**Precocious Interleukin 21 expression in CD4+ T cells of naïve mice defines a novel stage of T-follicular helper cell development**

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**SUMMARY**

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, spleen and blood of naïve mice. Molecular profiling, phenotypic characterizations, and capacity to fully mature after immunization establish nTFH cells as early TFH 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 readying them for rapid responses to foreign and autoantigenic challenges. Understanding this developmental pathway may suggest ways to enhance immunization or inhibit autoimmune diseases.

**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_12); [Ettinger et al., 2008](#_ENREF_15); [Jain et al., 2015](#_ENREF_19); [Spolski and Leonard, 2014](#_ENREF_44)). 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_9); [Nurieva et al., 2008](#_ENREF_40); [Vogelzang et al., 2008](#_ENREF_50)). 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_11); [Tangye et al., 2013](#_ENREF_47); [Vinuesa et al., 2005](#_ENREF_49)). 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_21); [Liu et al., 2012](#_ENREF_31); [Nurieva et al., 2009](#_ENREF_41); [Yu et al., 2009](#_ENREF_57)). 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 (TH) cells ([Choi et al., 2013](#_ENREF_8); [Lu et al., 2011](#_ENREF_33); [Luthje et al., 2012](#_ENREF_34)). Homing and positioning within GCs are defining features of fully matured GC TFH. However, IL21-expressing TFH-like cells, termed extrafollicular TFH (ETFH), are also found in the circulation andin extrafollicular lymphoid sites, where they help extrafollicular B cells differentiate into antibody-secreting plasmablasts and contribute to autoimmune diseases ([Lee et al., 2011](#_ENREF_25); [Odegard et al., 2008](#_ENREF_42)).

Given the importance of TFH and their signature cytokine, IL21, in health and disease, there is a need for deeper understanding of the ontogeny of TFH. Current understandings of TFH development are based largely on studies of TFH induced in cells of adult mice by immunization, infection, or stimulation *in vitro.* Accordingly, a pre-TFH ICOShiCXCR5+ stage in which IL21 is not expressed is established by the cytokine and costimulatory milieu in which naive T cells are first activated by antigens presented on DC ([Barnett et al., 2014](#_ENREF_2); [Choi et al., 2011](#_ENREF_7)). IL6 and IL12b (in humans) and upregulation of ICOS, IL6R, and CXCR5 are thought to be important for early TFH differentiation ([Akiba et al., 2005](#_ENREF_1); [Choi et al., 2011](#_ENREF_7); [Crotty, 2014](#_ENREF_11)). These extrinsic signals activate a transcriptional network governed largely by, BCL6, and associated early acting TFH-determining factors, including ASCL2, TCF1 and LEF1 ([Choi et al., 2015](#_ENREF_6); [Liu et al., 2014](#_ENREF_30); [Xu et al., 2015](#_ENREF_55)), and repress alternative TH fates by limiting BLIMP1, FOXO1 and FOXP1 ([Johnston et al., 2009](#_ENREF_21); [Stone et al., 2015](#_ENREF_45); [Wang et al., 2014](#_ENREF_52)). TFH programming is then reinforced 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 ETFH or increase expression of CXCR5 as they enter B cell follicles, clonally expand and fully mature to CXCR5hiPD1+ GC TFH expressing increasing levels of IL21 ([Lee et al., 2011](#_ENREF_25); [Odegard et al., 2008](#_ENREF_42)).

Here we investigate the natural ontogeny of nTFH through use of novel IL21-Venus Fluorescent Protein (VFP) reporter mouse. We show that IL21 is expressed within weeks of birth by a major subset of activated CD4 T cells in the spleens, circulation, and thymi of naïve mice. These IL21-expressing cells arise intrathymically in an AIRE-dependent manner as the earliest activated predecessors of mature TFH. These “natural” TFH (nTFH) are clonally diverse but develop even with stringent restriction of their TCRs. We further show that the thymic development of nTFH is strictly dependent on AIRE, while peripheral nTFH are a major target of suppression by TREG as their frequencies and maturation are increased strikingly in FoxP3-deficient mice. These studies link IL21 to early TFH development and function and show that the TFH program is engrained naturally.

