells with the highest expression in CXCR5+ PD1+ TFH, and with few cells expressing the NKT cell marker, NK1.1 (**Figure S1E** and data not shown). To examine the anatomic localization of VFP+ cells, we generated B6.*Sle1.Yaa* VFP mice that spontaneously develop germinal centers (GC) and stained spleens from mice with progressed disease. The results showed that VFP+ cells localized primarily to GC with some cells in the red pulp and T cell zone (**Figure S1F)**. VFP reporter mice immunized with DNP-KLH had similar accumulations of VFP+ cells in GC (data not shown). Taken together, these results demonstrated that the VFP reporter accurately marked cells expressing IL21 at the transcriptional and protein levelsand identified cells expressing IL21 after immunization that are consistent with prototypical mature TFH.

**Precocious IL21 expression is a property of a major population of activated CD4 T cells that arise in young naïve mice.**

Using this experimentally validated IL21-VFP reporter, we sought to investigate the patterns of IL21 expression by CD4 T cells in young unimmunized mice. FACS analysis of spleen cells from naïve IL21-VFP mice revealed appreciable and highly reproducible populations of VFP+ CD4T cells (0.5-3.5%) at 2 to 4 wks of age (**Figure 1A and 1B**). The splenic frequencies increased to ~10% at 17 wks of age and were paralleled by lower frequencies in blood at each time point (**Figure 1B**). Consistent with an activated state, the majority of VFP+ cells expressed high levels of CD44. A fraction, ranging in multiple experiments from 20-40%, also expressed elevated levels of ICOS. Additionally, CXCR5 expression was limited to a lesser population of VFP+ cells (less than 45%)(**Figure 1C** and data not shown). The possibility that NK T cells were the source of early expression of IL21 was excluded because a genetic deficiency in CD1d1 did not impact the frequencies of VFP+ CD4+ T cells from naïve mice (data not shown). These results indicate that IL21 expression is a feature of a novel, naturally activated, CD44+ CD4 T cells regardless of their expression of ICOS and CXCR5.

We next compared the frequencies of splenic CD4 T cells naturally expressing IL21 to those expressing alternative T-helper cytokines ­­­­­­­–IL10, IL17a and IFN– as revealed by IL17-GFP ([Lee et al., 2012](#_ENREF_26)), IL10-GFP ([Madan et al., 2009](#_ENREF_34)) and IFN-YFP ([Reinhardt et al., 2009](#_ENREF_48)) cytokine reporters. Reporter expression was mainly restricted to CD4 T cells showing an activated CD44+ phenotype. The frequencies of IL21-VFP+ cells from 4 and 8 wk old naïve mice were considerably higher than the frequencies of cells expressing alternative cytokines (**Figure 1D**). Given the fact that natural (n) CD4 TREG are known to develop in appreciable frequencies in naïve mice ([Chatenoud, 2011](#_ENREF_6)), we also compared the frequencies of VFP+ and FoxP3-GFP-expressing CD4 T cells. The frequencies of the two populations were surprisingly similar (**Figure 1D**). The results overall showed that expression of IL21 is a property of a major fraction ofthe earliest CD4 T cells to be activated spontaneously in naïve mice, rivaled only by FoxP3+ nTREG.

**Naturally occurring IL21+ CD4T cells are not dependent on CXCR5 or B cells but are influenced by IL6, IL10, IL21 and IFN1.**

Cognate engagements with B cells and upregulation of CXCR5 are thought to be required for TFH precursors to mature to the point that IL21 is expressed under most circumstances ([Barnett et al., 2014](#_ENREF_2); [Goenka et al., 2011](#_ENREF_18)). To directly address these requirements for naturally occurring IL21+ CD4 T cells, we determined the frequencies of splenic IL21-expressing CD4T cells in *Ighm-/-*, *Cxcr5-/-*and wild-type (WT) IL21-VFP reporter mice (**Figure 2A and 2B**). The results showed that the frequencies of VFP+ cells were not impacted by deficiencies in B cells or CXCR5.

