Elsevier Editorial System(tm) for Journal of Allergy and Clinical Immunology
Manuscript Draft

Manuscript Number:

Title: Monogenic mutations differentially impact the quantity and quality of T follicular helper cells in human primary immunodeficiencies

Article Type: Original Article

Section/Category: Immune Deficiencies, Infection, and Systemic Immune Disorders

Keywords: T follicular helper cells; humoral immunity, primary immunodeficiencies; cytokine signaling

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Manuscript Region of Origin: AUSTRALIA

Abstract: Background: T follicular helper (Tfh) cells underpin T-cell dependent humoral immunity and the success of most vaccines. Tfh cells also contribute to human immune disorders such as autoimmunity, immunodeficiency and malignancy. Understanding the molecular requirements for the generation and function of Tfh cells will provide strategies for targeting these cells to modulate their behavior in the setting of these immunological abnormalities.
Objective: To determine the signaling pathways and cellular interactions required for the development and function of Tfh cells in humans.
Methods: Human primary immunodeficiencies (PIDs) resulting from monogenic mutations provide a unique opportunity to assess the requirement for particular molecules in regulating human lymphocyte function. Circulating Tfh (cTfh) cell subsets, memory B cells and serum Ig levels were quantified and functionally assessed in healthy controls as well as patients with PIDs resulting from mutations in STAT3, STAT1, TYK2, IL21/R, IL10R, IFNGR1/2, IL12RB1, CD40LG, NEMO, ICOS or BTK.
Results: Loss-of function (LOF) mutations in STAT3, CD40LG, NEMO, ICOS or BTK reduced cTfh frequencies. STAT3, IL21/R LOF and STAT1 gain-of function (GOF) mutations skewed cTfh differentiation towards a phenotype characterized by over-expression of IFNγ and programmed death -1 (PD-1). IFNγ inhibited cTfh function in vitro and in vivo, corroborated by hypergammaglobulinemia in patients with IFNGR1/2, STAT1 and IL12RB1 mutations.
Conclusion: Specific mutations impact the quantity and quality of cTfh cells, highlighting the need to assess Tfh cells in patients by multiple criteria, including phenotype and function. Furthermore, IFNγ

functions in vivo to restrain Tfh-induced B cell differentiation. These findings shed new light on Tfh biology and explain compromised humoral immunity in some PIDs.

Monogenic mutations differentially impact the quantity and quality of T follicular helper cells in human primary immunodeficiencies

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This work was funded by project and program grants from the National Health and Medical Research Council (NHMRC) of Australia (to EKD, SGT, CSM, DAF, MCC), the German Federal Ministry of Education and Research (BMBF 01EO1303, to BG and KW), and Rockefeller University Center for 541 Clinical and Translational science (5UL1RR024143, to JLC). CSM is a recipient of a Career Development Fellowship and SGT a Principal Research Fellowship from the NHMRC of Australia.

The authors declare no conflicts of interest

ABSTRACT

Background: T follicular helper (Tfh) cells underpin T-cell dependent humoral immunity and the success of most vaccines. Tfh cells also contribute to human immune disorders such as autoimmunity, immunodeficiency and malignancy. Understanding the molecular requirements for the generation and function of Tfh cells will provide strategies for targeting these cells to modulate their behavior in the setting of these immunological abnormalities.

Objective: To determine the signaling pathways and cellular interactions required for the development and function of Tfh cells in humans.

Methods: Human primary immunodeficiencies (PIDs) resulting from monogenic mutations provide a unique opportunity to assess the requirement for particular molecules in regulating human lymphocyte function. Circulating Tfh (cTfh) cell subsets, memory B cells and serum Ig levels were quantified and functionally assessed in healthy controls as well as patients with PIDs resulting from mutations in *STAT3*, *STAT1*, *TYK2*, *IL21, IL21R*, *IL10R*, *IFNGR1/2*, *IL12RB1*, *CD40LG*, *NEMO*, *ICOS* or *BTK*.

Results: Loss-of function (LOF) mutations in *STAT3*, *CD40LG*, *NEMO*, *ICOS* or *BTK* reduced cTfh frequencies. *STAT3*, *IL21/R* LOF and *STAT1* gain-of function (GOF) mutations skewed cTfh differentiation towards a phenotype characterized by over-expression of IFNγ and programmed death - 1 (PD-1). IFNγ inhibited cTfh function *in vitro* and *in vivo*, corroborated by hypergammaglobulinemia in patients with *IFNGR1/2*, *STAT1* and *IL12RB1* mutations.

Conclusion: Specific mutations impact the quantity and quality of cTfh cells, highlighting the need to assess Tfh cells in patients by multiple criteria, including phenotype and function. Furthermore, IFNγ functions *in vivo* to restrain Tfh-induced B cell differentiation. These findings shed new light on Tfh biology and explain compromised humoral immunity in some PIDs.

Key Messages

• Loss-of function (LOF) mutations in *STAT3*, *CD40LG*, *NEMO*, *ICOS* or *BTK* reduce cTfh frequencies.

• *STAT3*, *IL21/R* LOF and *STAT1* gain-of function mutations skew cTfh differentiation towards a phenotype typified by over-expression of IFNγ and PD-1.

• IFNγ negatively regulates Ig production *in vivo*

Capsule summary

Analysis of patients with monogenic mutations identified molecular requirements for the generation, and function of Tfh cells. As Tfh cells are critical for long-lived Ab responses, our findings provide insight into mechanism underlying impaired humoral immunity in some primary immunodeficiencies.

Key words

• T follicular helper cells; humoral immunity, primary immunodeficiencies; cytokine signaling

Abbreviations

Tfh: T follicular helper cell; cTfh: circulating Tfh; TD: T-dependent; SLE: systemic lupus erythematosus; RA: rheumatoid arthritis; GCs: germinal centres' LOF: loss-of function; GOF: gain-of function; PIDs: primary immunodeficiencies

INTRODUCTION

Naïve $CD4^+$ T cells differentiate into distinct populations of effector cells with specialized functions. Such fine-tuning ensures the generation of appropriate immune responses that efficiently clear pathogens and generate long-term protective immunity following infection or vaccination[1]. The $CD4^+$ T cells responsible for mediating the differentiation of naïve B cells into memory cells and plasma cells, thereby providing effective humoral immunity against T-dependent (TD) antigen (Ag), are T follicular helper (Tfh) cells[2-5]. Tfh cells express elevated levels of CXCR5, PD-1, Bcl-6, and several molecules involved in T-cell/B-cell interactions and localize to follicles of secondary lymphoid tissues[2-4]. Differentiation of naïve $CD4^+$ T cells into Tfh cells is a complex process requiring integration of signals delivered by dendritic cells, B cells, cytokines, specific signaling pathways and transcription factors[2-5]. The critical role of Tfh cells in eliciting long-lived humoral immunity is evidenced by impaired generation of germinal centers (GCs), memory B cells and Abs to TD Ag in mice and humans who lack genes that promote Tfh formation[2-6]. Ab-mediated autoimmune conditions can also be caused by dysregulated Tfh function[7-9]. Thus, delineating molecular requirements underlying Tfh generation and function are important in understanding how these cells operate and in identifying pathways that could be targeted in the settings of vaccination, immunodeficiency, or autoimmunity.

Although studies of mice and some human immune disorders have taught us a great deal about Tfh cells, our understanding of human Tfh biology remains incomplete. This is largely due to limited access to lymphoid tissues where Tfh cells are located. However, progress has been made by studying circulating $CD4^+CXCR5^+$ T cells as correlates of tissue Tfh cells. Subsets of circulating Tfh (cTfh) cells have been reported, with $CCR6^+$, $CCR6^+PD\text{-}1^+$ or $CCR7^{lo}PD\text{-}1^{hi}$ subsets being superior to other subsets in providing B cell help[10, 11]. These subsets correlated with antibody (Ab) responses to influenza virus following vaccination in young adults, but not older adults[12], are increased in autoimmune diseases[8, 10, 13-16], and decreased in HIV-infection[11]. Similarly, $CD4^+CXCR5^+PD1^+$ $CXCR3^-$ T cells were identified as the circulating counterpart of lymphoid Tfh cells and their

149 frequencies positively correlated with neutralizing Abs in HIV infection[17]. Although these studies
150 generally confirmed that PD-1^{+}CXCR3^{-}/CCR6^{+} cTfh cells are a reliable correlate of Tfh cells in human
151 lymphoid tissue, the identity of the circulating B-helper human CD4^{+} T cells remains contentious, as
152 other studies demonstrated that CXCR5^{+}CXCR3^{+} *or* even CXCR5^{-} CD4^{+} T cells exhibit detectable B-
153 helper function[11, 15, 19, 20] and correlate with influenza vaccine responsiveness[18, 19].
154
155 To assess the molecular requirements for the generation and function of human cTfh cells, we
156 investigated >100 individuals with 14 different monogenic mutations that underlie primary
157 immunodeficiencies (PIDs). Our findings identify mutations that have distinct quantitative and/or
158 qualitative effects on human cTfh cells, providing an explanation for humoral immune defects in some
159 PIDs as well as insights into mechanisms regulating human Tfh differentiation and function.
160

Methods

Human samples

Peripheral blood mononuclear cells (PBMCs) were isolated from healthy controls (Australian Red Cross) and PID patients. Human spleens were obtained from cadaveric organ donors (NSW Organ Transplant Registry). All studies were approved by Institutional Human Research Ethics Committees.

Antibodies and Reagents

eFluor660-anti-IL-21, PerCP-Cy5.5-anti-IFNγ, FITC-anti-CD45RA, biotin-PD-1 were from eBiosciences. Alexa647-anti-CXCR5 and anti-pSTAT1, APC-anti-CD10, APC-Cy7-anti-CD4, BV605-anti-IgG, PE-anti-pSTAT3 and anti-CCR6, Pe-Cy7-anti-CD25 and anti-CD27, PerCpCy5.5-anti-CD127, biotin-anti-IgA, SA-PerCpCy5.5, and recombinant IFNγ were from Becton Dickinson. BV421-anti-CXCR3, Pacific Blue-anti-CD20 and SA-BV605 were from Biolegend.

