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

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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

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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*, *IL10R*, *CD40LG*, *NEMO*, *ICOS* or *BTK* reduced cTfh frequencies. *STAT3*, *IL21/R* LOF and *STAT1* gain-of function 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* LOF 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 the integrated signaling pathways required for their generation, maintenance and effector function, and explain compromised humoral immunity in some PIDs.

Keywords

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

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 much about Tfh cells, our understanding of human Tfh biology remains incomplete, 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

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decreased in HIV-infection[11]. Similarly, $CD4^+CXCR5^+PD1^+$ $CXCR3^-$ T cells were identified as the circulating counterpart of lymphoid Tfh cells and their frequencies positively correlated with neutralizing Abs in HIV infection[17]. Although these studies generally confirmed that PD-$1^+CXCR3^-/CCR6^+$ cTfh cells are a reliable correlate of Tfh cells in human lymphoid tissue, the identity of the circulating B-helper human $CD4^+$ T cells remains contentious, as other studies demonstrated that $CXCR5^+CXCR3^+$ *or* even $CXCR5^-$ $CD4^+$ T cells exhibit detectable B-helper function[11, 15, 19, 20] and correlate with influenza vaccine responsiveness[18, 19]

To assess the molecular requirements for the generation and function of human cTfh cells, we investigated >110 individuals with 14 different monogenic mutations that underlie primary immunodeficiencies (PIDs). Our findings identify mutations that have distinct quantitative and/or qualitative effects on human cTfh cells, providing an explanation for humoral immune defects in some PIDs as well as insights into mechanisms regulating human Tfh differentiation and function.

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.

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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 (50×10^3/200μl/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. 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 significantly higher levels of *TBX21* (T-bet), *GATA3* and *RORC* (RORγt) than naïve cells (Figure 1E–G). There was no significant 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].

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Delineation of memory CD4$^+$ T cells into defined populations of Th1, Th2, Th17 and cTfh 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 cTfh 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, of the cytokines examined, IL-10 and IL-21 were the only ones produced by cTfh cells at levels significantly greater than naïve cells (Figure 1L–O; $p<0.005$), consistent with the B-cell helper function of these cytokines[28]. The proportions of cTfh cells expressing IL-10 and IL-21 were also significantly greater than naïve cells ($p<0.005$), Th1 cells ($p<0.05$), Th2 cells ($p<0.05$) and Th1/Th17 cells ($p<0.05$), but not Th17 cells. 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 inflamed tissues[29]. *TBX21*, *GATA3* and *RORC* expression also significantly correlated with their respective production of Th1, Th2 and Th17 cytokines (Figure 1P–S).

Having successfully characterized Th1, Th2, Th17 and cTfh cells in peripheral blood, we next investigated cTfh cells using a similar approach: dividing them into subpopulations according to CCR6 and CXCR3[10] (Figure 2A, B) and assessing cytokine production and B-cell helper function. The different cTfh subsets produced lower levels of cytokines than memory (CD45RA$^-$CXCR5$^-$) cells, however IL-4 and IL-13 were enriched in the CXCR3$^-$CCR6$^-$ (double negative; DN) subset, IL-17A/F and IL-22 in the CCR6$^+$CXCR3$^-$ (hereafter referred to as CCR6$^+$) subset, and IFNγ in the CXCR3$^+$CCR6$^-$ ("CXCR3$^+$") and CXCR3$^+$CCR6$^+$ (double positive; DP) subsets (Figure 2C–F). In contrast, IL-10, IL-21 (Figure 2F) and CXCL13 (Figure 2G), which are produced by Tfh cells[25, 30], were comparable in all cTfh subsets. Total cTfh cells induced greater B-cell differentiation than naïve and memory cells (Figure 2H) as well as Th1, Th2, Th17 and Th1/17 memory cell subsets (Figure 2I). Amongst cTfh subsets, the CCR6$^+$ population consistently induced most Ig secretion by co-cultured B cells over the CXCR3$^+$, DN and DP subsets (Figure 2J). Thus, consistent with recent studies, blood CD4$^+$CXCR5$^+$ T cells have Tfh function, with the CCR6$^+$ subset being most effective[10, 11, 17].

Mutations causing PIDs impact CD4$^+$ T cell function and B-cell memory formation

These data established the ability to identify effector functions of human CD4$^+$ T cell subsets, thereby providing a framework to determine consequences of gene mutations on CD4$^+$ T cell differentiation *in vivo*. We examined CD4$^+$ T cell subsets and function in PID patients with mono- or bi-allelic mutations in surface receptors or cytokine signaling

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pathways that may impact humoral immunity in the context of Tfh formation and long-lived protective Ab responses[4].

LOF mutations in *CD40LG*, *NEMO*, *IL12RB1* or *TYK2* compromised IFNγ production by memory $CD4^+$ T cells (Figure 3A, see Table E1 in this articles Online Repository), consistent with known roles in eliciting Th1 responses[31]. Production of Th2 cytokines was enhanced by *IL12RB1*, *IFNGR1/2* or *TYK2* LOF mutations, confirming Th1 cells suppress Th2 cells[32], as well as by LOF mutations in *IL10R*, *IL21/R* or *STAT3* and GOF mutations in *STAT1* (Figure 3B, Table E1). This revealed novel roles for IL-10, 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; Table E1). *STAT3*, *NEMO*, *ICOS*, *IL12RB1* or *TYK2* LOF mutations strongly diminished Th17 cytokines (Figure 3C) and *RORC* expression (not shown). *STAT1* GOF and *IL21/R* LOF mutations also reduced Th17 cytokines but to a lesser extent than these other mutations (Figure 3C, Table E1). These findings are consistent with susceptibility of some individuals with these mutations to *Candida* infection (*STAT3*$_{LOF}$, *STAT1*$_{GOF}$, *IL21R*, *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, Table E1), mirroring intact immunity against *Candida* spp in these patients[31, 35], while *IL10R* mutations resulted in excessive production of IL-17A/F and IL-22 (Table E1). IL-10 production was compromised by *STAT3*, *TYK2, IL10R* and *IL21R* LOF and *STAT1* GOF mutations, but unaffected by other mutations, while IL-21 was reduced by *STAT1* GOF, *IL21R*, *IFNGR1/2* and *NEMO* LOF mutations (Figure 3D, Table E1).