We then tested the cytokine requirements for this natural VFP+ CD4 T cell population by evaluating possible requirements for cytokines, *Il6*, *Il21r, Il10*, *Il12b*, and *Ifn1,* previously reported to control the development of mature TFH ([Eto et al., 2011](#_ENREF_16); [Nakayamada et al., 2014](#_ENREF_42); [Ray et al., 2014](#_ENREF_47); [Tangye et al., 2013](#_ENREF_55)). After crossing IL21-VFP reporter to mice homozygous for knockout alleles of *Il6*, *Il21r, Il10*, *Il12b*, and *Ifnar1*, we evaluated frequencies for VFP+ CD4 T cells. The frequencies of VFP+ CD4 T cells arising spontaneously (**Figure 2C**) or after immunization (data not shown) were comparable in mice competent or deficient in expression of IL12b (IL12p40). In contrast, naïve reporter mice deficient in IL10 had significantly increased frequencies VFP+ cells, and naïve IL21-VFP mice deficient in expression of IFNAR1 had lower frequencies of IL21+ CD4 T cells (**Figure 2C**). Finally, mice lacking either IL6 or IL21R had significantly lower frequencies of IL21+ CD4 T cellsthan WT controls while mice doubly deficient in IL6 and IL21R had the most substantial reduction (**Figure 2D**). These results showed the TFH-directing cytokines IL6, IL21, and IFN1 promote and IL10 retards expansion of this IL21+ population. Taken together, the results suggest that IL21 expression identifies a novel naturally arising TFH-like population that is supported by tonic IL6, IL21 and IFN1 signaling and without need for CXCR5 or B cells.

**Comparative RNA-seq profiling of natural CD4 T cell populations.**

To gain insights into the molecular processes that distinguish this IL21-expressing TFH-like population from other naturally occurring CD4 T cell subpopulations, we performed paired-end RNA-seq on FACS-purified splenic CD4 T cells from 4 wk old IL21-VFP reporter mice based on the following criteria: naïve VFP- ICOSlo/- (N); activated VFP- ICOShi cells (ACT); and VFP+ ICOShi cells (IL21-ACT) (**Figure S2A**). Confirming the fidelity of the IL21 reporter and the sorting strategy, *Il21* and *VFP* were expressed coordinately and almost exclusively by the IL21-ACTpopulation, whereas *Icos* and *Cd44* transcripts were elevated to equivalent levels in the ACT and IL21-ACT populations but were minimal for N cells (**Figure S2B**).

To address in an unbiased manner how the overall transcriptional patterns of these natural CD4 T cell populations compared to those of T helper cell subpopulations generated in response to overt antigenic challenge, we extracted published gene expression data of polyclonal mature TFH, GC TFH, TH1, and Naïve T cells isolated from mice 8 days after acute infection with LCMV and performed hierarchical clustering to compare their gene expression patterns to our N, ACT, and IL21-ACT cells (Yusuf et al., 2010) (**Figure 3A top and S3)**. We similarly analyzed recent RNAseq data derived from ‘early’ IL2R Blimp1- TFH and IL2R+ Blimp1+ TH1 cells isolated from adoptively transferred naïve SMARTA TCR transgenic CD4+ T cells 3 days after acute LCMV infection ([Choi et al., 2015](#_ENREF_8)) (**Figure 3A bottom)**. IL21-ACT cells correlated highly to all subcategories of TFH generated following infection, while ACT cells correlated highly to TH1.

We then sought to identify genes whose expression patterns most reliably discriminated among N, ACT and IL21-ACT cells. Technical noise inherent to low read counts was minimized by only including genes having >20 transcripts per million (TPM) in at least one population. Of the 6996 genes meeting this criteria, 471 genes showed significantly higher expression level in one of the three cell populations. These included with 148 for N; 165 for ACT; and 158 for IL21-ACT (**Figure 3B, Dataset S1**). Functional gene enrichment analysis of these signature clades using David (https://david.ncifcrf.gov/) showed that the IL21-ACT population was most enriched for genes associated with T cell development, differentiation, activation, and TCR signaling. In contrast, gene enrichment of ACT cells, which included all other types of naturally activated CD4 T cells, were more restricted to generic lymphocyte and cytokine signaling pathways (**Figure 3B, Dataset S1).** These results suggested that IL21-ACT cells stand out in the extent to which they are engaged in T cell activation and signaling processes.