Lymphocyte phenotyping and isolation

T cells: PBMCs were incubated with mAbs to CD4, CD45RA, CD127, CD25, CXCR5, CXCR3, CCR6 and PD-1 and proportions of regulatory T cells ($CD4^+CD127^{lo}CD25^{hi}$), total memory ($CD4^+CD45RA^-$), cTfh ($CD4^+CD45RA^-CXCR5^+$), as well as subsets of non-cTfh memory and cTfh cells defined according to CXCR3 and CCR6 expression were determined[10, 20]. To isolate these subsets, Tregs were excluded and the remaining population sorted into naïve ($CD45RA^+$ $CXCR5^-CXCR3^-$ $CCR6^-$), non-Tfh memory ($CD45RA^-CXCR5^-$) and cTfh cells. Subsets of non-cTfh and cTfh cells were identified according to differential CXCR3 and CCR6 expression[10]. All populations were sorted on a FACS ARIA (Becton Dickinson) to > 98% purity.

B cells: PBMCs were incubated with mAbs to CD20, CD27, CD10, IgG and IgA, and the frequency of total memory ($CD20^+CD27^+CD10^-$) and switched memory B cells determined[21, 22].

Expression of phospho-STATs

Epstein Barr virus transformed lymphoblastoid cell lines (EBV-LCLs) established from healthy donors, *IFNGR2*$_{LOF}$, or *STAT1*$_{LOF}$ were stimulated with IFNγ or IL-21 for 30 mins. Cells were fixed, permeabilised and stained for anti-pSTAT1 and anti-pSTAT3[21].

Analysis of CD4$^+$ T cell function *in vitro*

Isolated CD4$^+$ T cell populations were cultured with T cell activation and expansion (TAE) beads (anti-CD2/CD3/CD28; Miltenyi Biotech) in 96 well round bottomed well plates. After 5 days, supernatants were harvested and production of IL-4, IL-5, IL-10, IL-13, IL-17A, IL-17F, IFNγ and TNFα determined by cytometric bead arrays (Becton Dickinson); secretion of IL-22 (eBioscience) and CXCL13 (R&D systems) was determined by ELISA. For cytokine expression, activated CD4$^+$ T cells were re-stimulated with PMA (100 ng/ml)/ionomycin (750 ng/ml) for 6 hours, with Brefeldin A (10 μg/ml) added after 2 hours. Cells were then fixed and expression of intracellular cytokines detected[20, 22, 23]. For gene expression, RNA was extracted, and transcribed into cDNA. Expression of *TBX21*, *GATA3*, *RORC* and *BCL6* was determined by qPCR and standardized to *GAPDH* [20, 24].

T-B cell co-culture assays and Ig determination

CD4$^+$ T cell subsets were treated with mitomycin C (100 μg/ml, Sigma) and then co-cultured at a 1:1 ratio (50 x 10^3/200μl/well) with allogeneic total splenic B cells[20, 22, 24]. In some experiments, exogenous IFNγ was added to the cultures. After 7 days Ig secretion was determined by ELISA[20, 21]. Serum IgG and IgM levels were determined by nephelometry.

Statistical analysis

Significant differences were determined using a one-way ANOVA (Prism; GraphPad Software).

RESULTS

Cytokine and transcription factor expression by human naïve and memory CD4$^+$ T cells

To determine the requirements for generating human Tfh cells *in vivo*, we first defined parameters that identify different CD4$^+$ T cell subsets in peripheral blood. Total memory cells were the predominant producers of all cytokines examined, producing ~5-100 fold higher levels of Th1 (IFNγ), Th2 (IL-4, IL-5, IL-13), and Th17 (IL-17A, IL-17F, IL-22) cytokines, as well as B-cell helper/Tfh cytokines (IL-10, IL-21), than naïve cells (Figure 1A-D). Memory CD4$^+$ T cells also expressed higher levels of *TBX21* (T-bet), *GATA3* and *RORC* (RORγt) than naïve cells (Figure 1E-G). There was no difference in *BCL6* expression between naïve and memory cells (Figure 1H), consistent with other studies reporting Bcl-6 levels are similar in circulating human CD4$^+$ T cell subsets[8, 10, 12, 15, 17, 25].

Delineation of memory CD4$^+$ T cells into defined populations of Th1, Th2, Th17 and Tfh cells and subsets

Human memory Th1, Th2, Th17 and cTfh cells can be defined according to differential expression of CXCR3, CCR6 and CXCR5[26, 27], with Th1 cells being CD45RA$^-$CXCR5$^-$CXCR3$^+$CCR6$^-$, Th17 cells CD45RA$^-$CXCR5$^-$CXCR3$^-$CCR6$^+$, Th2 cells CD45RA$^-$CXCR5$^-$CXCR3$^-$CCR6$^-$, and Tfh cells CD45RA$^-$CXCR5$^+$ (Figure 1I-K). In contrast, CD45RA$^+$ naïve cells lack these chemokine receptors (Figure 1I, J). We extended these findings by demonstrating Th1 cells were enriched for IFNγ secretion (Figure 1L), Th2 cells produced the greatest amounts of IL-4, IL-5 and IL-13 (Figure 1M), while Th17 cells secrete the most IL-17A, IL-17F and IL-22 (Figure 1N). Importantly, the highest proportion of IL-21-expressing cells was detected in the cTfh subset, which also contained a greater proportion of IL-10-expressing cells than Th1 and Th2 subsets (Figure 1O). Indeed, these were the only cytokines produced by cTfh cells at levels significantly greater than naïve cells (Figure 1L-O), consistent with the B-cell helper function of these cytokines[28]. The memory CD4$^+$ T cell population co-expressing CCR6 and CXCR3 (termed Th1/17 cells) produced both Th1 and Th17, but only low levels of Th2, cytokines (Figure 1L-O). Thus, these cells represent pro-inflammatory cells, consistent with their detection in

239 inflamed tissues[29]. *TBX21*, *GATA3* and *RORC* expression also correlated with their respective
240 production of Th1, Th2 and Th17 cytokines (Figure 1P-S).
241
242 Having successfully characterized Th1, Th2, Th17 and cTfh cells from peripheral blood, we next
243 investigated cTfh cells using a similar approach by dividing them into subpopulations according to
244 CCR6 and CXCR3[10] (Figure 2A, B) and assessing cytokine production and B-cell helper function. The
245 different cTfh subsets produced lower levels of cytokines than non-Tfh memory ($CD45RA^-CXCR5^-$)
246 cells, however IL-4 and IL-13 were enriched in the $CXCR3^-CCR6^-$ subset, IL-17A/F and IL-22 in the
247 $CCR6^+CXCR3^-$ (hereafter referred to as $CCR6^+$) subset, and IFNγ in the $CXCR3^+CCR6^-$ ("$CXCR3^+$")
248 and $CXCR3^+CCR6^+$ subsets (Figure 2C-F). In contrast, IL-10, IL-21 (Figure 2F) and CXCL13 (Figure
249 2G), which are preferentially produced by Tfh cells[25, 30], were comparable in all cTfh subsets. Total
250 cTfh cells induced greater B-cell differentiation than naïve and $CXCR5^-$ memory $CD4^+$ T cells (Figure
251 2H) as well as Th1, Th2, Th17 and Th1/17 memory cell subsets (Figure 2I). Amongst cTfh subsets, the
252 $CCR6^+$ population consistently induced most Ig secretion by co-cultured B cells over the $CXCR3^+$,
253 $CXCR3^-CCR6^-$ and $CXCR3^+CCR6^+$ subsets (Figure 2J). Thus, consistent with recent studies, blood
254 $CD4^+CXCR5^+$ T cells have Tfh function, with the $CCR6^+$ subset being most effective[10, 11, 17].
255
256 **Mutations causing PIDs impact $CD4^+$ T cell function and B-cell memory formation**
257 These data established the ability to identify effector functions of human $CD4^+$ T cell subsets, thereby
258 providing a framework to determine consequences of gene mutations on $CD4^+$ T cell differentiation *in*
259 *vivo*. We examined $CD4^+$ T cell subsets and function in PID patients with mono- or bi-allelic mutations
260 in surface receptors or cytokine signaling pathways that may impact humoral immunity in the context
261 of Tfh formation and long-lived protective Ab responses[4].
262
263 LOF mutations in *STAT1*, *CD40LG*, *NEMO*, *IL12RB1* or *TYK2* compromised IFNγ production by
264 memory $CD4^+$ T cells (Figure 3A), consistent with known roles in eliciting Th1 responses[31].
265 Production of Th2 cytokines was enhanced by *IL12RB1*, *IFNGR1/2* or *TYK2* LOF mutations,
266 confirming Th1 cells suppress Th2 cells[32], as well as by LOF mutations in *IL21*/*IL21R* and *STAT3* and

GOF mutations in *STAT1* (Figure 3B). This revealed novel roles for IL-21 and STAT3 as regulators of human Th2 immunity, which differ to studies in mice[33, 34]. B cells also clearly regulate CD4+ T cell differentiation, revealed by reduced IFNγ and increased Th2 cytokines in B-cell deficient individuals with BTK mutations (Figure 3A, B). *STAT3*, *NEMO*, *ICOS*, *IL12RB1* or *TYK2* LOF mutations strongly diminished Th17 cytokines (Figure 3C) and *RORC* expression (not shown). *STAT1* GOF and *IL21/IL21R* LOF mutations also reduced Th17 cytokines and *RORC* but to a lesser extent than these other mutations (Figure 3C). These findings are consistent with susceptibility of some individuals with these mutations to *Candida* infection ($STAT3_{LOF}$, $STAT1_{GOF}$, *IL12RB1*, *NEMO*)[31, 35], and the requirement for ICOS in human Th17 cell generation[36]. In contrast, $STAT1_{LOF}$ mutations had no or partial effect on IL-17A/F or IL-22 (Figure 3C), mirroring intact immunity against *Candida* spp in these patients[31, 35]. IL-10 production was compromised by *STAT3*, *TYK2* and *IL21R* LOF and *STAT1* GOF mutations, but unaffected by other mutations, while IL-21 was reduced by *STAT1* GOF, *IL21R* and *NEMO* LOF mutations (Figure 3D).