**RESULTS**

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

The IL21-VFP knock-in reporter allele was produced by introducing a construct containing an internal ribosomal entry site (IRES)-VFP LoxP-flanked *NeoR* selection cassette into non-coding exon 5 of the mouse *Il21* locus by targeted transgenesis in C57BL6/N (B6)-derived embryonic stem (ES) cells. Founder mice transmitting a properly targeted ES cell clone were crossed tocre mice to genetically excise the *NeoR* selection cassette (**Figure S1A and S1B**). Heterozygous and homozygous B6.IL21-VFP (IL21-VFP) mice lacking the *NeoR* selection cassette 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 *in vitro* with antibodies to CD3 and CD28. *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 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 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 by FACS 11 days later. 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; few cells expressed 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 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). Thus, the VFP reporter accurately marked cells expressing IL21 at the transcriptional and protein levelsand identified cells expressing IL21 after immunization that are phenotypically prototypical mature TFH.

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

Using the validated IL21-VFP reporter, we investigated the patterns of IL21 expression by CD4 T cells in young unimmunized mice. FACS analysis of spleen cells from 2-4 wk old naïve IL21-VFP mice revealed highly reproducible populations of VFP+ CD4T cells (0.5-3.5%) (**Figure 1A and 1B**). The splenic frequencies increased to ~10% at 17 wks and were paralleled by lower percentages in blood at each time point (**Figure 1B**). Consistent with an activated state, more than 80% of VFP+ cells expressed high levels of CD44 with elevated levels of ICOS detected on 20-40% of cells. Additionally, CXCR5 expression was limited to less than 35% of VFP+ cells (**Figure 1C** and data not shown). NK T cells were not the source of IL21 because a genetic deficiency in *Cd1d1* did not impact the frequencies of VFP+ CD4+ T cells naïve mice (data not shown). These results indicate that expression of IL21 is a feature of 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 TH cytokines ­­­­­­­–IL10, IL17a and IFN– as revealed by IL17-GFP, IL10-GFP and IFN-YFP cytokine reporters. Reporter expression was mainly restricted to CD4 T cells showing an activated CD44+ phenotype. The frequencies of VFP+ cells were considerably higher than those expressing alternative cytokines in age matched naïve mice (**Figure 1D**). Since natural (n) CD4 TREG develop in appreciable frequencies in naïve mice, we also compared VFP and FoxP3-GFP expressing CD4 T cells. The frequencies of the two populations were surprisingly similar (**Figure 1D**). These results showed that IL21 is expressed by a significant fraction ofnaturally activated CD4 T cells that arise neonatally, rivaled only in frequency by FoxP3+ TREG.

**Development of naturally occurring IL21-VFP+ CD4T cells is not dependent on CXCR5 or B cells but is controlled by IL6, IL10, IL21 and IFN1 and BCL6.**

Antigen-induced preTFH typically require cognate engagements with B cells and upregulation of CXCR5 to mature sufficiently to express IL21 ([Barnett et al., 2014](#_ENREF_2); [Goenka et al., 2011](#_ENREF_16)). To address such requirements for naturally occurring IL21+ CD4 T cells, we determined their splenic frequencies in naïve *Ighm-/-*, *Cxcr5-/-*and wild-type (WT) IL21-VFP reporter mice (**Figure 2A and 2B**). The results showed that B cells or CXCR5 were not required for the development of this VFP+ population.