As presented in a 3-way scatterplot (**Figure 3C**) and gene-selective transcriptional comparisons (**Figure 3D**), IL21-ACT showed increased expression compared with N and ACT cells of several prototypical TFH transcription factors, including *Bcl6, Maf, Fosb, E2f2,* and *Tox,* while downregulating the BLIMP1-encoding gene, *Pdrm1.* The increased expression of prototypical TFH markers, including *Sostdc1, Btla*, *Cd200*, *Slamf6*, *Gpm6b,* and *Cxcr5,* provided further support for TFH relatedness. IL21-ACT cells uniquely expressed *Il21* and were quite restricted in expression of other “effector” cytokine genes, with only *Ifng* expressed at appreciable levels that were reduced compared with the ACT population.

Furthermore, and consistent with a heightened activation state, IL21-ACT cells were distinguished from N and ACT by the upregulation of TCR co-signaling genes, *Cd4*, *Cd28* and *Lag3*, and cell cycling genes (**Figure 3D**). Additional genes with selectively increased expression in IL21-ACT cells are included in **Dataset S1.**

In contrast, ACT cells showed expression profiles inclusive of those for TH1, TH2, NKT and TREG cells (**Figure 3E**), while transcriptional support for previously described nTH17 cells ([Kim et al., 2011](#_ENREF_23); [Marks et al., 2009](#_ENREF_36)) was not apparent (**Supplementary Dataset 1**). The overall results strongly support the existence of an actively engaged, natural TFH population, hereafter referred to as nTFH, that emerges only weeks after birth in naïve mice already capacitated with much of the central transcriptional machinery, cell surface receptors, and IL21 required for effector functions.

**nTFH have a diverse TCR repertoire but stringent restriction does not alter their generation.**

The fact that nTFH emerged from the naïve T cell pool at surprisingly high frequencies could be explained by TCR usage bias akin to that found for NKT cells ([Bendelac et al., 1994](#_ENREF_3); [Hsieh et al., 2012](#_ENREF_20); [Takahama et al., 1991](#_ENREF_54); [Vicari and Zlotnik, 1996](#_ENREF_57)) or TREG or, alternatively, by clonally diverse TCRs that are susceptible to triggering by low affinity self-antigens ([Moran and Hogquist, 2012](#_ENREF_40)). To discriminate between these possibilities, we mined the RNAseq data described above to compare TCR usage of ACT IL21 (nTFH) to naive (N) and activated (ACT) CD4 T cell populations. The *Trav* and *Trbv* repertoires were quite similar for naïve and nTFH, while the repertoire of ACT cells was more biased, including substantially increased usages of *Trav11* and *Trav11b* (**Figure 4A**). Thus, despite their precocious appearance and activation state, nTFH have a TCR repertoire not readily distinguished from naïve CD4 T cells. As this lack of biased TCR usage was more consistent with TCR activation through promiscuous, low affinity TCR engagements, we reasoned that limitation of the CD4 T cell repertoire to a single TCR might still permit nTFH development. To test this possibility, we analyzed TCRα-deficient IL21-VFP mice expressing the ovalbumin-specific OT2 transgene. FACS comparisons of these cohorts at 4 and 14 wk of age showed that while the frequencies of ICOS+ T cells were markedly lower in 14 wk old *Tcr*α-/- OT2 mice, appreciable populations of nTFH were still present at both time points (**Figure 4B**). The frequencies of nTFH were also similar in OT2 TCR transgenic and TCR-intact IL21-VFP mice (data not shown). Therefore, stringent restriction of their normally broad TCR repertoire did not compromise the generation of nTFH.

**Adoptively transferred nTFH persist and can differentiate into mature TFH after immunization**. To determine if nTFH are short or long-lived, we sort-purified nTFH and adoptively transferred them to allotype-marked Tcrα-/- mice. VFP+ cells were readily detected by FACS in PBL of recipient mice 2 wks after transfer and persisted through wk 6. (**Figure 5A**). Phenotypic analyses of VFP+ cells at each of these time points showed that the majority of CD4 T cells retained their CD44+CXCR5+PD1- nTFH phenotype with some exhibiting the more mature CD44+CXCR5+PD1- pre-TFH phenotype. Essentially no mature CXCR5+PD1+ TFH were found (**Figure 5B**). Taken together, these data indicated that adoptively transferred nTFH can persist for at least 6 wk with most maintaining their original identity.