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*, *IL10R*, *ICOS*, *CD40LG* and *NEMO* LOF, and *STAT1* GOF, mutations (Figure 3E). It is thus likely that signaling via IL-10R and IL-21R underlie the paucity of memory B cells in STAT3-deficient patients. Although memory B cells were reduced in patients with *STAT3* LOF, *STAT1* GOF, *IL10R* and *NEMO* mutations, 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 most of 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 *BTK*-deficient 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, Table E1). The reduction in $CCR6^+$ $CD4^+$ T cells due to *STAT3*

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mutations likely reflects the inability of mutant STAT3 to induce *CCR6*, which has been shown to be a direct target of STAT3 in mice[37]. 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, 38, 39] - as well as in *IL10R* or *NEMO* (Figure 4A, B). In our cohort of *CD40LG*-deficient patients, three had clinically milder disease than those with classic HIGM (ie detectable levels of IgG/A, less severe infections, later age of diagnosis). These patients had normal cTfh frequencies, thus correlating with disease severity (Figure 4A, B). While there were trends for fewer cTfh when signaling through IL-21R, IL-12R or TYK2 was compromised, these differences were not significant (Figure 4A, B). None of the other mutations examined impacted Tfh formation, as determined by quantifying circulating $CD4^+CXCR5^+$ T cells. In addition to Tfh cells that promote B-cell differentiation, there is also a population of cells termed T follicular regulatory (Tfr) cells that restrain Tfh function to regulate production of specific Abs[40]. These cells can be defined as $CXCR5^+$ cells within the population of Tregs. Although specific gene mutations impacted the formation of cTfh cells (Figure 4A, B), the proportions of $CXCR5^+$ Tregs (putative Tfr cells) in all groups of PID patients were similar to those in healthy controls. Thus, the effects of the gene mutations studied here are specific to cTfh cells, suggesting distinct origins of cTfh and cTfr cells. This is consistent with Tfr cells arising from Tregs, which are largely unaffected by the mutations studied here (Table 1), rather than Tfh cells[40].

$STAT3_{LOF}$ 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 DN cTfh cells, while the DP 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,

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predominantly yielding a population with limited B-cell helper capacity, which is consistent with impaired humoral immunity in *STAT3*$_{LOF}$ and *STAT1*$_{GOF}$ individuals[21, 41].

IFNγ restrains Tfh-induced B cell differentiation

IFNγ can impede Ig secretion by human PBMCs[42–46]. Taken together with 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, it was important to demonstrate that IFNγ directly signals in human B cells. IFNγ induced STAT1 phosphorylation in EBV-LCLs from healthy controls, but not *STAT1* or *IFNGR*-deficient individuals, whilst IL-21-induced STAT3 activation was unaffected by such mutations (Figure 5A). 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 also 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 Tfh cells[24, 47], 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 increased 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 significantly greater expression on CXCR3$^+$ and CXCR3$^+$CCR6$^+$ subsets compared to CCR6$^+$ and CCR6$^-$CXCR3$^-$ subsets (Figure 6C). Despite this, PD-1 was significantly higher on all cTfh subsets from *STAT3*$_{LOF}$ patients, and for most cTfh subsets

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from *STAT1*$_{GOF}$ and *IL21R*-deficient patients compared to controls (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 are required for Ag-specific Ab responses[48]. While it was initially proposed that Th2 cells mediated 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, 49]. Furthermore, targeting Tfh cells may constitute 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, 49]. However, other studies found that $CXCR3^+$ cTfh[18] or $CD4^+CXCR5^-$ T cells[19] predicted Ab responses following vaccination. Thus, despite substantial advances in our understanding of human Tfh biology, the exact correlates between successful Ab responses and $CD4^+$ T cells subsets remain controversial and present a roadblock to translating these findings to the clinic.

Here we examined 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, 38, 39]. Our finding of reduced cTfh cells in NEMO-deficiency revealed another similarity between disease due to *CD40LG* and *NEMO* mutations, consistent with NEMO being required for CD40 signalling[35]. The significant reduction in cTfh cells in IL-10R-deficiency was unexpected, as IL-10/IL-10R-signalling in mice reduced Tfh formation[4]. This 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]. The deficits in cTfh cells in these groups of PID patients are likely a direct effect of the mutation, rather than being secondary to infection, as cTfh cells were detected at normal frequencies in *STAT1* and *IFNGR1/2*-deficient individuals. Our findings highlight that interactions between $CD4^+$ T cells, DCs and B cells are required for Tfh formation[2–5]. It is likely that CD40L/CD40/NEMO signaling in B cells contributes to the maintenance of cTfh cells in humans. However, a T-cell intrinsic role for NEMO cannot be discounted. The importance of B cells is also evident from studies reporting diminished cTfh frequencies in patients with severe B-cell deficiency due to *E47*[50] or *NFKB2*[51] mutations. Our data also shed light on the division of labor between STAT3-activating cytokines in generating cTfh cells, as well as on the functionality and requirements for cTfh subset formation. Mutations in *STAT3* or *IL10R*, but not *IL21*/*IL21R*, had comparable deleterious effects on cTfh frequencies, implying IL-10/STAT3 signaling is important in establishing the pool of cTfh cells. However, $STAT3_{LOF}$, $STAT1_{GOF}$ and $IL21/R_{LOF}$ mutations, but not IL10R-deficiency, reduced $CCR6^+$ cTfh cells most likely due to STAT3 directly regulating CCR6 expression[37]. The resultant population of cTfh cells in these individuals was skewed to a $CXCR3^+IFN\gamma^{++}IL\text{-}10^{low}$ phenotype

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resembling STAT3-deficient murine Tfh cells, which also adopt a Th1 fate[52]. This revealed a dominant role for IL21/STAT3 signaling in specifying cTfh subsets. These data demonstrate that specific mutations cause qualitative changes in cTfh cells that would not be apparent by quantifying $CD4^+CXCR5^+$ T cells.