We then examined whether cytokine signals – IL6, IL10, IL21R, IL12b (IL12p40), and IFN1­­– that have been shown to control induced TFH ([Eto et al., 2011](#_ENREF_14); [Nakayamada et al., 2014](#_ENREF_39); [Ray et al., 2014](#_ENREF_43); [Suto et al., 2008](#_ENREF_46); [Tangye et al., 2013](#_ENREF_47));Cucak, 2009 #18}, also control this VFP+ population. After crossing the IL21-VFP reporter onto mice homozygous for knockout alleles of *Il6*, *Il21r, Il10*, *Il12b* and *Ifnar1*, we evaluated percentages of splenic VFP+ CD4 T cells. VFP+ CD4 T cells arising spontaneously (**Figure 2C**) were not affected by a deficiency in IL12b (IL12p40). In contrast, naïve reporter mice deficient in IL10 showed significant increases in VFP+ CD4 T cells while a deficiency in IFNAR1 caused a reduction (**Figure 2C**). Consistent with synergy between IL6 and IL21 ([Eto et al., 2011](#_ENREF_14)), mice lacking IL6 showed only a modest reduction in VFP+ CD4 T cells, while a deficiency in IL21R caused significant reductions, and mice doubly deficient in IL6 and IL21R showed the greatest reduction (**Figure 2D**). Finally, given the importance of BCL6 in TFH development, we tested if it was required for the development of VFP+ CD4 T cells. CD4-cre transgenic mice homozygous for a floxed knockout allele of *Bcl6* had few or no VFP+ cells (**Figure 2D**); those in which some VFP+ cells were detected are likely due to incomplete excision of *Bcl6* by the CD4-cre transgene. The results suggest that precocious IL21 expression marks a natural TFH-like population that depends on BCL6 and tonic signals from the same cytokines that control development of more mature stages of induced TFH.

**Comparative RNAseq 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 RNAseq on FACS-purified splenic CD4 T cells from 4 wk old IL21-VFP reporter mice based on the following criteria: naïve VFP- ICOSlo/- cells (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 IL21-ACTcells, whereas *Icos* and *Cd44* transcripts were equivalent in the ACT and IL21-ACT populations but were minimal for N cells (**Figure S2B**).

To address how the transcriptional patterns of these natural CD4 T cell subpopulations compared to induced TH subpopulations of adult mice generated after antigenic challenge, we extracted published gene expression data of polyclonal mature TFH, GC TFH, TH1 and naïve cells isolated from mice 8 days after acute infection with LCMV ([Yusuf et al., 2010](#_ENREF_58)) and performed hierarchical clustering to compare their gene expression patterns to those of our N, ACT, and IL21-ACT populations (**Figure 3A top)**. We similarly analyzed RNAseq data derived from ‘early’ IL2R Blimp1- TFH and IL2R+ Blimp1+ TH1 cells from adoptively transferred naïve SMARTA TCR transgenic CD4+ T cells 3 days after acute LCMV infection ([Choi et al., 2015](#_ENREF_6)) (**Figure 3A bottom)**. IL21-ACT cells clustered most closely with TFH generated 3 or 8 days following infection, while ACT cells clustered with TH1 cells.

We then sought to identify expression patterns of genes that most reliably discriminated the N, ACT and IL21-ACT subpopulations. 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 these criteria, 471 genes showed significantly higher expression level in one of the three cell populations (**Table S1)**. These included with 148 for N; 165 for ACT; and 158 for IL21-ACT Functional gene enrichment analysis of these signature clades using DAVID (https://david.ncifcrf.gov/) showed that IL21-ACT cells were most enriched for genes associated with T cell development, differentiation, activation, proliferation and adhesion. In contrast, ACT cells, which included all other types of naturally activated VFP- CD4 T cells, were more restricted to generic lymphocyte and cytokine signaling pathways (**Figure 3B, Table S2).** These results indicated that IL21-ACT cells stood out in the extent of their engagement in T cell activation and signaling processes.

As presented by a 3-way scatterplot (**Figure 3C**) and gene-selective transcriptional comparisons among N, ACT and IL21-ACT (**Figure 3D; Table S3)**, IL21-ACT cells showed increased expression of several prototypical TFH transcription factors, including *Bcl6, Maf, Fosb, E2f2,* and *Tox* while downregulating the BLIMP1-encoding gene, *Pdrm1.* Increased expression in IL21-ACT cells of prototypical TFH markers, including *Sostdc1, Btla*, *Cd200*, *Slamf6*, *Gpm6b,* and *Cxcr5,* provided further support for TFH relatedness. Consistent with an increased activation state, IL21-ACT cells were distinguished from N and ACT by heightened expression of signaling related genes, *Cd4*, *Cd28* and *Lag3*, and cell cycling genes (**Figure 3D**). Additional genes with selectively increased expression in IL21-ACT cells are included in **Table S3.** IL21-ACT cells were also quite restricted in effector cytokines expressed; in addition to *Il21*, only *Ifng* was expressed at appreciable levels that were less than in the ACT population.