Since a major objective was to establish regulators of human lymphocyte differentiation in the context of TD B-cell function, we assessed PID patients for memory B cells. There were marked reductions in memory B cells in patients with *STAT3*, *IL21/R*, *ICOS*, *CD40LG* and *NEMO* LOF mutations, and *STAT1* GOF mutations (Figure 3E). Although memory B cells were reduced in patients with *STAT3* LOF, *STAT1* GOF and *NEMO*-deficient patients, the residual memory cells still underwent class switching. In contrast, a lack of CD40L, ICOS or IL-21/IL-21R signaling impaired isotype switching. The frequencies of memory B cells were unaffected by LOF mutations in *STAT1*, *TYK2*, *IL12RB1* or *IFNGR1/2*, however there was a trend for more switched memory B cells in these patients (Figure 3F). Together, these data demonstrated the validity of using PID patients as models for defects in B and $CD4^+$ T cell differentiation and cytokine production.

Specific gene mutations compromise the generation of human circulating Tfh cells

We next used these patients to examine the relationship between PID-associated gene mutations and phenotypically-defined subsets of $CD4^+$ T cells. Th1 cells were significantly increased in $STAT3_{LOF}$ and $STAT1_{GOF}$ patients, while Th17 cells were significantly reduced in these as well as in *NEMO*, *ICOS* or *IL12RB1*-mutant patients (Table 1), consistent with poor production of IL-17A/IL-17F, and IL-22 by memory cells from most of these patient groups (Figure 3C). Th2- and Th1/17-phenotype cells were unaffected by most of the mutations examined (Table 1). When cTfh cells were measured, significant reductions were observed in patients with mutations in *STAT3*, *CD40LG*, *BTK* and *ICOS* - consistent with previous studies[20, 37, 38] - as well as *NEMO* (Figure 4A, B). In our cohort of *CD40LG*-deficient patients, three individuals had clinically milder disease than those with classic HIGM (ie detectable levels of IgG/A, less severe infections, later age of diagnosis). Strikingly, these patients had normal cTfh frequencies, thereby correlating disease severity with cTfh cells (Figure 4A, B). There were also fewer cTfh cells in patients' lacking IL-21/R, IL10R, IL12RB1 or TYK2, however these were not significantly different to controls (Figure 4A, B). None of the other mutations impacted Tfh formation, as determined by quantifying circulating $CD4^+CXCR5^+$ T cells.

$STAT3_{LOF}$ or $STAT1_{GOF}$ mutations skew cTfh differentiation to a non-helper phenotype

When we analyzed cTfh subsets defined by CXCR3 and CCR6 expression[10](Figure 2D), we found significant reductions in $CCR6^+$ cTfh cells – the most proficient B-helper cTfh population (Figure 2H-J)[10] - in patients with $STAT3_{LOF}$, $STAT1_{GOF}$ and *IL21/IL21R* mutations, and corresponding increases in $CXCR3^+$ cTfh cells in $STAT3_{LOF}$ and $STAT1_{GOF}$ individuals (Figure 4C, D). The loss of $CCR6^+$ cTfh cells in these patients was even more striking when ratios of $CCR6^+$ to $CXCR3^+$ cTfh cells were calculated (ie controls: 1.6; $STAT3_{LOF}$: 0.18 [$p<0.001$]; $STAT1_{GOF}$: 0.6 [$p<0.05$]; IL21/IL21R: 0.6). *NEMO* mutations resulted in more $CCR6^-CXCR3^-$ cTfh cells, while the $CCR6^+CXCR3^+$ subset was not affected by any mutations examined (Figure 4D). Thus, not only did $STAT3_{LOF}$ mutations compromise cTfh generation, they also prevented formation of the cTfh subset most capable of inducing B-cell help.

This perturbation to cTfh differentiation was mirrored by *STAT1*$_{GOF}$ and to a lesser extent *IL21/IL21R*$_{LOF}$ mutations (Figure 4A-D).

These data suggest that cTfh cells from patients with *STAT3*$_{LOF}$ and *STAT1*$_{GOF}$ mutations would exhibit skewed cytokine production. Indeed, cTfh cells from *STAT3*$_{LOF}$ individuals exhibited 4-fold fewer IL-17A-expressing and 4-fold more IFNγ-expressing cells than healthy controls (Figure 4E). Although IL-21 was expressed by comparable frequencies of control and *STAT3*$_{LOF}$ cTfh cells, IL-10 secretion by STAT3-deficient cTfh cells was markedly reduced (6-fold) (Figure 4E). *STAT1*$_{GOF}$ mutant cTfh cells exhibited similar, but less extreme, perturbations to cytokine production (Figure 4E). Thus, altered phenotypes of cTfh cells in some PIDs correlated with altered function with respect to cytokine production, demonstrating these mutations not only impact cTfh generation but also their quality, predominantly yielding a population with limited B-cell helper capacity, consistent with impaired humoral immunity in *STAT3*$_{LOF}$ and *STAT1*$_{GOF}$ individuals[21, 39].

IFNγ restrains Tfh-induced B cell differentiation

IFNγ can impede Ig secretion by human B cells[40-44]. Based on this and our findings of skewed differentiation of *STAT3*$_{LOF}$ and *STAT1*$_{GOF}$ cTfh cells to an IFNγ-secreting subset, we hypothesized that IFNγ production by CXCR3^{+} cTfh cells would compromise their B-helper function (Figure 2J)[10]. To investigate this, we first established that IFNγ directly activates human B cells. IFNγ phosphorylated STAT1 in EBV-LCLs from healthy controls, but not *STAT1* or *IFNGR*-deficient individuals (Figure 5A). In contrast, IL-21-induced STAT3 activation was unaffected by such mutations. Next, we co-cultured cTfh and allogeneic B cells with or without exogenous IFNγ. Exogenous IFNγ suppressed TD differentiation of normal B cells by 40-50%, but had no effect on Ig secretion by *IFNGR1*- or *STAT1*-deficient B cells (Figure 5B), demonstrating B-cell intrinsic signaling via IFNγR1/STAT1 mediates the repressive effect of IFNγ on Tfh-induced B-cell differentiation.

If IFNγ plays a substantial role in regulating Ig production *in vivo*, mutations in molecules regulating its production (ie *IL12RB1*) or signaling (ie *IFNGR1* or *STAT1*) should cause hypergammaglobulinemia. Indeed, 60% (9/15), 38% (6/16) and 53% (17/32) of patients with *IFNGR1/2*, *STAT1* and *IL12RB1* LOF mutations, respectively, had serum IgG levels greater than age-matched controls (Figure 5C), while 60% and 25% of *IL12RB1* or *STAT1*-deficient patients had elevated serum IgM (Figure 5D). Notably, several patients whose Ig levels fell within the normal range were actually at the upper end of normal (Figure 5C, D). Thus, a key function of IFNγ *in vivo* is to suppress Ig production.

Mutations in *STAT3*, *STAT1* and *IL21R* cause aberrant expression of PD-1 on CD4$^+$ T cells

Although PD-1 is highly expressed on lymphoid Tfh cells[24, 45], only a subset of cTfh cells exhibit elevated PD-1[9, 11, 12, 15, 17]. PD-1^{hi} cTfh cells have been used as a biomarker of humoral immunity in health and disease[8, 11, 12, 14, 15, 17]. As cTfh development was perturbed by different PID-causing mutations, we examined PD-1 in these patients. PD-1 was expressed at low levels on naïve CD4$^+$ T cells irrespective of genotype, and induced on non-cTfh memory cells from healthy controls and patients (Figure 6A, B). Non-Tfh memory cells from *STAT1*$_{GOF}$ and *IL21/R*$_{LOF}$ and cTfh cells from *STAT3*$_{LOF}$ and *STAT1*$_{GOF}$ individuals expressed significantly more PD-1 than controls (Figure 6A, B). None of the other mutations affected PD-1 expression, suggesting signaling via STAT3/STAT1, possibly downstream of IL-21, regulates PD-1 on memory CD4$^+$ T cells.

As *STAT3*$_{LOF}$, *STAT1*$_{GOF}$ and to a lesser extent *IL21R* mutations skewed cTfh differentiation towards a CXCR3$^+$ phenotype and upregulated PD-1, we tested whether these two observations were related. PD-1 was expressed by all cTfh subsets from healthy controls, with greater expression on CXCR3$^+$ and CXCR3$^+$CCR6$^+$ subsets compared to CCR6$^+$ and CCR6$^-$CXCR3$^-$ subsets (Figure 6C). Amongst the different patients, PD-1 was higher on all cTfh subsets compared to controls, and this increase was most pronounced for CXCR3$^+$ cTfh cells (Figure 6C, D). Thus, *STAT3*$_{LOF}$, *STAT1*$_{GOF}$ or *IL21/IL21R* mutations dysregulate PD-1 expression.

Discussion

It was first reported in 1965 that thymus-derived cells were required for Ag-specific Ab responses[46]. While it was initially proposed that Th2 cells mediate B-cell differentiation[32], it is now clear that this is mediated by Tfh cells. For this reason, Tfh cells are being used as a biomarker for humoral immunity[8-15, 17, 18, 47]. Furthermore, targeting Tfh cells may be a viable approach of enhancing the efficacy of some vaccines and treating diseases caused by autoantibodies[2, 4, 5, 7]. Tracking Tfh cells in humans requires clear understanding of their circulating counterparts. Recent studies revealed heterogeneity within circulating $CD4^+CXCR5^+$ T cells, with $CCR7^{lo}PD1^+$ or $CXCR3^-/CCR6^+PD1^+$ subsets emerging as the most efficient helpers for B-cell differentiation[8, 11, 17]. Notably, frequencies of these subsets correlated with *in vivo* Tfh behavior, such as generating neutralizing Abs following infection/vaccination[12, 17], excessive activity in autoimmunity[8, 9, 13-15], or impaired function in HIV infection[11, 47]. However, other studies found that $CXCR3^+$ cTfh[18] or $CD4^+CXCR5^-$ T cells[19] correlate with Ab responses following vaccination. Thus, despite substantial advances in our understanding of human Tfh biology, this remains controversial and presents a roadblock to translating these findings to the clinic.