The mechanism underlying greater helper function of $CCR6^+$, and corresponding poor function of $CXCR3^+$, cTfh cells is unknown. Although it was reported that $CCR6^+$ cTfh cells produce more IL-21 than other subsets[10], this was not confirmed by us or others[11, 13, 17] and is unlikely to explain their ability to promote B-cell differentiation. Rather, based on several lines of evidence including our data, we propose this results from reduced IFNγ production. First, exogenous IFNγ reduced Ig production by human PBMCs[43, 44] or in B/Tfh cell co-cultures[45]. This was not observed for *IFNGR1* or *STAT1*-deficient B cells, demonstrating a B-cell intrinsic inhibitory effect of IFNγ. Second, *Mycobacterium leprae*-specific IgG levels negatively correlated with IFNγ production by PBMCs in response to *M leprae* Ags[42]. Third, serum Ig was elevated in many patients with mutations in the IFNγ signaling pathway. Fourth, there were trends for increased class switched memory B cells in individuals with *IFNGR1/2*, *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. As IFNγ is induced by IL-12/IL-12R/Tyk2 signalling and itself signals through STAT1, it is likely that the similar phenotypes detected in patients with *IL12RB1*, *TYK2*, *IFNGR1/2* and *STAT1* LOF mutations reflect the contribution of a common pathway to the regulation of B-cell differentiation and Ig secretion.

The abundance of IFNγ-producing cTfh cells in $STAT3_{LOF}$ and $STAT1_{GOF}$ patients, together with reduced $CCR6^+$ cTfh cells, would thus contribute to impaired humoral immune responses[21, 41]. This would be further compounded by reduced IL-10 production, an important cytokine for B cell differentiation in the context of Tfh/B cell interactions[22, 28, 45]. $CCR6^+$ cTfh cells are also reduced in HIV-infection[11], where Tfh cells exhibit impaired B-helper function[11, 49, 53]. The similarities in cTfh dysregulation in HIV infection and PIDs identifies intrinsic (specific gene mutations) and extrinsic/environmental (chronic viral infection/pathogen exposure) factors as modifiers of Tfh differentiation and function. Thus, it would be interesting to determine whether Tfh cells in HIV^+ patients also have skewed cytokine profile with increased IFNγ and reduced IL-10. Our findings regarding IFNγ and impaired humoral immunity also explain the paradoxical data of Ueno and colleagues that cTfh cells are reduced in IL-12Rβ1-deficiency, yet these patients have intact if not heightened Ab responses to vaccination or infection[54]. Here, while impaired IL-12R signaling may reduce cTfh cells[54], there will also be less IL-12-induced IFNγ production, resulting in a qualitative change in the cTfh cytokine repertoire, with reduced IFNγ-mediated suppression of B cell responses.

A defining feature of Tfh cells is elevated PD-1 expression[47]. Although most cTfh cells express low levels of PD-1, a subset with elevated PD-1 corresponds to the most efficient population of B-helper cells[8, 11, 12, 17]. Given our finding of fewer cTfh cells in $STAT3_{LOF}$ patients, and skewing of cTfh subsets in $STAT1_{GOF}$ or $STAT3_{LOF}$ individuals to a phenotype with reduced B-helper function, it may be predicted that their cTfh cells would have reduced

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PD-1. However, this was not the case, as PD-1 was significantly increased on cTfh cells in these individuals, raising several important possibilities. First, as PD-1 engagement impedes Tfh formation *in vivo*[55–57], exaggerated signaling through over-expressed PD-1 on *STAT1*$_{GOF}$ and *STAT3*$_{LOF}$ cTfh cells may further impair function. Interestingly, the PD-1 ligand PD-L1 is aberrantly expressed on CD4$^+$ T cells from *STAT1*$_{GOF}$ patients[41], revealing a possible autocrine mechanism of restrained cTfh function. Similarly, while PD-1 is expressed comparably on Tfh cells from healthy donors and HIV-infected individuals, more GC B cells from HIV-infected individuals expressed PD-L1 than healthy donors[53]. Importantly, this heightened expression impaired Tfh cell IL-21 production, suppressing effector function[53, 58]. PD-1^{hi}-expressing Tfh cells also undergo accelerated death compared to cells expressing lower levels of PD-1[59]. Thus, dysregulated expression of PD-1 or its ligand(s) could compromise Tfh function or survival in these PIDs[41, 53, 58, 59]. Interestingly, cTfh cells in STAT3-deficient individuals also expressed CD57 (MC Cook et al; submitted). While CD57 is expressed by a large proportion of Tfh cells in lymphoid tissues, it is usually absent from cTfh cells in healthy controls[9, 17, 24]. Expression of CD57 by Tfh cells has been associated with an exhausted/senescent phenotype, and a susceptibility to rapidly undergo death relative to CD57$^-$ CD4$^+$ T cells[60] (MC Cook et al; submitted). Thus, it is possible that cTfh cells in some PIDs correspond to “exhausted” cells, and this would further impact their function in the setting of humoral responses in these individuals. These possibilities are currently being investigated. Second, our data caution against solely quantifying cTfh cells with respect to PD-1 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, cTfh cells in these and other immunopathologies should be assessed for phenotype *and* function. The importance of doing so is highlighted by a recent study reporting impaired cTfh function in elderly individuals even though their cTfh cells expressed sufficient levels of IL-21 and ICOS[12]. Third, STAT3 signaling during Tfh differentiation is required to not only generate the CCR6$^+$ subset, but also regulate PD-1 and minimize suppression through PD-1/PD-L1 interactions. The mechanism underlying STAT3-mediated PD-1 repression is unknown but may involve IL-21 as *IL21R* deficiency partially recapitulated this defect. Interestingly, as STAT3 can protect Tfh cells from the inhibitory effects of IFNα[52], and IFNα induces PD-1 on murine CD4$^+$ T cells[61], 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, 41]. 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.