Thus, an active and engaged natural TFH population, hereafter referred to as nTFH, emerges within weeks of birth already capacitated with much of the central transcriptional machinery, cell surface receptors, and IL21 required for TFH effector functions. nTFH were distinguished from ACT cells, whose expression signatures were consistent with populations of TH1, TH2, NKT and TREG also arising in naïve mice (**Figure 3E**), while transcriptional support for previously described nTH17 cells ([Kim et al., 2011](#_ENREF_22); [Marks et al., 2009](#_ENREF_35)) was not apparent (**Table S3**).

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

The generation of nTFH in surprisingly high frequencies could be explained by their having focused TCR usage akin to that found for CD1d1-dependent iNKT cells or, alternatively, by clonally diverse TCRs that are unusually sensitive to triggering by diverse self-peptide (p)/MHC ligands. To investigate these possibilities, we used our RNAseq data to evaluate *Trav* and *Trbv* usage patterns of the naïve (N), activated (ACT) and IL21-ACT (nTFH) populations. While ACT cells showed bias, including substantially increased usages of *Trav11* and *Trav11d*, the usages of naïve and nTFH were quite similar (**Figure 4A**). If nTFH are activated by self-p/MHC ligands, we reasoned that limitation of the CD4 T cell repertoire to a single TCR known be supported by basal self-p/MHC stimulation might be sufficient to actuate nTFH. 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*a-/- OT2 mice, appreciable populations of nTFH were still present at both time points (**Figure 4B**). The frequencies of nTFH were also similar to those of OT2 TCR transgenic and TCR-intact IL21-VFP mice (data not shown). The ability of nTFH to develop even when their TCR repertoire is markedly restricted is consistent with them being activated by basal self-p/MHC ligands.

**nTFH are long lived and differentiate into mature TFH after immunization.**

To determine if nTFH are long-livedand capable of differentiating into full TFH, we adoptively transferred sort-purified nTFH to allotype-marked Tcr-/-mice. VFP+ cells were readily detected by FACS in PBL of recipient mice 2 wk after transfer and persisted through wk 6. (**Figure 5A**). Phenotypic analyses at each of these time points showed that many transferred VFP+ CD4 T cells retained the nTFH phenotype with some exhibiting the more mature phenotype of CXCR5+ PD1- pre-TFH cells. Essentially no mature CXCR5+ PD1+ TFH were found (**Figure 5B**). The data showed that adoptively transferred nTFH persist for at least 6 wk with many maintaining their original identity.

We then examined how transferred nTFH would respond to antigenic challenge by immunizing recipient mice with DNP-KLH. FACS analyses of spleen cells performed 10d 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 these cohorts 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 their frequencies in unimmunized mice (**Figure 5D**). The results showed that nTFH cells respond to antigenic stimulation by expanding and readily differentiating into fully mature TFH.

Finally, to determine if naïve VFP- CD4 T cells can differentiate into nTFH, we adoptively transferred purified naïve CD4+ CD44lo CD62+ CXCR5- PD1- VFP- T cells from IL21-VFP Ly5.2 allotype mice into Ly5.1 allotype marked Tcr-/- mice (**Figure S3A-C**). The recipients exhibited VFP+ cells with the majority being CXCR5- PD1- nTFH.

**IL6, IL21 and AIRE promote nTFH development in the neonatal thymus.**

CD4 T cells positively and negatively selected by self-p/MHC ligands in the thymus are released to the periphery in a naïve state. However, a fraction of them, including nTREG, are activated in the thymus ([Chatenoud, 2011](#_ENREF_5); [Dons et al., 2012](#_ENREF_13)). 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 detected at increased levels in older mice (**Figure 6A and B**). Using reporter mice, we also examined the frequencies of thymic single positive CD4 T cells in 4-6 wk old mice expressing , IL17, IFNγ, IL21 and FoxP3 (**Figure 6C**). Only IL21-VFP+ and Foxp3-GFP+ cells were found in significant frequencies, with FoxP3+ CD4 T cells dominating (**Figure 6C**). To determine if CD4+CD8- thymocytes expressing IL21 share cell surface markers with peripheral nTFH, we analyzed expression of CD44, ICOS, CD5 and CD3e in VFP+ CD4 T cells from both sites. Only ICOS was differentially expressed compared with splenic nTFH, with the great majority of thymic nTFH expressing unusually high levels of ICOS (**Figure 6D)**.These differences suggest that ICOS signaling supports nTFH in the thymus but is less critical in the periphery**.**