We have now examined healthy controls and a wide spectrum of PIDs to delineate quantitative and qualitative consequences of gene mutations on cTfh cells. Consistent with previous studies, cTfh cells were reduced by mutations in *CD40LG*, *ICOS*, *BTK* or *STAT3*[20, 37, 38], as well as *NEMO*, revealing another similarity between disease due to CD40LG and NEMO-deficiency[35]. These deficits are likely a direct effect of the mutation, rather than secondary to infection, as cTfh cells persisted at normal frequencies in STAT1 and IFNGR1/2-deficient individuals. These findings highlight that interactions between $CD4^+$ T cells, DCs and B cells are required for Tfh formation[2-5]. The importance of B cells is also evident from studies reporting diminished cTfh frequencies in patients with severe B-cell deficiency due to *E47*[48] or *NFKB2*[49] mutations. There were trends for fewer cTfh when signaling through IL-10R, IL-21R, IL-12R or TYK2 was compromised, but these differences were not significant. Thus, while these pathways may contribute to Tfh formation, their functional deficiency can be compensated by alternate signals. These findings support data from mice demonstrating that

while IL-21R deficiency reduces Tfh cells under some conditions, a greater defect occurs when both IL-21 and IL-6 is blocked[50, 51]. However, the finding of reduced Tfh cells in IL-10R-deficient humans was unexpected, as IL-10/IL-10R-signalling in mice reduced Tfh formation[4]. While this needs to be confirmed in additional patients, it points to possible species-specific differences in the role of IL-10 in regulating human and murine Tfh cells, which parallels the distinct effects of IL-10 on B-cell differentiation in these species[28].

Our findings also shed substantial light on the functionality and requirements cTfh subsets. $STAT3_{LOF}$, $STAT1_{GOF}$ and $IL21/R_{LOF}$ mutations reduced $CCR6^+$ cTfh cells, skewing the population to a $CXCR3^+IFN\gamma^{++}IL\text{-}10^{low}$ phenotype resembling STAT3-deficient murine Tfh cells that exhibited a Th1 fate[52]. This demonstrates that specific mutations cause qualitative changes in cTfh cells that would not be apparent by quantifying $CD4^+CXCR5^+$ T cells. The mechanism underlying the greater helper function of $CCR6^+$, and corresponding poor function of $CXCR3^+$, cTfh cells is unknown. Although it was reported that $CCR6^+$ cTfh cells produce most IL-21 compared to other subsets[10], this has not been confirmed[11, 13, 17] and is unlikely to explain their ability to promote B-cell differentiation. Rather, based on several lines of evidence including data presented here, we propose this results from reduced IFNγ production. First, exogenous IFNγ reduced Ig production in cultures of human PBMCs[41, 42] or B/Tfh cell co-cultures[43]. This was not observed for *IFNGR1* or *STAT1*-deficient B cells, demonstrating a B-cell intrinsic inhibitory effect of IFNγ. Second, levels of *Mycobacterium leprae*-specific IgG negatively correlated with *in vitro* IFNγ production by PBMCs in response to *M leprae* Ags[40]. Third, serum Ig levels were elevated in many patients with mutations in the IFNγ signaling pathway. Fourth, there were trends for increased frequencies of class switched memory B cells in individuals with *IFNGR1/2*, *IL12RB/TYK2* or $STAT1_{LOF}$ mutations. Overall, IFNγ appears to attenuate Ag-specific Ab responses in humans *in vivo*, thereby explaining the poor helper function of $CXCR3^+$ cTfh cells.

The abundance of IFNγ-producing cTfh cells in $STAT3_{LOF}$ and $STAT1_{GOF}$ patients, together with reduced $CCR6^+$ cTfh cells, would contribute to impaired humoral immune responses[21, 39]. This would

426 be further compounded by reduced cTfh IL-10 production, which induces Ig secretion in the context of
427 Tfh/B cell interactions[22, 28, 43]. $CCR6^+$ cTfh cells are also reduced in HIV-infection[11], where cTfh cells
428 exhibit impaired B-helper function[11, 47, 53]. It would be interesting to determine whether cTfh cells in
429 HIV^+ patients also have skewed cytokine profile with increased IFNγ and reduced IL-10. Our findings
430 regarding IFNγ and impaired humoral immunity explains the paradoxical data of Ueno and colleagues
431 that cTfh cells are reduced in IL-12Rβ1-deficiency, yet these patients exhibit intact if not heightened
432 Ab responses following vaccination or infection[54]. Here, while impaired IL-12R signaling may reduce
433 cTfh cells[54], there will also be less IL-12-induced IFNγ production, resulting in a qualitative change in
434 the cTfh cytokine repertoire, with reduced IFNγ-mediated suppression of B cell responses.
435
436 A defining feature of Tfh cells is elevated PD-1 expression[45]. Although most cTfh cells have low levels
437 of PD-1, a subset with elevated expression corresponds to the most efficient population of B-helper
438 cells[8, 11, 12, 17]. Given our finding of fewer cTfh cells *STAT3*$_{LOF}$ patients, and skewed cTfh subsets in
439 *STAT1*$_{GOF}$ or *STAT3*$_{LOF}$ individuals to a phenotype consistent with reduced B-helper function, it may be
440 predicted that their cTfh cells would have reduced PD-1. However, this was not the case, as PD-1 was
441 significantly increased on cTfh cells in these individuals. This raises several important points. First, as
442 PD-1 engagement impedes Tfh formation *in vivo*[55-57], exaggerated signaling through over-expressed
443 PD-1 on *STAT1*$_{GOF}$ and *STAT3*$_{LOF}$ cTfh cells may further contribute to impaired function[58].
444 Interestingly, PD-L1, a PD-1 ligand, is aberrantly expressed on $CD4^+$ T cells from *STAT1*$_{GOF}$ patients[39],
445 revealing a possible autocrine mechanism of restrained cTfh function. Similarly, while PD-1 is
446 expressed comparably on Tfh cells from healthy donors and HIV-infected individuals, more GC B cells
447 from HIV-infected individuals expressed PD-L1 than healthy donors[53]. Importantly, heightened PD-L1
448 on GC B cells in HIV^+ individuals impaired Tfh cell IL-21 production, suppressing effector function[53].
449 Similarly, B cells expressing the highest levels of PD-L1 most efficiently suppressed Tfh function,
450 including IL-10 production[58]. Thus, dysregulated expression of PD-1 or its ligand(s) could compromise
451 Tfh function[39, 53, 58]. Second, our data caution against solely quantifying cTfh cells with respect to PD-1
452 as a biomarker for humoral immunity. While this correlation has been established in vaccination[12], HIV

infection[11, 17] and autoimmunity[8, 9, 15, 16], it may be misleading in some monogenic PIDs. Thus, to infer defects in cTfh cells in these and other immunopathologies, they should be assessed for phenotype *and* function. This is highlighted by a recent study reporting impaired cTfh function in elderly, but not young, individuals even though cTfh cells from these groups exhibited similar IL-21 and ICOS expression[12]. Third, STAT3 signaling during Tfh differentiation is required to not only generate the $CCR6^+$ subset, but also regulate PD-1 to minimize suppression through PD-1/PD-L1 interactions. The mechanism underlying STAT3-mediated PD-1 repression is unknown but may involve IL-21 as IL-21R deficiency partially recapitulated this defect. Interestingly, STAT3 can protect Tfh cells from the inhibitory effects of IFNα[52]. Since IFNα induces PD-1 on murine $CD4^+$ T cells[59], elevated PD-1 on $STAT3_{LOF}$ cTfh cells may result from heightened IFNα signaling in the absence of STAT3.

Intriguingly, our findings also revealed overlapping cellular phenotypes due to $STAT3_{LOF}$ and $STAT1_{GOF}$ mutations including reduced production of Th17 cytokines by memory $CD4^+$ T cells, skewed differentiation of and PD-1 expression by cTfh cells, and fewer memory B cells. These shared functional defects are consistent with similar clinical features of these distinct molecular entities, such as mucocutaneous candidiasis and impaired Ab responses[21, 35, 39]. While the mechanism underlying these common features is unknown, the data suggest that hypermorphic STAT1 suppresses STAT3, creating a situation mimicking STAT3-deficiency in STAT3-sufficient cells. Notably, there was greater variability in these defects in $STAT1_{GOF}$ patients, suggesting some mutations are more deleterious than others, consistent with the broader clinical phenotypes of affected individuals. Further studies will be required to elucidate exactly how $STAT1_{GOF}$ intersects with STAT3 to regulate function. Collectively, our study provides important insights into the molecular requirements and signaling pathways regulating human Tfh function and mechanisms for poor humoral immunity in some PIDs, such as elevated IFNγ but reduced IL-10 production and aberrant PD-1 expression. These defects would co-operate to limit cTfh function by suppressing B-cell help via IFNγ[40-44] and PD-1 engagement impairing IL-10 and IL-21 production[53, 58]. Identifying these pathways potentially provides opportunities to manipulate Tfh function not only in immunodeficiency, but also autoimmunity.