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Collectively, our study provides important insights into the molecular requirements and signaling pathways regulating human cTfh 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[42–46] and PD-1 engagement impairing survival or production of IL-10 and IL-21[53, 58, 59]. Identifying these pathways potentially provides opportunities to manipulate Tfh function not only in immunodeficiency, but also autoimmunity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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

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Key Messages

- Loss-of function (LOF) mutations in *STAT3*, *IL10R*, *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 IFN-γ and PD-1.
- IFN-γ negatively regulates Ig production *in vivo*

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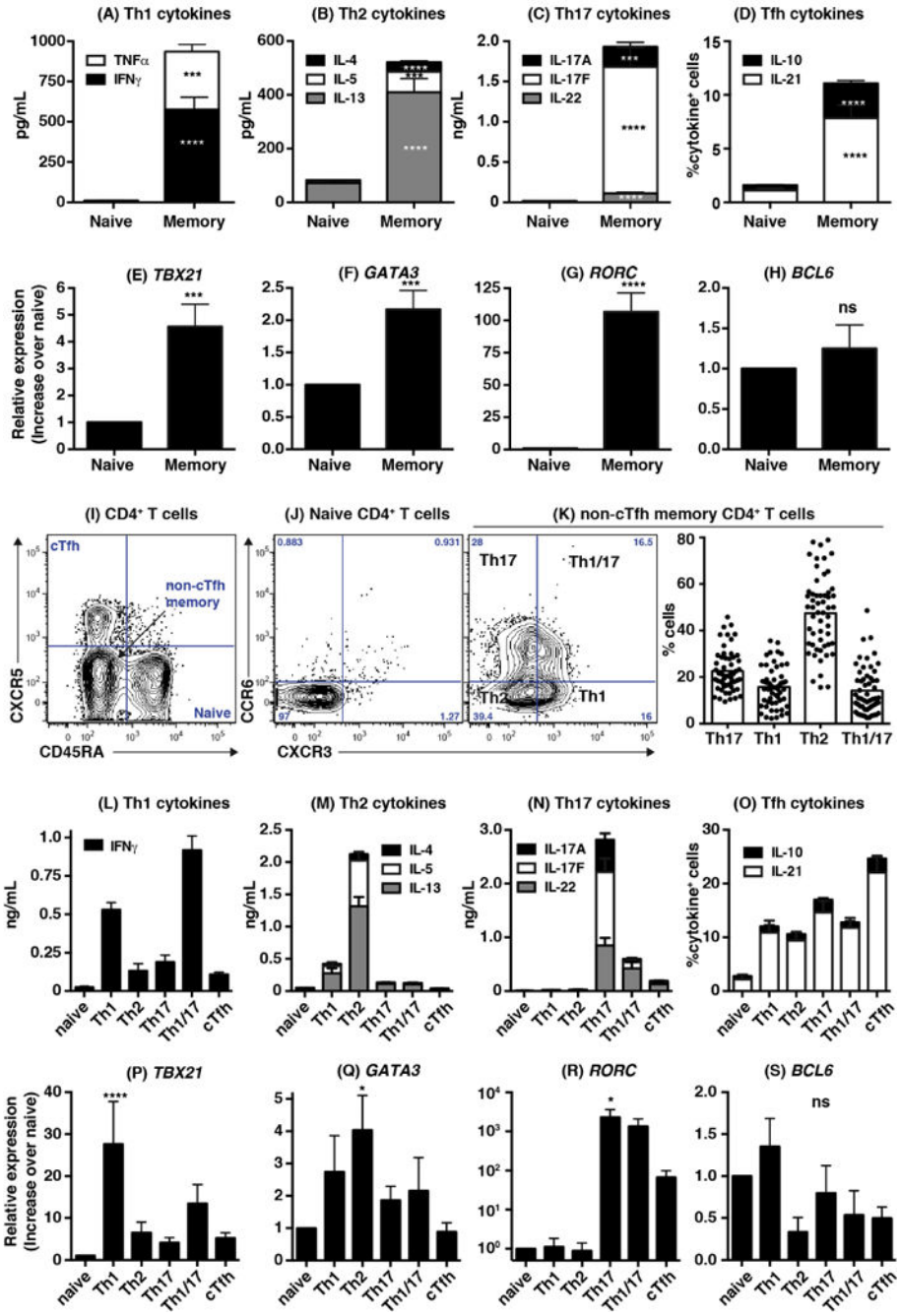

Figure 1. Identification of effector subsets within populations of human memory CD4$^+$ T cells

(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

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($CXCR3^+CCR6^+$) 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. Significant differences (one-way ANOVA) between naïve and memory $CD4^+$ T cells or subsets are indicated.