We then examined how the transferred nTFH would respond to antigenic challenge by immunizing recipient mice with DNP-KLH. FACS analyses of spleen cells performed 10 days later showed that the frequency of VFP+ cells in immunized recipients was twice that of non-immunized mice (**Figure 5C**). Phenotypic analyses of VFP+ cells from immunized and non-immunized mice showed that antigenic challenge had little or no effect on the frequencies of nTFH or pre-TFH while the frequencies of full CXCR5+PD1+ TFH were significantly increased over the frequencies of these cells in unimmunized mice (**Figure 5D**). These results showed that nTFH cells are responsive to foreign antigen stimulation causing them to readily differentiate into fully mature TFH.

**nTFH develop in the neonatal thymus through IL6, IL21 and AIRE.**

Classically, T cells selected positively and negatively by self-peptide (p)/MHC ligands in the thymus are released to the periphery in a naïve state. However, it is increasingly appreciated that a fraction of them, including nTREG, are activated through this selection process in thymi ([Chatenoud, 2011](#_ENREF_6); [Dons et al., 2012](#_ENREF_15)). We performed FACS analyses of thymocytes and spleen cells from WT and naïve IL21-VFP mice at 2 d, 2 wk and 4 wk of age. Very few nTFH were present in the thymi or spleens of 2 d old mice (**Figure 6A and B**) but were increasingly detected at later ages (**Figure 6A and B**). For comparison, we also examined thymic frequencies of CD4 T cells expressing , IL17, IFNγ, IL21 and FoxP3 using the appropriate reporter mice at 4-6 wks of age (**Figure 6C**). Only IL21+ and Foxp3+ CD4 T cells were found in significant frequencies, with FoxP3+ CD4 T cells clearly dominating (**Figure 6C**). To determine if CD4+ thymocytes expressing IL21 share cell surface markers with peripheral nTFH, we analyzed the patterns of expression of CD44, ICOS, CD5 and CD3e in VFP+ CD4 T cells from both sites. However, and in contrast to peripheral nTFH, high ICOS expression was a feature of the great majority of thymic nTFH, suggesting that ICOS signaling is heightened during thymic nTFH activation (**Figure 6D).**

Having previously shown that IL6 and IL21 cooperatively support peripheral nTFH (**Figure 2D**), we asked if the cytokines contribute to the development of thymic nTFH. Comparisons of frequencies of nTFH in IL21-VFP mice that were WT or deficient in expression of IL6, IL21R or both, was consistent with the additive contributions of IL6 and IL21 signaling in the earliest stages of nTFH development in the thymus (**Figure 6E**).

The transcriptional regulator, AIRE, acts in the thymus in a gene dosage-dependent manner to help safeguard central tolerance. AIRE forces expression of peripheral tissue antigens (PTA) in medullary thymic epithelial cells (TEC) during stages of T cell development in which selection can occur ([Liston et al., 2003](#_ENREF_29); [Malchow et al., 2013](#_ENREF_35); [Mathis and Benoist, 2009](#_ENREF_37)). Thus, newly formed conventional T cells with strong reactivity for PTA can be deleted before they escape to the periphery and cause tissue specific autoimmunity (XXX). However, effects of AIRE on regulatory T cells are more nuanced and more consistent with positive selection ([Liston et al., 2003](#_ENREF_29); [Malchow et al., 2013](#_ENREF_35); [Mathis and Benoist, 2009](#_ENREF_37)). To examine the effects of AIRE on development of thymic nTFH, we used FACS to determine the frequencies of nTFH among thymocytes from 6-8 wk old IL21-VFP mice in which the functional copies of AIRE were varied. Frequencies of nTFH found in WT mice were substantially reduced in *Aire+/-* mice and only slightly further reduced in *Aire-/-* mice, indicating that the AIRE supports thymic nTFH development in a highly dosage sensitive manner (**Figure 6F**). These results suggest a critical role for AIRE in the positive selection of thymic nTFH. This finding, combined with the fact that thymic nTFH are supported by IL6/IL21 signals and are evident soon after birth, strongly suggest that TFH programming is a proximal manifestation of T cell selection in the thymus.

**AIRE and FoxP3 restrict expansion and maturation of peripheral nTFH.**

Having shown that AIRE is required for thymic nTFH development, we sought to understand how AIRE influences nTFH at peripheral sites. Splenic frequencies of VFP+ CD4 T cells from *Aire-/-, Aire-/+*and WT showed that AIRE suppresses the peripheral nTFH in a highly dosage sensitive manner and inverse to that found in the thymus (**Figure 7A**).