Table 1: Tregs and $CD4^+CXCR5^-$ Memory subsets in human PIDs

Genetic diseases	Tregs	Total memory	$CD4^+CXCR5^-$ memory cells			
			Th1 ($CXCR3^+$ $CCR6^-$)	Th2 ($CXCR3^-$ $CCR6^-$)	Th17 ($CXCR3^-$ $CCR6^+$)	Th1/17 ($CXCR3^+$ $CCR6^+$)
Controls (n=59-64)	6.2 ± 0.4	51.4 ± 2.0	15.2 ± 1.1	47.0 ± 2.0	23.5 ± 1.2	14.2 ± 1.2
STAT3 (n=19-22)	7.4 ± 0.7	29.5 ± 3.9 ***	34.9 ± 4.5 ****	45.9 ± 5.3	5.1 ± 0.7 ****	10.0 ± 1.6
$STAT1_{GOF}$ (n=15-17)	5.7 ± 1.0	43.8 ± 4.4	29.5 ± 4.4 *	54.8 ± 5.0	6.7 ± 0.7 ****	10.1 ± 2.2
$STAT1_{LOF}$ (n=6)	7.5 ± 1.1	38.0 ± 6.1	22.4 ± 6.2	37.9 ± 7.6	20.5 ± 4.4	19.2 ± 5.0
IL-21/R (n=5)	5.8 ± 1.1	32.2 ± 7.1	25.1 ± 11.2	57.6 ± 10.5	12.0 ± 3.3	5.3 ± 1.1
IL10R (n=2)	10.8 ± 3.2	40.9 ± 10.1	36.5 ± 34.6	38.1 ± 8.9	29.6 ± 28.4	4.1 ± 0.5
CD40LG (n=8)	3.6 ± 0.8	25.9 ± 3.8 **	6.9 ± 2.0	70.7 ± 6.0 *	17.9 ± 2.8	4.6 ± 2.0
NEMO (n=9-12)	9.0 ± 1.7	40.2 ± 7.6	2.6 ± 1.6	85.2 ± 4.0 ****	10.7 ± 2.3 **	1.4 ± 0.7 **
BTK (n=6-11)	4.9 ± 0.7	30.0 ± 5.2 *	12.3 ± 3.6	71.4 ± 6.5	11.2 ± 1.8 *	5.2 ± 1.6
ICOS (n=6)	8.8 ± 0.9	53.1 ± 7.1	30.5 ± 6.3	50.1 ± 4.5	10.4 ± 1.4 *	8.9 ± 1.7
IL12RB1 (n=3)	4.1 ± 1.8	16.4 ± 3.0 *	12.8 ± 2.1	67.8 ± 7.6	16.3 ± 8.4	3.0 ± 1.2
IFNGR1/2 (n=4-7)	15.8 ± 5.0 ****	49.1 ± 7.0	15.7 ± 3.6	55.9 ± 5.8	16.8 ± 2.6	11.5 ± 2.9
TYK2 (n=4)	5.4 ± 0.6	56.2 ± 10.5	18.3 ± 5.6	43.4 ± 5.3	18.8 ± 4.6	19.5 ± 5.1

PBMCs from healthy controls or patients with specific gene mutations were labeled with mAb against CD4, CD127, CD25, CD45RA, CXCR5, CXCR3 and CCR6. The proportions of Tregs amongst all $CD4^+$ T cells, the proportions of memory cells within the non-Treg cell population, and the proportions of $CD4^+CD45RA^-CXCR5^-$ memory cells with a Th1 ($CXCR3^+CCR6^-$), Th2 ($CXCR3^-CCR6^-$), Th17 ($CXCR3^-CCR6^+$) or Th1/17 ($CXCR3^+CCR6^+$) phenotype were then determined by flow cytometric analysis. The values represent mean ± SEM for the indicated number of healthy controls or patients. Significant differences (one-way ANOVA) between healthy controls and patients are indicated.

Figure Legends

Figure 1: Identification of effector subsets within populations of human memory CD4$^+$ T cells

(A-H): Naïve and memory CD4$^+$ T cells were sorted from healthy controls and stimulated with TAE (anti-CD2/CD3/CD28) beads. Secretion/expression of the indicated cytokines (A-D; mean ± SEM; n=25-27) or transcription factors (E-H; mean ± SEM; n=12-19) were determined after 5 days.

(I) Resolving blood naïve (CD45RA$^+$CXCR5$^-$), non-Tfh memory (CD45RA$^-$CXCR5$^-$) and cTfh (CD45RA$^-$CXCR5$^+$) cells from healthy controls. (J, K) CXCR3 and CCR6 expression on naive and non-Tfh memory cells; (K) depicts % of Th1 (CXCR3$^+$CCR6$^-$), Th2 (CXCR3$^-$CCR6$^-$), Th17 (CXCR3$^-$ CCR6$^+$) and Th1/17 (CXCR3$^+$CCR6$^+$) subsets amongst the non-Tfh memory population (n=55-58).

(L-S) Secretion/expression of the indicated cytokines (L-O; mean ± SEM; n=10-15) or transcription factors (P-S; mean ± SEM; n=7-10) by naïve, Th1, Th2, Th17, Th1/Th17 and cTfh subsets after 5 days of culture with TAE beads.

Figure 2: Delineation of subsets of human circulating Tfh cells

(A, B) cTfh cells were defined as CD45RA$^-$CXCR5$^+$ CD4$^+$ T cells. CXCR3$^+$CCR6$^-$, CXCR3$^-$CCR6$^-$, CXCR3$^-$CCR6$^+$ and CXCR3$^+$CCR6$^+$ subsets were then detected (n=55-58).

(C)-(G): Naïve, non-Tfh memory, total cTfh and CXCR3$^+$CCR6$^-$, CXCR3$^-$CCR6$^+$ CXCR3$^-$CCR6$^-$ (DN), and CXCR3$^+$CCR6$^+$ (DP) cTfh subsets were sorted from peripheral blood and stimulated with TAE beads. After 5 days, secretion or expression of the indicated cytokines were determined (n=5-6).

(H)-(J): purified subsets of blood CD4$^+$ T cells were co-cultured with allogeneic B cells and TAE beads. IgM, IgG and IgA secretion was determined after 7 days.

Figure 3: Defects in cytokine production due to monogenic mutations causing different PIDs.

(A-D) memory CD4$^+$ T cells were sorted from healthy controls or patients with the indicated gene mutations then stimulated with TAE beads for 5 days. (A) IFNγ, (B) IL-4, IL-5, IL-13 (Th2 cytokines), (C) IL-17A, IL-17F, IL-22 (Th17 cytokines), (D) IL-10, IL-21 (Tfh cytokines) production was determined (mean ± SEM). Controls: n=29-32; *STAT3*$_{LOF}$: n=6; *STAT1*$_{GOF}$: n=7-9; *STAT1*$_{LOF}$: n=5-7;

IL21/R: n=5; *CD40LG*: n=4; *NEMO*: n=5-6; *BTK*: n=6; *ICOS*: n=4; *IL12R*: n=5; *IFNGR1/2*: n=6; *TYK2*: n=3.

(E-F) proportions of total (E) and class switched (F) memory B cells in healthy controls and patients were determined.

Figure 4: Effect of monogenic mutations on the generation of human cTfh cells

(A, B) cTfh cells were identified amongst the population of non-Tregs as $CD45RA^{-}CXCR5^{+}$ $CD4^{+}$ T cells. cTfh frequencies in healthy donors (n=64) and patients with mutations in *STAT3* (n=23), *STAT1* (GOF [n=16] and LOF [n=6]), *IL-21R/IL21* (n=5), *IL10R* (n=2), *CD40LG* (n=10), *NEMO* (n=11), *BTK* (n=11), *ICOS* (n=6), *IL12RB1* (n=8), *IFNGR1/2* (n=4) or *TYK2* (n=4) were determined.

(C, D) proportions of $CXCR3^{+}CCR6^{-}$, $CXCR3^{-}CCR6^{+}$, $CXCR3^{-}CCR6^{-}$ or $CXCR3^{+}CCR6^{+}$ subsets amongst total cTfh cells.

(E) Production of IL-17A, IFNγ, IL-21 and IL-10 by sorted cTfh cells from healthy donors, $STAT3_{LOF}$ or $STAT1_{GOF}$ patients (mean ± SEM; n=3-4).

Figure 5: IFNγ suppresses B-cell differentiation *in vitro* and *in vivo*

(A) Phosphorylation of STAT1 or STAT3 in EBV-LCLs from healthy controls (red histograms) or individuals with *IFNGR2* (blue) or *STAT1* (green) mutations in response to IFNγ or IL-21. Grey histogram: response of unstimulated cells from healthy controls.

(B) cTfh cells from healthy controls were co-cultured with allogeneic normal, IFNγR1- or STAT1-deficient B cells in the absence or presence of exogenous IFNγ. Ig secretion was determined after 7 days. Values represent the mean % Ig secretion (± SEM) in the presence of IFNγ relative to it absence (defined as 100%).

(C, D) serum levels of IgG and IgM in individuals with *IFNGR1/IFNGR2*, *IL12RB1* or $STAT1_{LOF}$ mutations. The solid upper and lower lines correspond to normal serum Ig levels in age-matched controls

Figure 6: Aberrant expression of PD-1 on cTfh cells from patients with $STAT3_{LOF}$, $STAT1_{GOF}$ and $IL21/R_{LOF}$ mutations

(A, B) PD-1 expression on naïve, non-Tfh memory and cTfh cells. Contour and histogram plots in (A) represent individual healthy controls or patients. The graph (B) depicts the average PD-1 expression (MFI ± SEM) for all individuals tested. (C, D) PD-1 expression on $CXCR3^{+}CCR6^{-}$, $CXCR3^{-}CCR6^{+}$, $CXCR3^{-}CCR6^{-}$ and $CXCR3^{+}CCR6^{+}$ cTfh subsets cells from healthy controls (n=36) or patients with $STAT3_{LOF}$ (n=12), $STAT1_{GOF}$ (n=14) and $IL21/R_{LOF}$ mutations (n=5).