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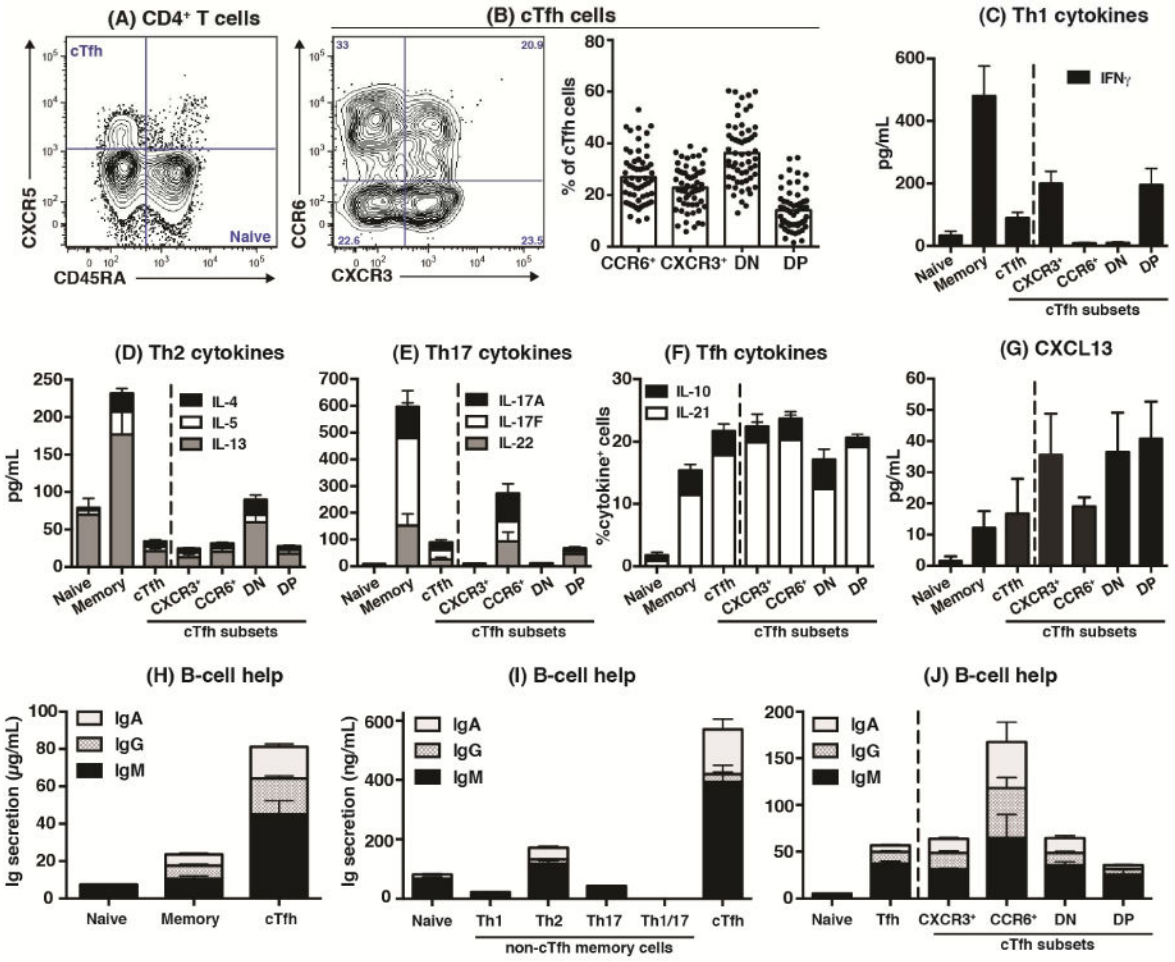

Figure 2. Delineating subsets of human circulating Tfh cells

(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.

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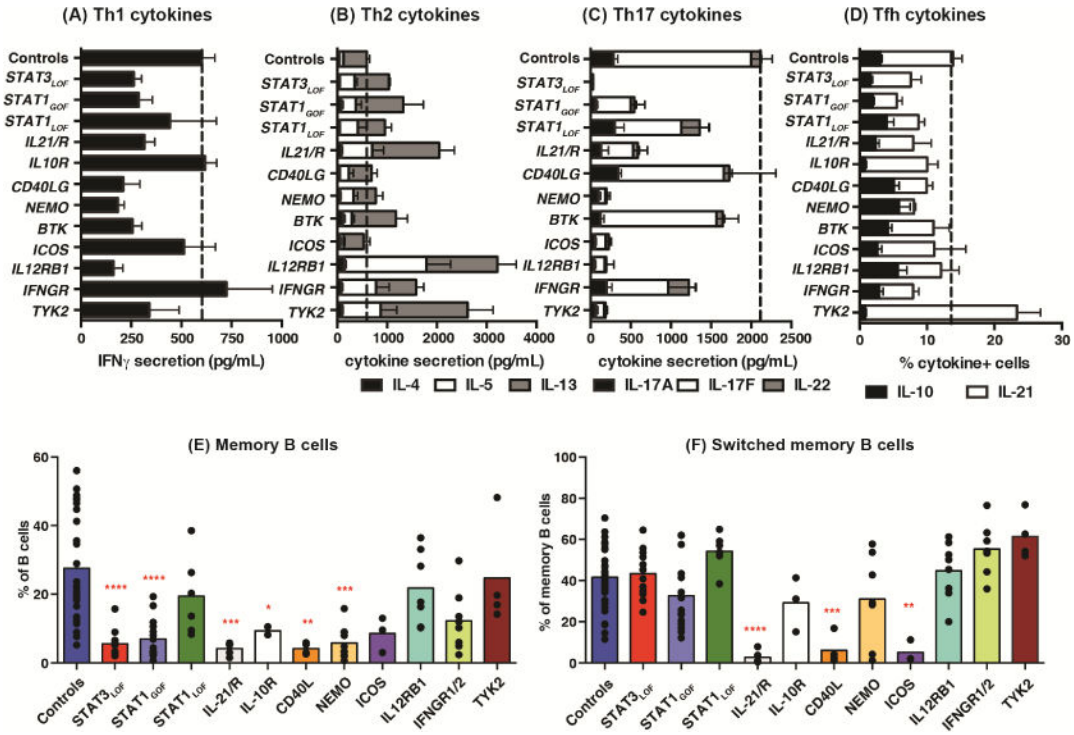