Having shown that IL6 and IL21 cooperatively support the development of peripheral nTFH (**Figure 2D**), we asked if these cytokines also 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 cytokine signaling pathways were consistent with contributions of both IL6 and IL21 signaling to the earliest stages of thymic nTFH development (**Figure 6E**).

A primary, but not exclusive, role of the transcriptional regulator, AIRE, is to help safeguard central tolerance by forcing the ectopic expression of peripheral tissue antigens (PTA) on medullary thymic epithelial cells (mTEC) and representation on DC for negative selection ([Liston et al., 2003](#_ENREF_29); [Mathis and Benoist, 2009](#_ENREF_36)). However, most recent findings support a role for AIRE in the thymic induction of nTREG ([Chan and Anderson, 2015](#_ENREF_4); [Kyewski and Feuerer, 2014](#_ENREF_24); [Yang et al., 2015](#_ENREF_56)). Studies have also shown that thymic development of nTH17 cells requires AIRE ([Jenkinson et al., 2015](#_ENREF_20)) and that AIRE deficiency reduces thymic iNKT cells ([Lindh et al., 2010](#_ENREF_28)). To examine the effects of AIRE on development of thymic nTFH, we determined the frequencies of nTFH among thymocytes from 6-8 wk old WT, *Aire+/-* and *Aire-/-* IL21-VFP mice. Frequencies of nTFH in WT mice were substantially greater than in *Aire+/-* and *Aire-/-* mice, indicating that AIRE supports thymic nTFH development in a highly dose dependent manner (**Figure 6F**). This finding suggests a critical role for AIRE in the induction of nTFH in the thymus.

**AIRE and FoxP3+ CD4 T cells restrict the expansion and maturation of peripheral nTFH**

Having shown that AIRE is required for thymic nTFH development, we asked if AIRE influences nTFH in the periphery. Splenic frequencies of VFP+ CD4 T cells from WT, *Aire+/-* and *Aire-/-* mice showed that AIRE normally suppresses peripheral nTFH, the oppositeof what was found in the thymus (**Figure 7A and B**).

As nTREG are the only other naturally activated CD4 T cell population with frequencies comparable to nTFH in young mice (**Figure 1D and 6C**), we asked if nTREG control nTFH. *Foxp3*-deficient (scurfy) mice lack TREG and consequentially develop a profound autoimmune disease by 3-4 wk of age. We generated *Foxp3*-/- IL21-VFP reporter mice and analyzed their male progeny at 2 wk of age before the onset of disease 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 in *Foxp3*-deficient mice (**Figure 7C**), with the most striking increase being in nTFH (**Figure 7D**). However, a deficiency in FoxP3 did not affect thymic nTFH frequencies (**Figure 7E).** Thus, newly developing thymic nTFH are insensitive to abundant nTREG, but in the periphery, TREG potently restrain the neonatal expansion and further differentiation of nTFH.

**DISCUSSION**

Through use of IL21-VFP reporter mice, we found that IL21 is functionally expressed by many of the earliest activated thymic and peripheral CD4 T cells that develop naturally in young naïve mice. While IL21 is usually associated with late stages of TFH development, we have shown that IL21 is a property of a population of TFH-related cells that arise naturally through endogenous stimuli and cytokines shortly after birth. These studies link IL21 to very early TFH development and function.