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Figure 1

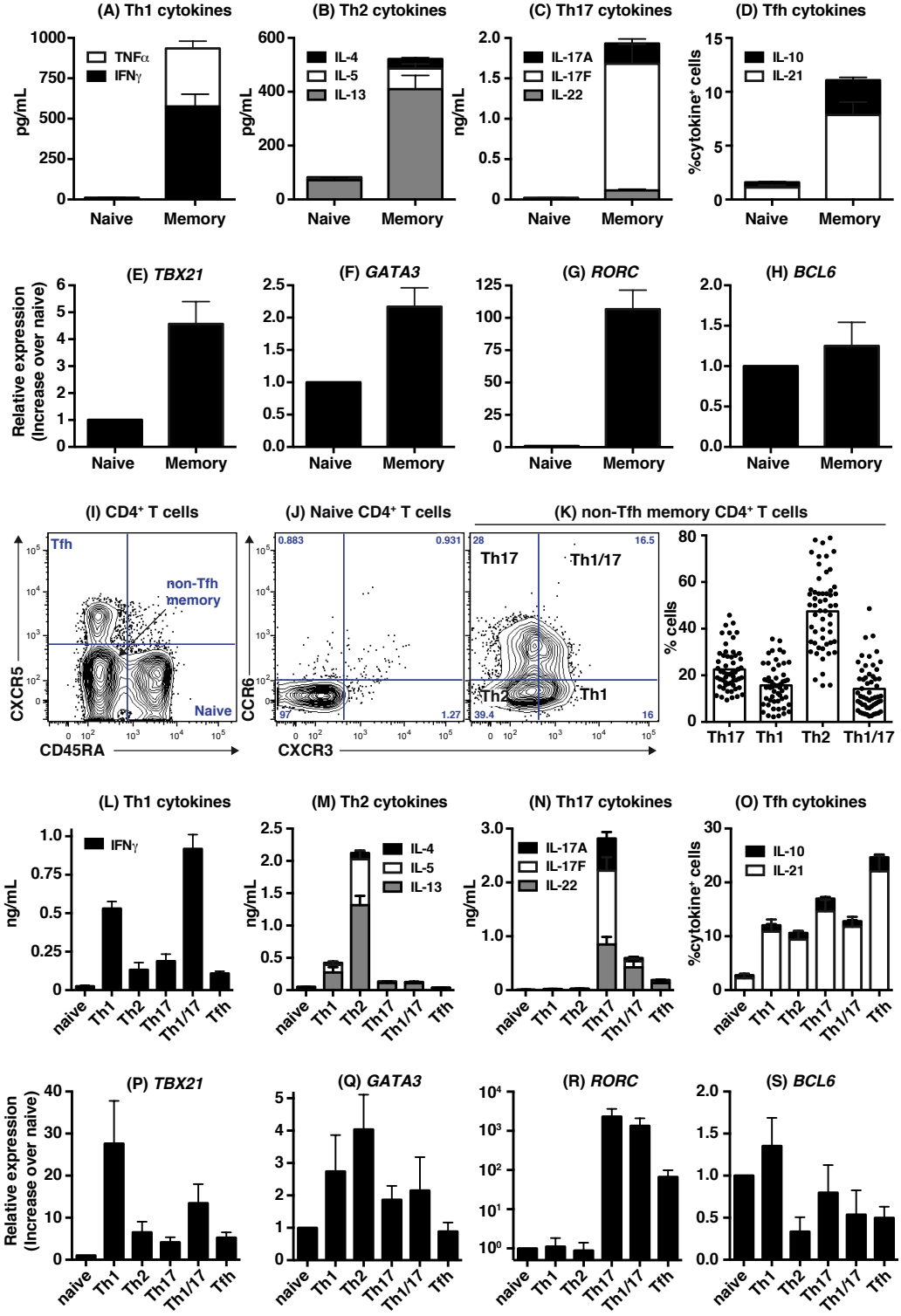

(A) Th1 cytokines
pg/mL
TNFα
IFNγ
Naive
Memory
(B) Th2 cytokines
pg/mL
IL-4
IL-5
IL-13
(C) Th17 cytokines
ng/mL
IL-17A
IL-17F
IL-22
(D) Th17 cytokines
%cytokine+ cells
IL-10
IL-21
(E) TBX21
Relative expression (Increase over naive)
(F) GATA3
(G) RORC
(H) BCL6
(I) CD4+ T cells
Tfh
non-Tfh memory
Naive
CXCR5
CD45RA
(J) Naive CD4+ T cells
0.883
0.931
97
1.27
CCR6
CXCR3
(K) non-Tfh memory CD4+ T cells
28
16.5
Th17
Th1/17
Th2
Th1
39.4
16
% cells
(L) Th1 cytokines
(M) Th2 cytokines
(N) Th17 cytokines
(O) Th17 cytokines
naive
Th1
Th2
Th17
Th1/17
Tfh
(P) TBX21
(Q) GATA3
(R) RORC
(S) BCL6

Figure 2

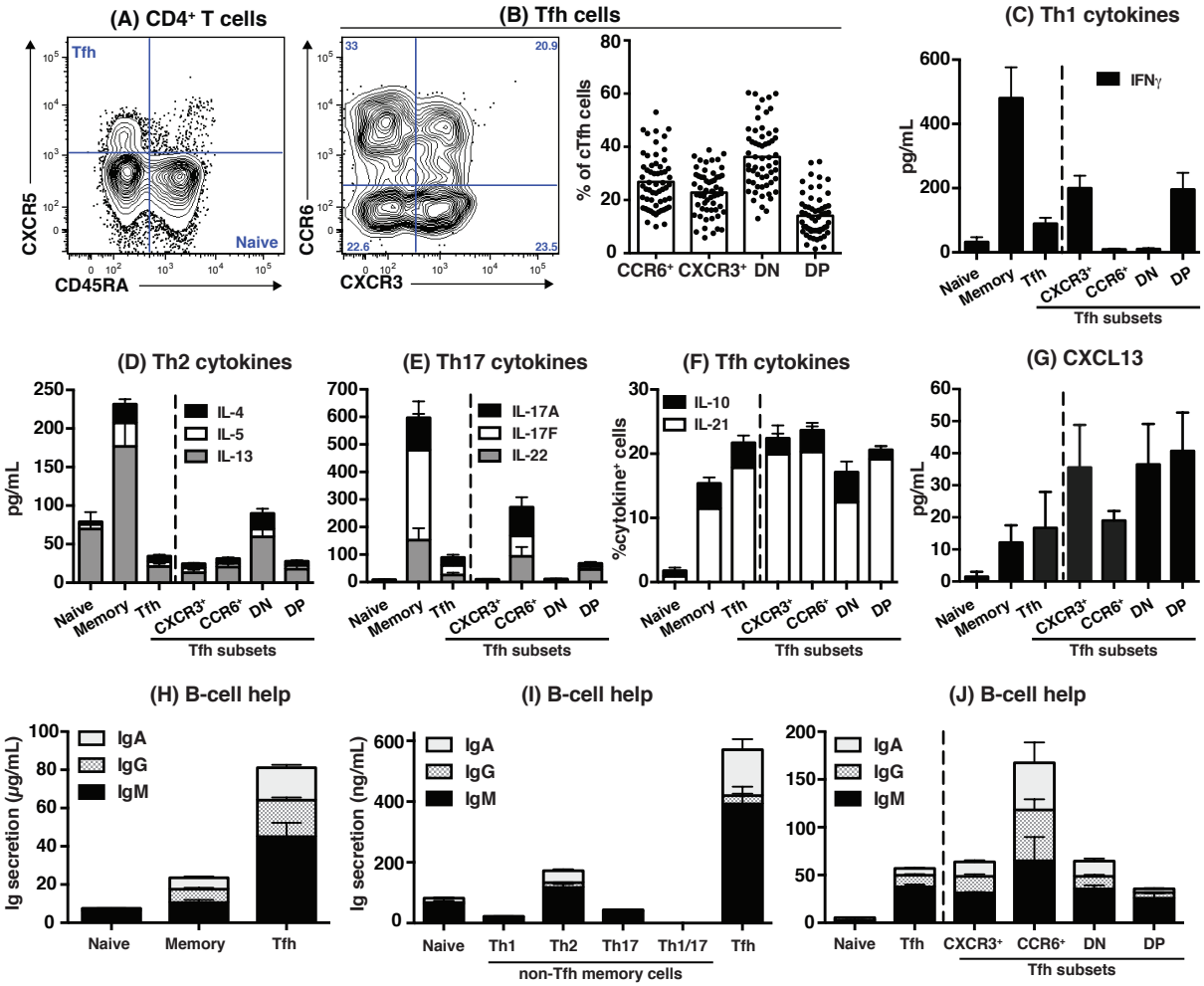
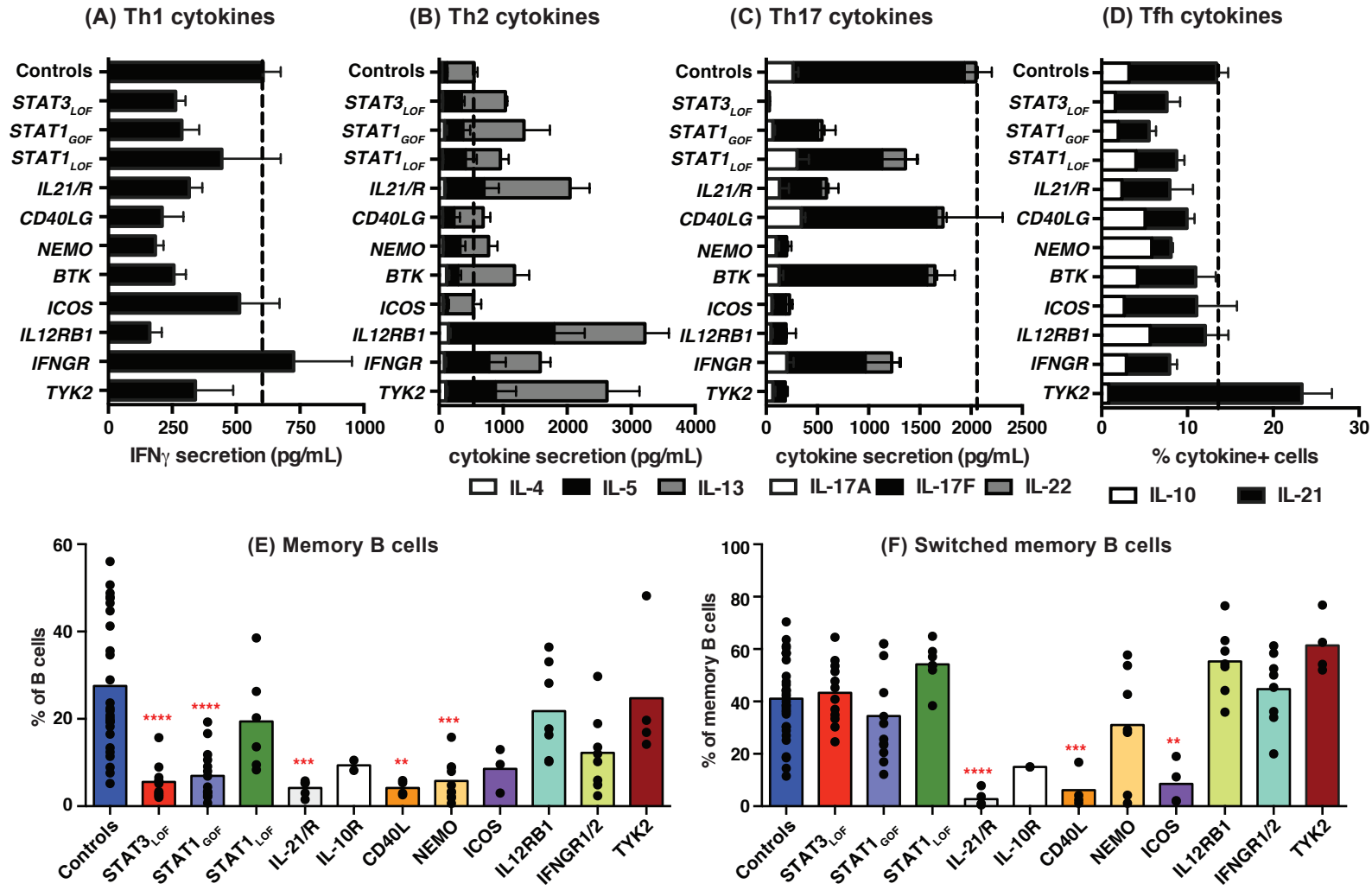