Given that nTREG are the only other naturally activated T cell population with frequencies comparable to nTFH (**Figure 1D and 6C**), we examined if nTREG cells control nTFH. FoxP3-deficient (scurfy) mice lack TREG and consequentially develop a profound autoimmune disease by 3-4 wk of age([Ramsdell and Ziegler, 2014](#_ENREF_46)). We generated *Foxp3*-/- IL21-VFP reporter mice and analyzed their male progeny at 2 wk of age before disease onset and at 4 wk of age when they display overt signs of disease. Substantial increases in the frequencies of splenic VFP+ cells at all stages of TFH differentiation were found, but with the most striking increase being in nTFH (**Figure 7B and C**). However, a deficiency in FoxP3 had no effect on thymic nTFH frequencies (**Figure 7D).** Thus, newly developing thymic nTFH were unaffected by an abundance of nTREG cells in that organ, but in the periphery, nTREG target nTFH and in doing so potently restrain their expansion and further differentiation.

**DISCUSSION**

Through use of novel IL21-VFP reporter mice, we found, surprisingly, that IL21 expression is a property of many of the earliest activated thymic and peripheral CD4 T cells that develop in naïve mice. We also show that these naturally occurring T cells are an early TFH that are controlled by cytokines, including their secretion of IL21, that typifies late stages of TFH differentiation. These findings show that IL21 is an integral to the earliest steps of TFH development and function.

Current models based on immunization/infection protocols position IL21 as a cytokine expressed by highly differentiated mature and GC TFH ([Crotty, 2014](#_ENREF_13); [Spolski and Leonard, 2014](#_ENREF_52)). TFH programming is initiated shortly after encounter with antigen-presenting DC. This IL21-negative pre-TFH stage is initiated by IL6/IL6R, ICOSL/ICOSand TCR/MHCII signaling and is characterized by the upregulation of ICOS, IL6R and CXCR5 on the cell surface ([Akiba et al., 2005](#_ENREF_1); [Choi et al., 2011](#_ENREF_9); [Crotty, 2014](#_ENREF_13)). The fact that the development of nTFH is unimpaired by the absence of B cells or CXCR5 is consistent with their activation through antigens presented on DC. However, while the cell surface expression of ICOS and CXCR5 is thought to a property of pre-TFH and required for TFH to achieve a sufficient stage of maturity to express IL21 ([Akiba et al., 2005](#_ENREF_1); [Crotty, 2014](#_ENREF_13)), we found that activation, marked by enhanced expression of CD44, was sufficient for IL21 expression by nTFH. These findings strongly suggest that nTFH are activated by DC but can be distinguished from previously defined pre-TFH by their expression of IL21 and failure to uniformly express ICOS or CXCR5. Furthermore, our results clearly show that nTFH persist after adoptive transfer and potently respond and differentiate after immunization into fully mature CXCR5+ PD1+ IL21-expressing TFH. While the IL21 expression phenotype was durable in the majority of transferred nTFH, some lost IL21 expression, possibly by conversion to effector memory cells or to alternative polarization states ([Luthje et al., 2012](#_ENREF_33)). We therefore conclude that nTFH comprise a stable pool of naturally activated T cells that are homeostatically maintained for a considerable period with the capacity to fully differentiate in response to foreign antigens.

Tonic signaling is a term used to describe signaling pathways that are operative consitutively in the steady state. It is well established that IL6, IL10, IL21 and IFN1 have substantial effect on T cell responses, including TFH, after induction by immunization/infection ([Cai et al., 2012](#_ENREF_5); [Eto et al., 2011](#_ENREF_16); [Nakayamada et al., 2014](#_ENREF_42); [Ray et al., 2014](#_ENREF_47)). Accordingly, IL6 has been shown to supply important signals for all stages of TFH induction, including early TFH development and their maturation to full and GC TFH ([Choi et al., 2011](#_ENREF_9); [Eto et al., 2011](#_ENREF_16)). IL21, in contrast, has only been considered to be a late stage growth and differentiation factor that is expressed at increasing levels as TFH encounter follicular and GC B cells ([Barnett et al., 2014](#_ENREF_2); [Crotty, 2011](#_ENREF_12)). Repressive effects of IL10 signaling on TFH have also been documented ([Cai et al., 2012](#_ENREF_5); [Crotty, 2014](#_ENREF_13)), and while there is evidence that IFN1 signals can be TFH-promoting, a recent study has shown that IFN1 signaling promotes TH1 responses and features of TFH, but does not include IL21 expression ([Nakayamada et al., 2011](#_ENREF_41); [Nakayamada et al., 2014](#_ENREF_42); [Ray et al., 2014](#_ENREF_47)). Our results show that all of these cytokines are biologically active and functionally consequential at an early age in naïve mice, with IL6, IL21 and IFN1 supporting and IL10 repressing nTFH. We conclude that these tonic cytokine signals play substantial roles in natural TFH development.