Based on current models, TFH programming is initiated upon encounter with antigen-presenting DC ([Crotty, 2014](#_ENREF_11); [Goenka et al., 2011](#_ENREF_16)). This IL21-negative, pre-TFH stage is initiated by IL6/IL6R, ICOSL/ICOS, and TCR/pMHCII signaling and is characterized by cell surface expression of ICOS, IL6R and CXCR5 ([Akiba et al., 2005](#_ENREF_1); [Barnett et al., 2014](#_ENREF_2); [Choi et al., 2011](#_ENREF_7); [Crotty, 2014](#_ENREF_11)). We have shown that nTFH develop in neonatal mice unimpaired by the absence of B cells or CXCR5, consistent with their activation through antigen-presenting DC. Moreover, while cell surface expression of ICOS and CXCR5 by pre-TFH was thought to be required for TFH to mature sufficiently to express IL21 ([Barnett et al., 2014](#_ENREF_2); [Choi et al., 2011](#_ENREF_7); [Crotty, 2014](#_ENREF_11)), we found that activation, marked by expression of CD44, was sufficient for expression of IL21 by nTFH. Furthermore, our results clearly showed that nTFH persist after adoptive transfer, indicating that nTFH are stably maintained. Finally, we showed that nTFH potently respond and differentiate after immunization into fully mature CXCR5+ PD1+ IL21-expressing TFH. However, while IL21 expression was stable 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_34)). We therefore conclude that nTFH comprise a major, stable pool of naturally activated cells that are homeostatically maintained for a considerable period of time with the capacity to differentiate to more mature TFH in response to foreign antigens.

Tonic signaling describes pathways that operate constitutively in the steady state. It is well established that IL6, IL10, IL21 and IFN1 have substantial effects on responses of T cells, including TFH, induced by immunization or infection ([Cai et al., 2012](#_ENREF_3); [Eto et al., 2011](#_ENREF_14); [Nakayamada et al., 2014](#_ENREF_39); [Ray et al., 2014](#_ENREF_43)). IL6 was shown to supply important signals for all stages of TFH, from early development to their maturation as full and GC TFH ([Choi et al., 2011](#_ENREF_7); [Eto et al., 2011](#_ENREF_14)). IL21, in contrast, has been considered to be only a late stage growth and differentiation factor ([Barnett et al., 2014](#_ENREF_2); [Crotty, 2011](#_ENREF_10)). Repressive effects of IL10 on TFH have also been documented ([Cai et al., 2012](#_ENREF_3); [Crotty, 2014](#_ENREF_11)), and while there is evidence that IFN1 signals can promote TFH, a recent study showed that IFN1 signaling promotes TH1 responses and features of TFH that do not include IL21 expression ([Nakayamada et al., 2011](#_ENREF_38); [Nakayamada et al., 2014](#_ENREF_39); [Ray et al., 2014](#_ENREF_43)). Our results document the importance of tonic signaling by these cytokines in controlling the earliest stages of nTFH development.

Our RNAseq profiling of CD4 T cells from young mice provides new insights into TH lineages that develop naturally. Expression profiles consistent with TFH, TH1, TH2 and NKT cells were evident, which we take as evidence that all of these populations develop naturally, but with nTFH being the preferred choice. In keeping with many earlier studies of induced TFH, BCL6 was critically required for the neonatal development of these cells. Further comparison of naïve, activated, and activated IL21 (nTFH) cells provided a unique glimpse into the genes that best distinguish nTFH from naïve CD4 cells and natural TH populations. The nTFH signature included many prototypical markers of mature TFH. However, the expression of several key transcriptional regulators was only incrementally increased in nTFH as compared to naïve cells (*e.g*, 2-fold for *Bcl6*). Thus, at least at the transcriptional level, actuation of the TFH program may be best explained by the combinatorial effect of factors that enhance the BCL6 regulatory axis. In adult mice, the transcription factors, TCF1 and LEF1 act upstream of BCL6 by inducing expression of IL6R, IL6ST and ICOS to promote early TFH development and have been shown to be substantially upregulated in induced TFH ([Choi et al., 2015](#_ENREF_6); [Xu et al., 2015](#_ENREF_55)). However, we found that *Tcf1* and *Lef1* were most highly expressed by naïve CD4 T cells, reduced in nTFH, and further reduced in ACT cells, suggesting that they repress TH1 differentiation. FOXO1 and FOXP1werereported to inhibit induced TFH ([Stone et al., 2015](#_ENREF_45); [Wang et al., 2014](#_ENREF_52); [Weber et al., 2015](#_ENREF_53)). However, we found that *Foxo1* and *Foxp1* are more highly expressed in naïve and nTFH than ACT cells. Whether these distinctions reflect intrinsic differences between natural vs. induced TFH development, higher levels of translational control, and/or methods of ascertainment remain to be determined.