(A) CD4+ T cells
Tfh
Naive
CXCR5
CD45RA
(B) Tfh cells
33
20.9
22.6
23.5
CCR6
CXCR3
% of cTfh cells
CCR6+ CXCR3+ DN DP
(C) Th1 cytokines
IFNγ
pg/mL
Naive Memory Tfh CXCR3+ CCR6+ DN DP
Tfh subsets
(D) Th2 cytokines
IL-4
IL-5
IL-13
pg/mL
Naive Memory Tfh CXCR3+ CCR6+ DN DP
Tfh subsets
(E) Th17 cytokines
IL-17A
IL-17F
IL-22
Naive Memory Tfh CXCR3+ CCR6+ DN DP
Tfh subsets
(F) Tfh cytokines
IL-10
IL-21
%cytokine+ cells
Naive Memory Tfh CXCR3+ CCR6+ DN DP
Tfh subsets
(G) CXCL13
pg/mL
Naive Memory Tfh CXCR3+ CCR6+ DN DP
Tfh subsets
(H) B-cell help
IgA
IgG
IgM
Ig secretion (μg/mL)
Naive Memory Tfh
(I) B-cell help
IgA
IgG
IgM
Ig secretion (ng/mL)
Naive Th1 Th2 Th17 Th1/17 Tfh
non-Tfh memory cells
(J) B-cell help
IgA
IgG
IgM
Naive Tfh CXCR3+ CCR6+ DN DP
Tfh subsets

Figure 3


(A) Th1 cytokines
(B) Th2 cytokines
(C) Th17 cytokines
(D) Tfh cytokines
Controls
STAT3LOF
STAT1GOF
STAT1LOF
IL21/R
CD40LG
NEMO
BTK
ICOS
IL12RB1
IFNGR
TYK2
IFNγ secretion (pg/mL)
cytokine secretion (pg/mL)
cytokine secretion (pg/mL)
% cytokine+ cells
IL-4
IL-5
IL-13
IL-17A
IL-17F
IL-22
IL-10
IL-21

(E) Memory B cells
% of B cells
Controls
STAT3LOF
STAT1GOF
STAT1LOF
IL-21/R
IL-10R
CD40L
NEMO
ICOS
IL12RB1
IFNGR1/2
TYK2

(F) Switched memory B cells
% of memory B cells
Controls
STAT3LOF
STAT1GOF
STAT1LOF
IL-21/R
IL-10R
CD40L
NEMO
ICOS
IL12RB1
IFNGR1/2
TYK2

Figure 4

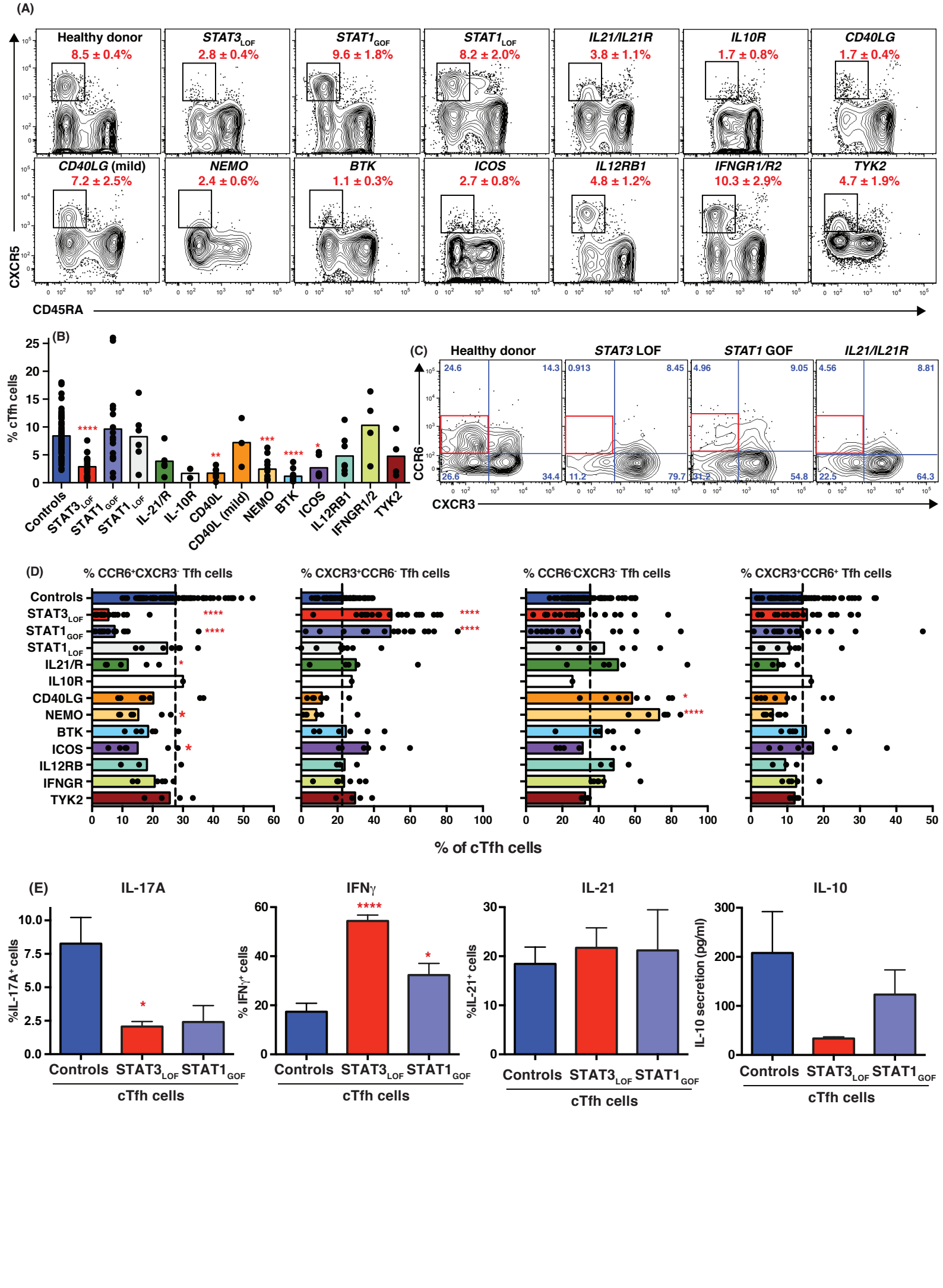

(A)
Healthy donor
8.5 ± 0.4%
STAT3LOF
2.8 ± 0.4%
STAT1GOF
9.6 ± 1.8%
STAT1LOF
8.2 ± 2.0%
IL21/IL21R
3.8 ± 1.1%
IL10R
1.7 ± 0.8%
CD40LG
1.7 ± 0.4%
CD40LG (mild)
7.2 ± 2.5%
NEMO
2.4 ± 0.6%
BTK
1.1 ± 0.3%
ICOS
2.7 ± 0.8%
IL12RB1
4.8 ± 1.2%
IFNGR1/R2
10.3 ± 2.9%
TYK2
4.7 ± 1.9%
CXCR5
CD45RA
(B)
% cTfh cells
Controls
STAT3LOF
STAT1GOF
STAT1LOF
IL-21/R
IL-10R
CD40L
CD40L (mild)
NEMO
BTK
ICOS
IL12RB1
IFNGR1/2
TYK2
(C)
Healthy donor
STAT3 LOF
STAT1 GOF
IL21/IL21R
24.6
14.3
26.6
34.4
0.913
8.45
11.2
79.7
4.96
9.05
31.2
54.8
4.56
8.81
22.5
64.3
CCR6
CXCR3
(D)
% CCR6+CXCR3- Tfh cells
% CXCR3+CCR6- Tfh cells
% CCR6-CXCR3- Tfh cells
% CXCR3+CCR6+ Tfh cells
Controls
STAT3LOF
STAT1GOF
STAT1LOF
IL21/R
IL10R
CD40LG
NEMO
BTK
ICOS
IL12RB
IFNGR
TYK2
% of cTfh cells
(E)
IL-17A
IFNγ
IL-21
IL-10
%IL-17A+ cells
% IFNγ+ cells
%IL-21+ cells
IL-10 secretion (pg/ml)
Controls
STAT3LOF
STAT1GOF
cTfh cells