Extrinsic signals, including cytokines, direct a transcriptional network by which naïve T cells differentiate into TFH as opposed to alternative T helper lineages. While there is a large body of information describing the molecular patterns that discriminate TFH from other T helper lineages after deliberate stimulation, our RNAseq profiling shed light into molecular processes through which naïve CD4 T cell lineages are established naturally. These studies indicate that lineages of TFH, TH1 and TH2 cells are already established in young naïve mice, but with TFH being a preferred choice. Other studies have documented natural populations of TH17 based on cellular and molecular criteria, including upregulated expression of *Rorc* and the induction of TH17 cytokines including *Il17* after stimulation ([Kim et al., 2011](#_ENREF_23); [Marks et al., 2009](#_ENREF_36)). Our RNAseq profiling of CD4 T cells from young naïve mice did not detect an appreciable TH17 signature, suggesting that nTH17 cells are underrepresented, possibly because they have not fully matured.

An alliance between nTFH and the TFH lineage was even more clearly established by whole transcriptome comparisons of our RNAseq data with datasets that include TFH induced after viral infection. Our 3-way comparison of naïve, activated, and activated IL21 CD4 T cells further afforded the unique opportunity to identify genes that best discriminated nTFH from alternative CD4 T cell populations. The nTFH signature included many prototypical markers of conventional TFH. Transcriptional factors commonly associated with TFH, including the master TFH regulatory hub, *Bcl6*, as well as *Maf*, *E2f2, Fosb, Nfatc1, Pou2af1* and *Tox,* were also part of the signature. However, rather than all or none, increases of many were only incremental in nTFH (*e.g*, 2-fold for *Bcl6*). Thus, at least at the transcriptional level, combinations of small differences that actuate the TFH program may best explain TFH commitment. Our results also provide clues as to how the transcription factors, TCF1, LEF1, FOXO1 and FOXP1 control early TFH development. *Tcf1* and *Lef1* have been shown to be substantially upregulated in TFH and act upstream of BCL6 by inducing expression of IL6R, IL6ST and ICOS to promote early TFH development ([Choi et al., 2015](#_ENREF_8)). However, our 3-way comparisons that included naïve CD4 T cells of neonatal mice showed that *Tcf1* and *Lef1* were most highly expressed by naïve CD4 T cells. FOXO1 and FOXP1have beenpreviously reported to inhibit early TFH development ([Stone et al., 2015](#_ENREF_53); [Wang et al., 2014](#_ENREF_60); [Weber et al., 2015](#_ENREF_62)). We found that *Foxo1* and *Foxp1* are more highly expressed in nTFH than ACT cells. These results are not consistent with their selective inhibitory effects on nTFH development, but are likely explained by post-translational differences between cell lineages.

Our identification and characterization of nTFH provides new clues into the roles of self-antigens in CD4 T cell selection and function. Self-peptide (p)/MHC ligands expressed and presented by thymic TEC and DC instruct and qualify the T cell repertoire through iterative TCR engagements ([Hogquist and Jameson, 2014](#_ENREF_19); [Moran and Hogquist, 2012](#_ENREF_40); [Weinreich and Hogquist, 2008](#_ENREF_63)). Classically, negative selection of potentially autoreactive T cell clones with high avidity for self-p/MHC is accomplished mainly by encounter of self-p/MHC ligands presented by cortical TEC and DC, whereas clones with weak avidity for self are selected and homeostatically maintained in the naïve state by basal self-p/MHC signaling (XXX). A class distinction between conventional naive T cells and so-called natural T helper populations (inclusive of NKT, TH17, nTFH cells) is that natural T cells develop and persist in an activated state. Our results, in contrast, are consistent with nTFH having a diverse TCR repertoire, but that limitation of the repertoire to near singularity by the OT2 TCR transgene does not compromise their development. We interpret these findings to suggest that nTFH are governed by the same basal self-p/MHC signals that select and maintain conventional naïve CD4 T cells but are activated because they are intrinsically hypersensitive to these signals. Our findings that nTFH are present in thymi of mice shortly after birth under control of AIRE (discussed below) indicates that these signals are closely adjoined with those required for T cell selection in the thymus.