Figure 3. Defects in cytokine production due to monogenic mutations causing different PIDs

(A–D) memory CD4$^+$ T cells were sorted from healthy controls or patients with the indicated gene mutations then stimulated with TAE beads. Production of (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) was determined after 5 days (mean ± SEM). Controls: n=26–35; *STAT3*$_{LOF}$: n=6; *STAT1*$_{GOF}$: n=7–9; *STAT1*$_{LOF}$: n=5–7; *IL21/R*: n=5; *IL10R*: n=2; *CD40LG*: n=4; *NEMO*: n=5–6; *BTK*: n=6; *ICOS*: n=4; *IL12R*: n=5; *IFNGR1/2*: n=6; *TYK2*: n=3. See Table E1 for more detailed results. (E–F) proportions of total (E) and class switched (F) memory B cells in healthy controls (n=37) and patients [*STAT3*$_{LOF}$: n=11–12; *STAT1*$_{GOF}$: n=16; *STAT1*$_{LOF}$: n=7; *IL21/R*: n=5; *IL10R*: n=3–4; *CD40LG*: n=4; *NEMO*: n=7–10; *ICOS*: n=3; *IL12R*: n=8; *IFNGR1/2*: n=7–8; *TYK2*: n=4] were determined. Significant differences (one-way ANOVA) between controls and patients are indicated.

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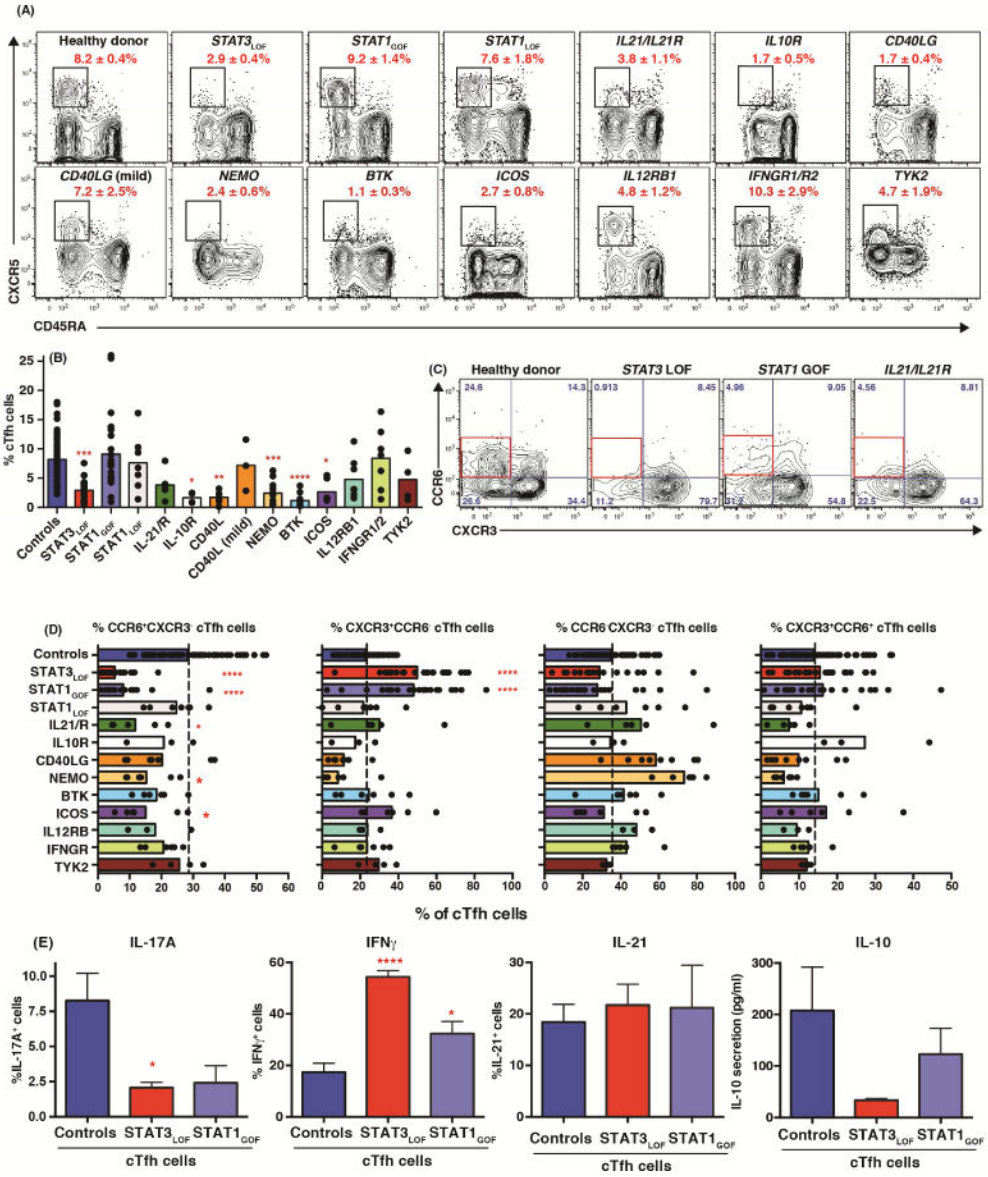

Figure 4. Effect of monogenic mutations on the generation of human cTfh cells

(A, B) cTfh cells were identified amongst the population of non-Tregs as $CD45RA^{-}CXCR5^{+}$ $CD4^{+}$ T cells. cTfh frequencies in healthy donors (n=72) and patients with mutations in *STAT3* (n=24), *STAT1* (GOF [n=22]; LOF [n=7]), *IL-21R/IL21* (n=5), *IL10R* (n=4), *CD40LG* (n=10), *NEMO* (n=11), *BTK* (n=11), *ICOS* (n=6), *IL12RB1* (n=8), *IFNGR1/2* (n=7) or *TYK2* (n=4) were determined. (C, D) proportions of $CXCR3^{+}CCR6^{-}$, $CXCR3^{-}CCR6^{+}$, $CXCR3^{-}CCR6^{-}$ or $CXCR3^{+}CCR6^{+}$ subsets amongst total cTfh cells [healthy donors: n=71; *STAT3:* n=21, *STAT1:* GOF n=20, LOF n=7; *IL-21R/IL21*: n=5; *IL10R*: n=3; *CD40LG*: n=8; *NEMO:* n=7; *BTK:* n=5; *ICOS:* n=6; *IL12RB1:* n=3; *IFNGR1/2:* n=6; *TYK2:* n=4]. (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). Significant differences (one-way ANOVA) between controls and patients are indicated.