Figure 5

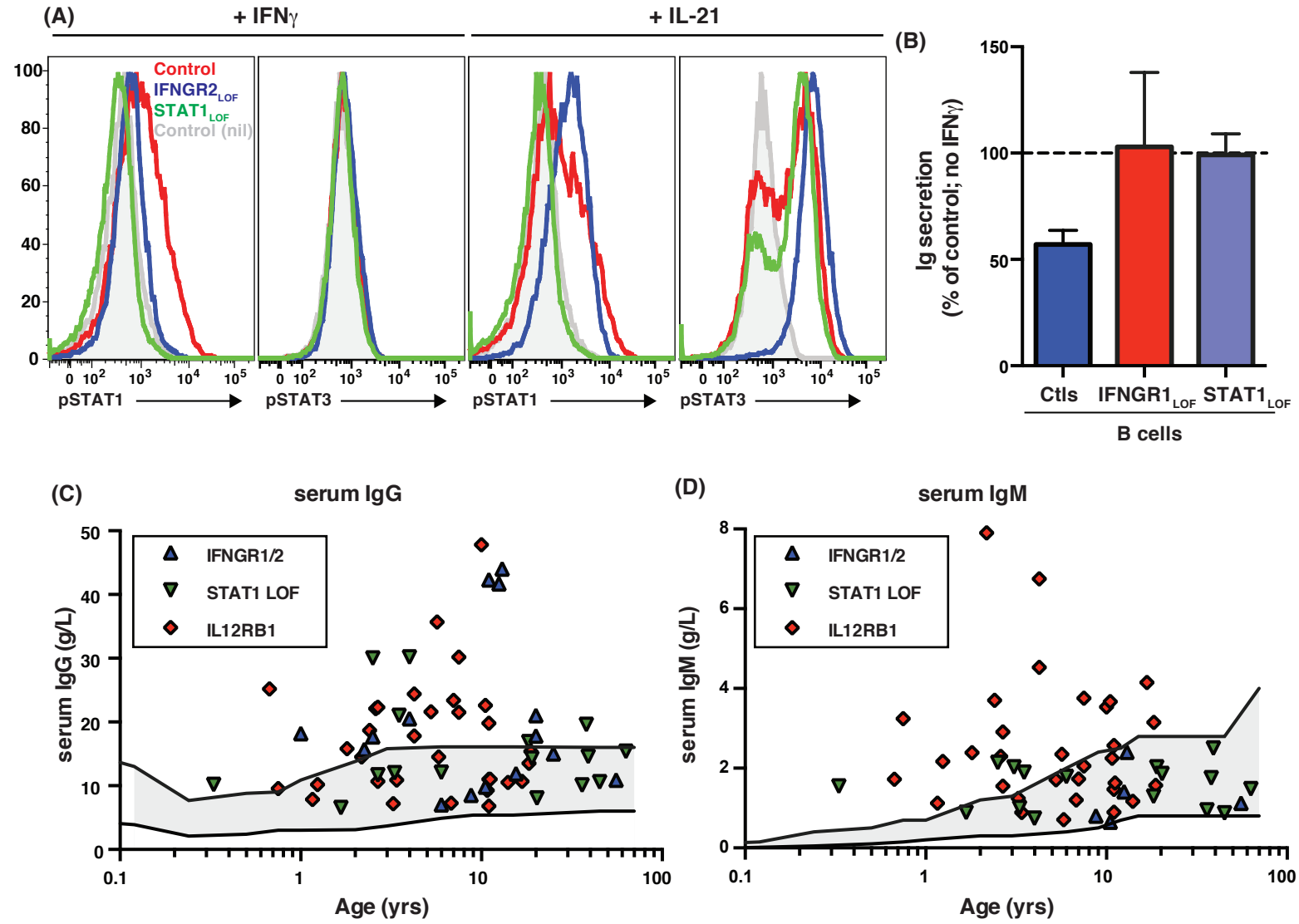
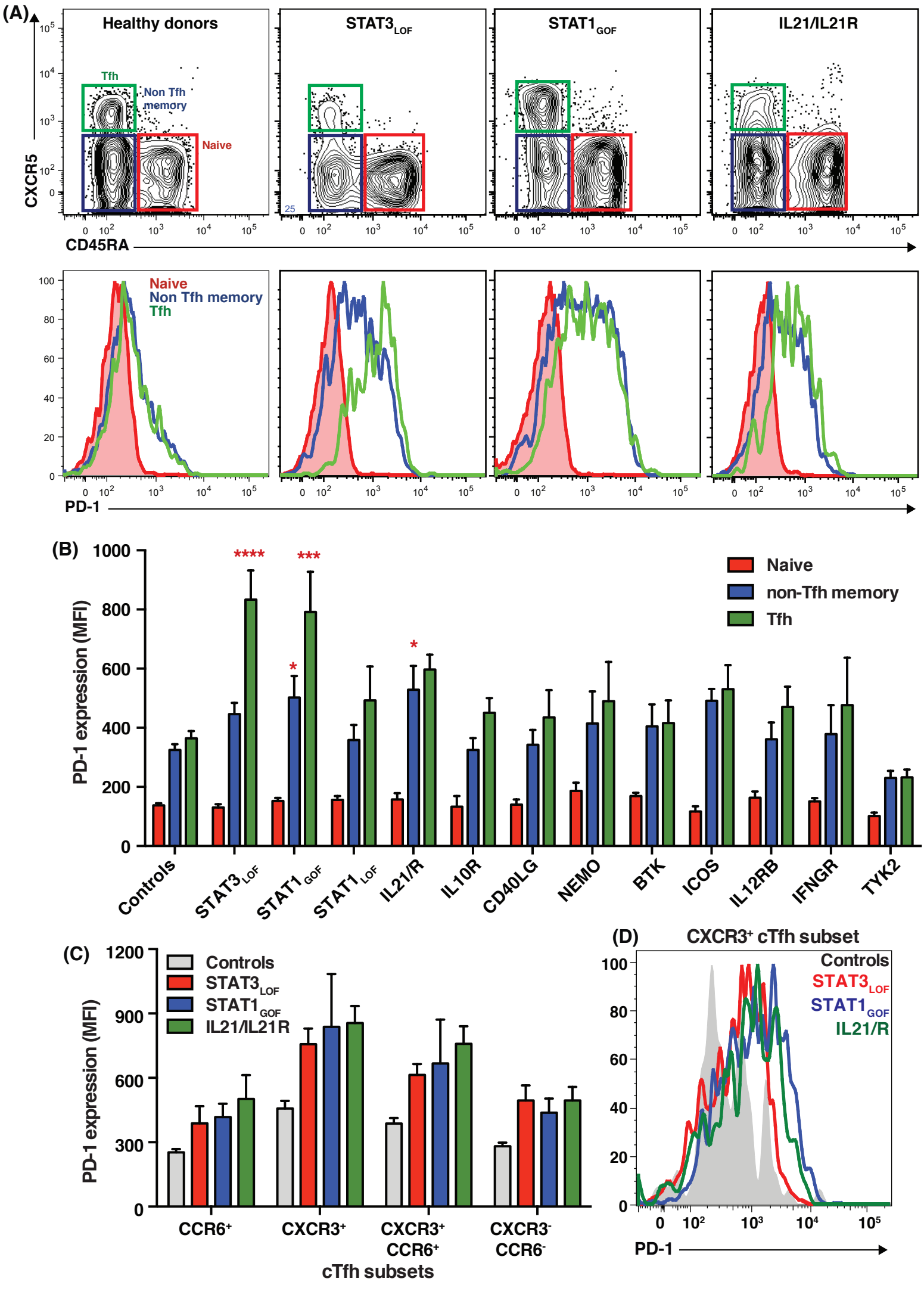

(A)
+ IFNγ
+ IL-21
Control
IFNGR2LOF
STAT1LOF
Control (nil)
pSTAT1
pSTAT3
pSTAT1
pSTAT3
(B)
Ig secretion (% of control; no IFNγ)
Ctls
IFNGR1LOF
STAT1LOF
B cells
(C)
serum IgG
IFNGR1/2
STAT1 LOF
IL12RB1
serum IgG (g/L)
Age (yrs)
(D)
serum IgM
IFNGR1/2
STAT1 LOF
IL12RB1
serum IgM (g/L)
Age (yrs)

Figure 6


(A)
Healthy donors
STAT3LOF
STAT1GOF
IL21/IL21R
Tfh
Non Tfh memory
Naive
CXCR5
CD45RA
PD-1
(B)
PD-1 expression (MFI)
Naive
non-Tfh memory
Tfh
Controls
STAT3LOF
STAT1GOF
STAT1LOF
IL21/R
IL10R
CD40LG
NEMO
BTK
ICOS
IL12RB
IFNGR
TYK2
(C)
Controls
STAT3LOF
STAT1GOF
IL21/IL21R
PD-1 expression (MFI)
CCR6+
CXCR3+
CXCR3+ CCR6+
CXCR3- CCR6-
cTfh subsets
(D)
CXCR3+ cTfh subset
Controls
STAT3LOF
STAT1GOF
IL21/R
PD-1