Participation of the transcriptional regulator AIRE in establishing central tolerance is well documented but incompletely understood ([Liston et al., 2003](#_ENREF_29); [Mathis and Benoist, 2009](#_ENREF_37)). Its mode for eliminating newly produced conventional T cells reactive to PTA is by forcing ectopic expression of peripheral tissue antigens (PTA) in medullary TEC, then PTA re-presented by medullary DC cause most efficient negative selection ([Liston et al., 2003](#_ENREF_29); [Mathis and Benoist, 2009](#_ENREF_37)). Our findings that AIRE limits the peripheral expansion of nTFH is consistent the prototypical role of AIRE in limiting autoimmunity. However, we also found that AIRE has the opposite effect in the thymus where it is required for the development of nTFH. This almost complete dependence on AIRE supports its critical role in the positive selection of nTFH. AIRE induces promiscuous gene expression in a mosaic manner with any given TEC expresses only 1-3% of PTAs (XXX). The extreme degree to which the gene dosage of AIRE controls thymic nTFH may suggest that mosaic expression can cause T cells that are ultimately capable of effector functions to be negatively selected by PTA on TEC, but impairs positive selection of nTFH and nTREG.

These behaviors show parallels with findings for FoxP3+ TREG ([Malchow et al., 2013](#_ENREF_35)) Both nTFH and TREG develop and are activated by weak basal stimuli that result in appreciable thymic and peripheral frequencies starting in early post-natal life that exceed other natural T cell populations. In agreement with recent reports ([Iwamoto et al., 2014](#_ENREF_21); [Wing et al., 2014](#_ENREF_64)), we have shown that the absence of FoxP3 causes a substantial increased in IL21-expressing TFH in the periphery. Our finding that the most striking increases in the absence of TREG were in the frequencies of nTFH rather than more differentiated TFH suggests that nTFH are a primary target of TREG. A key function of TREG may thus be to constrain nTFH that arise in parallel through similar thymic selection processes but with opposing functions.

Our identification nTFH may shed light on the origins of more mature forms 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, resulting in full differentiation to TFH in GCs. In contrast, self-reactivity, rather than high affinity driven clonal selection to foreign antigens, is thought to be a general property of autoimmune disease-promoting CD4 T cells ([Koehli et al., 2014](#_ENREF_24)). 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 humans and BXSB.*Yaa* disease in mice, in which IL21-producing TFH and related TFH-like cells are important pathogenic drivers ([Bubier et al., 2009](#_ENREF_4); [McPhee et al., 2013](#_ENREF_38); [Sawalha et al., 2008](#_ENREF_49); [Schmitt et al., 2013](#_ENREF_50); [Spolski and Leonard, 2008](#_ENREF_51), [2014](#_ENREF_52); [Tangye et al., 2013](#_ENREF_55); [Terrier et al., 2012](#_ENREF_56); [Vinuesa et al., 2005](#_ENREF_58); [Wang et al., 2011](#_ENREF_61)).

**METHODS**

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

B6.Cg-*Il21tm1.1Hm*/HmDcr mice were generated by Ozgene Pty. Ltd. (Bentley WA), under the direction of HCM, 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, inserted downstream of IRES-Venus, was flanked by loxP sites so that it could be deleted by Cre recombinase (extend details in Supplemental Experimental Procedure).

***Mice***

Mice were maintained in a specific pathogen-free mouse colony

at The Jackson Laboratory and are on a C57BL6/J background. All mice were bred to B6.Cg-*Il21tm1.1Hm*/HmDcr and used in the experiments (see Supplemental Experimental Procedure for further mouse information). All experiments were performed under Protocol 01022 approved by the Institutional Animal Care and Use Committee of the Jackson Laboratory.

***Immunization and In vitro stimulation***

Mice were immunized intraperitoneally with 200µL of trinitrophenol-conjugated keyhole limpet hemocyanin (TNP-KLH) emulsified in complete Freund’s adjuvant and analyzed 7-11 days after immunization. For in vitro stimulation, total splenocytes were isolated from naïve B6.IL21-VFP mice, red blood cells were lysed and cells were cultured in 6 well plates with 5 ug/ml of anti-CD3 and 1 ug/ml of anti-CD28 for 24 hours in 5% FBS-supplemented DMEM. Cells were then stained and sorted for being CD4+VFP- and CD4+VFP+.