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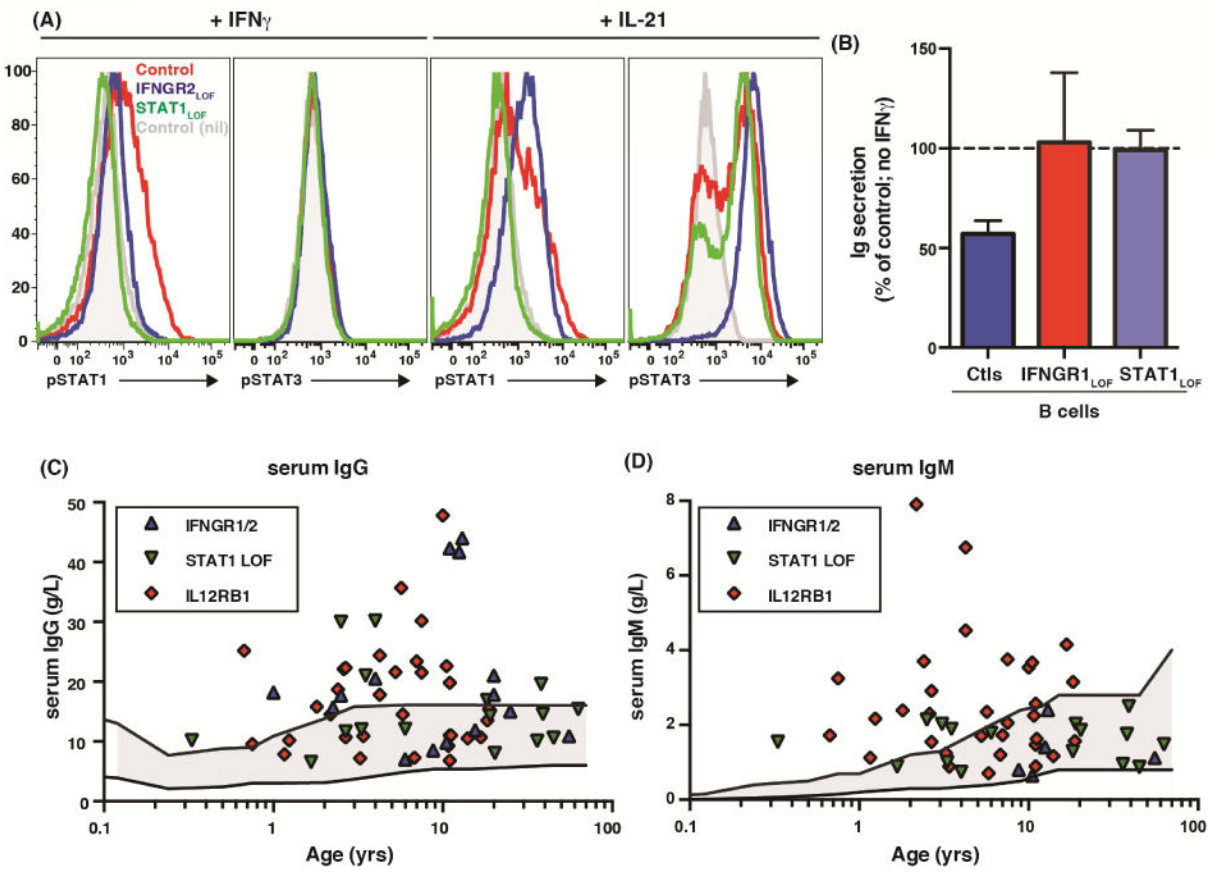