Classically, thymocytes with high TCR affinity for self-p/MHC ligands are eliminated, while those with weak but sufficient affinities are positively selected and then maintained in the periphery in the naïve state through continued basal self-p/MHC signaling. The extent to which inherent TCR affinities and abundance of positively selecting self-p/MHC ligands control the clonal composition of the naïve T cell repertoire and its ultimate ability to foment responses to foreign antigens is increasingly recognized ([Hogquist and Jameson, 2014](#_ENREF_17); [Lo and Allen, 2014](#_ENREF_32); [Tubo and Jenkins, 2014](#_ENREF_48); [Vrisekoop et al., 2014](#_ENREF_51)). The fate of T cells surviving negative selection while maintaining more appreciable self-reactivity than naïve T cells is a further complication, but with deviation to nTREG being an outcome ([Chan and Anderson, 2015](#_ENREF_4); [Hogquist and Jameson, 2014](#_ENREF_17); [Kyewski and Feuerer, 2014](#_ENREF_24))**.** A defining feature of so-called natural T cell populations, including nTREG and nTFH, is that they are clonally diverse and already activated. The fact that *activated* nTFH are found in thymi shortly after birth suggests that they, like nTREG, are hypersensitive to the same self-p/MHC signals that positively select naïve T cells. The extent to which AIRE influences nTFH reinforces the role of self-p/MHC signals in the development and activation of this population. Our finding that AIRE limits peripheral expansion of nTFH is consistent with the prototypic role of AIRE in restraining autoimmunity. However, we also found that AIRE has the opposite, highly dosage dependent effect in the thymus. This almost complete copy number dependence on AIRE supports its critical role in the thymic selection of nTFH, possibly in a more extreme manner than its positive influence on TREG.

Overall, the ontogeny and behavior of nTFH has striking parallels with Foxp3+ nTREG. Both nTFH and nTREG are clonally diverse and are spontaneously activated by natural stimuli in the thymus and periphery in frequencies that exceed other natural T cell populations in early post-natal life. AIRE supports their thymic development but limits their capabilities in the periphery. In agreement with recent reports ([Iwamoto et al., 2014](#_ENREF_18); [Wing et al., 2014](#_ENREF_54)), we showed that the absence of FoxP3 causes a substantial increase 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 peripheral nTFH are a primary target of TREG in young mice. Thus, a key function of TREG may be to constrain nTFH that arise through similar selection processes but with opposing functions.

Our identification of nTFH arising neonatally 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. Peripheral nTFH maintained in an activated state by basal TCR/self-pMHC stimulation may be poised for rapid responses to foreign antigens, permitting rapid antigen-driven clonal selection by B cells and follicular dendritic cells resulting in full differentiation to GC TFH. In contrast, self-reactivity, rather than high affinity-driven clonal selection to foreign antigens, is thought to be a property of autoimmune disease-promoting CD4 T cells ([Koehli et al., 2014](#_ENREF_23)). Genetic and environmental factors that cause the polyclonal expansion and further differentiation of this already self-reactive nTFH population may underlie autoimmune disorders in which IL21-producing TFH and related TFH-like cells are important pathogenic drivers.

**METHODS**

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

The bicistronic IL21-VFP knock-in allele (*Il21tm1.1Hm*) was generated in C57BL/6N ES cells by Ozgene Pty. Ltd. (Bentley WA) under the direction of HCM part of a contract with NIAID, NIH. Genotypic confirmation of precise targeted transgenesis was established by Southern blot and DNA sequence analyses at Ozgene. Genetic excision of the *loxP*-PGK-neo selection cassette-*loxP* was genetically excised by crossing to cre-transgenic “deletor” mice. Following genetic confirmations by DNA sequencing, mice carrying the knock-in allele were monitored using a three primer PCR assay with the following primers: *a,* F-AATGCATTTCTTTCACTTCCATGTT; *b,* R-TTAGTTAATGGGCGAAAGGATCTTA; *c*, F-AACGAGAAGCGCGATCACAT. The WT band (primers a and 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.