**ELISA**

Total splenocytes isolated from naïve B6.IL21-VFP mice were culture for 36 hours with with 5 ug/ml of anti-CD3 and 1 ug/ml of anti-CD28 in 5% FBS-supplemented DMEM. Cells were then sorted for being CD4+VFP- and CD4+VFP+. VFP- and VFP+ cells were then cultured independently in 5% FBS-supplemented DMEM. Supernatant was collected after 24hrs and used for ELISA for IL21, IL17, IL2, IL10 and IFNy. See supplemental experimental procedures for expanded protocol

***FACS***

Analytical and preparative FACS was performed by established procedures after FMO gating([McPhee et al., 2013](#_ENREF_38)). Antibodies are listed in **Supplementary Table 1**. Samples were run on a four-laser/13- color BD LSRII analytical cytometer (BD Biosciences) and analyzed with FlowJo software version 8 or 9 (Tree Star). Cell sorting was performed using a FACSAria (BD Bioscience).

***Adoptive Transfer***

Splenocytes from 4–6 wk old IL21-VFP mice were pooled and CD4 T cells were isolated via negative cell depletion (see supplemental experimental procedure). Cells were then sorted for populations N, ACT and ACT IL21 based on gating set by CXCR5 and PD1 FMO controls. Cells were then injected into recipient B6.CD45.1 *Tcrα-/-* mice via the tail vein.

***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. SuperScript III Platinum Two-Step qRT-PCR Kit with Syber Green (Invitrogen) was used for RT-PCR (primers listed in **Supplementary Table 2**) according to manufacturer’s protocol. Samples were run in triplicate on the ViiA Real Time PCR system (Life Technologies) and mean expression was normalized to 18sRNA using the Ct method.

***RNAseq – Can anything go into supplemental methods?***

Total RNA from FACS-purified VFP-ICOS- (N), VFP-ICOS+(ACT), and VFP+ICOS+(ACT IL21) CD4+ cell pools were extracted with Qiagen RNeasy Mini Kits (Qiagen, Hilden, Germany) in 2 biological 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, and aligned to the GRCm38.73 assembly transcriptome with Bowtie. Transcript expression levels were estimated in transcripts per million (TPM) using RSEM([Li and Dewey, 2011](#_ENREF_28)). Differential expression analysis across the three conditions (*i.e.* sorted populations) was conducted with EBSeq([Leng et al., 2013](#_ENREF_27)). Signature genes for the three sample populations were defined by three criteria: maximum mean expression in the signature population; mean TPM greater than 20 in the signature population; and differential expression in the signature population relative to the other two populations (*P* < 0.05, pairwise t-test). 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 combined with the mouse transcriptome file.

***Comparison of RNAseq profiles with antigen-induced TFH* microarray datasets XULONG & GREG please update.**

Gene expression profiles of the 8-day Naïve CD 4 T cells, TH1 cells, TFH cells, GC TFH cells by Yusuf et al. and the 3-day TFH, TH1 cells by Choi et al. were downloaded from gene expression omnibus (GSE21380 and GSE67334). The 8-day and 3-day samples were measured by microarray and RNA-seq, respectively. To compare the gene expression profiles of these cell populations with our N, ACT, and IL21-ACT cells: firstly, gene expression levels by microarray and RNA-seq were transformed into log2 intensity and log2 TPM scales; secondly, platform and study differences were corrected by subtracting the first principle component of the combined samples for each transcript; and thirdly hierarchical clustering was performed on the combined samples based 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. Histology images were viewed with an Olympus BX41 microscope (10-100X objectives) and photographed with an Olympus DP71 camera. DP controller software (Version 3.3.1.292) was used for image acquisition.

***Statistical analyses***

Data was analyzed by non-parametric statistics with GraphPad Prism v6. Details in supplemental experimental procedures***.***

**Author Contributions**

E.B.A., H.C.M., G.W.C. and D.C.R. wrote the paper. E.B.A., H.C.M., G.W.C. and D.C.R. designed experiments. E.B.A, X.W, G.W.C, T.J.S., G.P., G.J.C., S.L.K., and S.J.performed experiments. H.W. provided advice, discussion and data analysis assistance critical to the work.

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