Figure 5. IFNγ suppresses B-cell differentiation *in vitro* and *in vivo*

(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

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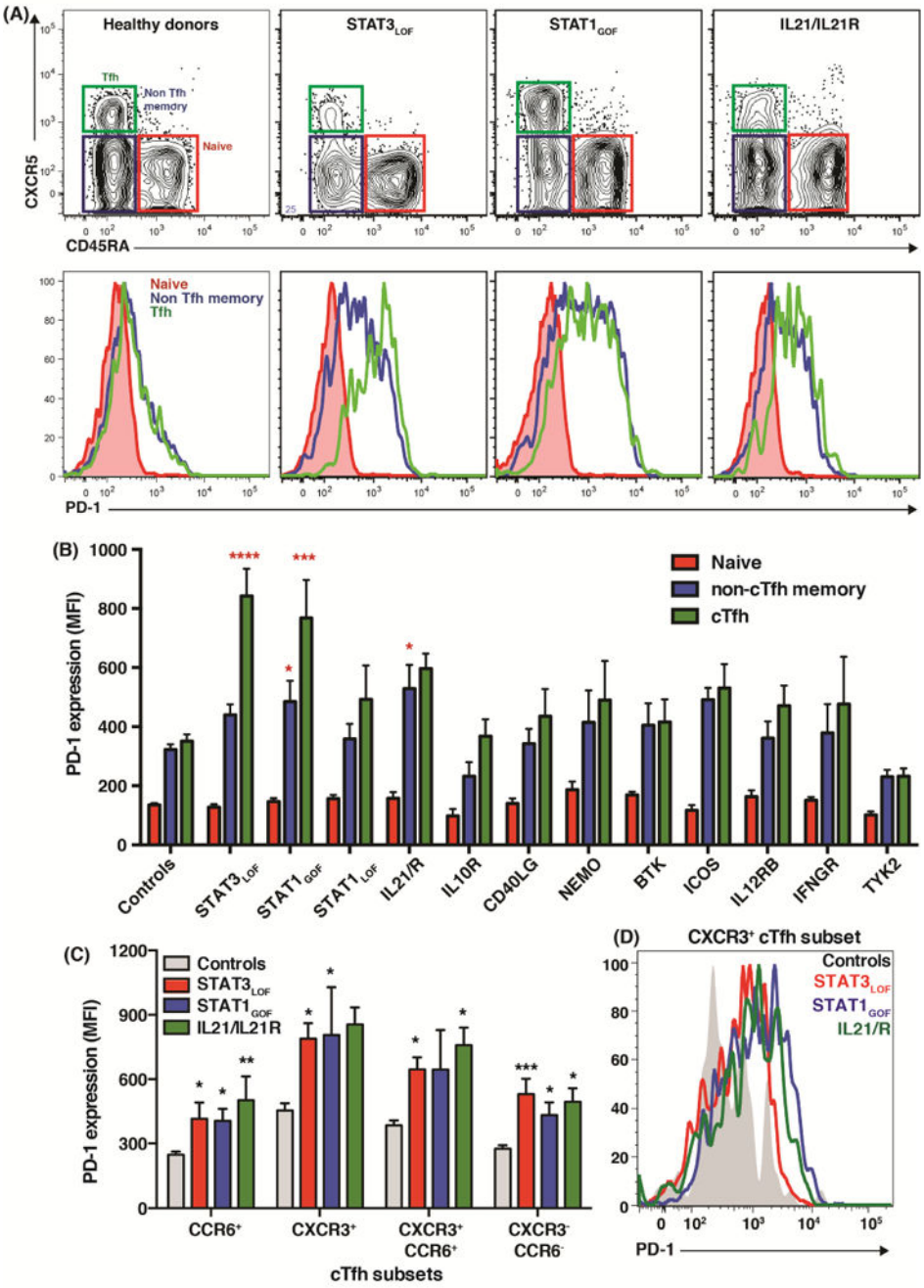

Figure 6. Aberrant expression of PD-1 on cTfh cells from patients with $STAT3_{LOF}$, $STAT1_{GOF}$ and $IL21/R_{LOF}$ mutations

(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 [healthy donors: n=45; *STAT3:* n=15; *STAT1:* GOF n=18, LOF n=7; *IL-21R/IL21*: n=5; *IL10R*: n=4; *CD40LG*: n=3; *NEMO:* n=5–6; *BTK:* n=5–6; *ICOS:* n=5; *IL12RB1:* n=4; *IFNGR1/2:* n=4; *TYK2:* n=4]. (C, D) PD-1 expression on $CXCR3^{+}CCR6^{-}$, CXCR3-$CCR6^{+}$, $CXCR3^{-}CCR6^{-}$ and $CXCR3^{+}CCR6^{+}$ cTfh subsets cells from healthy controls (n=25) or patients with $STAT3_{LOF}$

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(n=10), $STAT1_{GOF}$ (n=10) or $IL21/R_{LOF}$ mutations (n=5). Significant differences (one-way ANOVA) between controls and patients are indicated.

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Table 1

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

Genetic diseases	Tregs	$CXCR5^+$ Tregs (%Tregs)	Total memory	$CD4^+CXCR5^-$ memory cells			
				Th1 ($CXCR3^+$ $CCR6^-$)	Th2 ($CXCR3^-CCR6^-$)	Th17 ($CXCR3^-CCR6^+$)	Th1/17 ($CXCR3^+$ $CCR6^+$)
Controls (n=47–71)	6.1 ± 0.3	8.2 ± 0.9	52.2 ± 1.8	15.5 ± 1.0	47.3 ± 1.8	23.0 ± 1.1	14.1 ± 1.1
STAT3$_{LOF}$ (n=12–22)	7.4 ± 0.7	8.7 ± 1.7	29.6 ± 3.7***	34.4 ± 4.3****	46.3 ± 5.1	5.3 ± 0.7****	10.0 ± 1.5
STA T1$_{GOF}$ (n=16–23)	5.6 ± 0.7	12.0 ± 1.6	41.0 ± 3.7	27.4 ± 3.4*	56.9 ± 3.7	6.9 ± 1.05****	9.5 ± 1.6
STA T1$_{LOF}$ =5–6)	7.5 ± 1.1	15.9 ± 5.5	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	6.6 ± 2.4	32.2 ± 7.1	25.1 ± 11.2	57.6 ± 10.5	12.0 ± 3.3	5.3 ± 1.1
IL10R (n=4)	6.4 ± 1.2	9.8 ± 5.3	31.5 ± 9.4	20.1 ± 17.0	52.8 ± 12.3	23.0 ± 10.9	4.1 ± 1.8
CD40LG (n=8)	3.6 ± 0.8	6.0 ± 1.4	25.9 ± 3.8**	6.9 ± 2.0	70.7 ± 6.0*	17.9 ± 2.8	4.6 ± 2.0
NEMO (n=8–12)	9.0 ± 1.7	8.3 ± 1.7	40.2 ± 7.6	2.6 ± 1.6	85.2 ± 4.0****	10.7 ± 2.3**	1.4 ± 0.7**
BTK (n=4–11)	4.9 ± 0.7	5.5 ± 1.1	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	10.1 ± 2.8	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	10.8 ± 2.2	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****	10.5 ± 1.5	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	3.3 ± 1.2	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 Tregs expressing CXCR5 (putative Tfr cells), 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.