***Mice***

All mice used were maintained on a B6/J background in a specific pathogen-free mouse colony at The Jackson Laboratory. See Supplemental Experimental Procedures for strains used and genotyping methods. 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 DNP-KLH emulsified in complete Freund’s adjuvant and analyzed 7-11 days after immunization. For in vitro stimulation, total splenocytes from naïve B6.IL21-VFP mice depleted of red blood cells by exposure to ACK buffer were cultured in 6 well plates with 5 µg/ml of anti-CD3 and 1 µg/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+. For serum cytokine analysis, splenocytes from naïve B6.IL21-VFP mice were cultured for 36h with 5 µg/ml of anti-CD3 and 1 µg/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 cultured independently in 5% FBS-supplemented DMEM. Supernatants were collected after 24h and cytokines were measured by ELISA (See Supplemental Experimental Procedures).

***Flow Cytometry***

Analytical and preparative FACS was performed by established procedures after FMO gating ([McPhee et al., 2013](#_ENREF_37)) using antibodies listed in Supplemental Experimental Procedures. Samples were run on a four-laser/13-color BD LSRII cytometer (BD Biosciences) and analyzed with FlowJo software version 8 or 9 (Tree Star).

***Cell purifications for RNAseq and CD4+ T cell transfers***

Samples for RNAseq were prepared from pooled spleen cells isolated from 4 wk old B6.IL21-VFP mice by negative cell depletion methods. CD4+ T cells were enriched by incubating Milltenyi streptavidin microbeads (Milltenyil; BD Bioscience) coupled with CD11b, CD11c, B220 and CD8 antibodies with total mouse splenocytes, followed by a pass through the autoMACS pro separator (Milltenyi) according to the manufacturer’s protocols. Purification of VFP-ICOS- (N), VFP-ICOS+(ACT), and VFP+ICOS+(ACT IL21) CD4+ T cellswas then performed by cell sorting based on VFP, anti-CD4 and anti-ICOS detection using a FACSAria (BD Biosciences).

***RNAseq and analysis***

Total RNA from 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). 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_27)). Differential expression analysis across the three conditions (*i.e.* sorted populations) was conducted with EBSeq ([Leng et al., 2013](#_ENREF_26)). 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 was added to the mouse transcriptome file. See Supplemental Experimental Procedures for comparisons of our RNAseq profiles with antigen-induced TFH microarray datasets.TCR usages of our samples were determined by aligning all RNAseq transcripts to Ensembl TR\_V\_genes and are reported as TPM. TCR\_J and TCR\_D segments were excluded because of difficulties in aligning short sequences. Transcripts with maximum expression <5 TPM were filtered out, resulting in a total of 68 *Tcrav* and *Tcrbv* genes.

***Statistical analyses***

Data were analyzed by non-parametric statistics (GraphPad Prism v6). The Mann-Whitney U test was used for 2 cohort comparisons and the Kruskal-Willis test was used for analyses comparing more than 2 groups.

**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.

**Acknowledgments**

We thank J. Ward (Global VetPathology) for assistance with IHC images; T. Duffy and W. Schott (The Jackson Laboratory) for flow cytometry assistance; D. Serreze (The Jackson Laboratory) for manuscript review; and the Gene Expression Service (The Jackson Laboratory) for RNA sequencing. Supported in part by the Alliance for Lupus Research (E.B.A., T.J.S., G.P., G.J.C. and D.C.R.), The Gina M. Finzi memorial student summer fellowship, Lupus Foundation of America (E.B.A.), The Jackson laboratory summer student program (S.L.K.), the Intramural Research Program of the NIH, NIAID (H.C.M., S.J., and H.W) and The Pyewacket Foundation (X.W. and G.W.C.).

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