Table 3. CD4$^+$ T-cell subsets in primary immunodeficiencies

PID	CD4$^+$ T cells	Naive CD4$^+$ T cells	Memory CD4$^+$ T cells	CXCR5$^+$CD45RA$^+$ CD4$^+$ T cells	CXCR5$^+$CD45RA$^-$ CD4$^+$ T cells
IL12RB1 (n = 6)	40 ± 4.4	71 ± 4.7	28 ± 4.7	1.6 ± 0.46	14 ± 2.8
STAT1 (n = 6)	40*	41 ± 10	56 ± 10	3.9 ± 0.68	11 ± 2.3
STAT3 (n = 14)	41 ± 3.1	65 ± 3.8	33 ± 3.7	1.5 ± 0.29 (n = 11)	8.7 ± 1.6 (n = 11)
TYK2 (n = 2)	41*	53 ± 24	43 ± 27	2.8 ± 1.2	15 ± 5.4
Controls	43 ± 1.4 (n = 54)	39 ± 1.7 (n = 70)	59 ± 1.7 (n = 70)	2.8 ± 0.24 (n = 61)	15 ± 1.1 (n = 61)

Values are mean ± SEM percentage.
PID indicates primary immunodeficiency.
*Analysis was only performed on 1 patient.

IL-21–inducing cytokines predominantly activate STAT1, STAT3, and STAT4 in human naive CD4$^+$ T cells (data not shown), suggesting that these transcription factors are important in inducing Tfh cells in humans. To investigate this further, we took advantage of additional primary immunodeficiencies that result from mutations in components of several cytokine-signaling pathways, namely *STAT1*, *TYK2*, and *STAT3*.

STAT1 is dispensable for IL-12–induced expression of IL-21 in human CD4$^+$ T cells

STAT1-deficient patients and healthy donors had comparable frequencies of naive and memory CD4$^+$ T cells (Figure 2A-D; Table 3). The proportions of CXCR5$^+$CD45RA$^-$ or CD45RA$^+$ Tfh cells in STAT1-deficient patients were also comparable with healthy donors (Figure 2A-B,E; Table 3). The ability of STAT1-deficient CD4$^+$ T cells to differentiate into IL-21$^+$ cells in response to Th1-polarizing conditions was then tested. Because of the limited numbers of cells available from these rare patients, total CD4$^+$ T cells were examined. When CD4$^+$ T cells from healthy donors or STAT1-deficient patients were activated under neutral conditions, IL-21 and IFNγ production could be detected; however, the frequencies of cytokine-expressing cells was lower in STAT1-deficient patients (Figure 2F,H; IL-21: normal, 9.1% ± 6.2%; STAT1, 2.7% ± 1.8%; IFNγ: normal, 11.3% ± 3.8%; STAT1, 1.6% ± 0.9%). Despite these differences in the nonpolarizing cultures, expression of IL-21 and IFNγ in both normal and STAT1-deficient CD4$^+$ T cells increased after culture with IL-12 (Figure 2F,H; IL-21: normal, 30.5% ± 7.9%; STAT1, 13.2% ± 6.7%; IFNγ: normal, 31.3% ± 7.9%; STAT1, 12.3% ± 2.5%). Although there appeared to be a reduced ability of IL-12 to enhance expression of IL-21 and IFNγ in the absence of STAT1, compared with normal CD4$^+$ T cells (Figure 2F,H), when the effect of IL-12 was expressed as fold-change relative to nonpolarizing cultures, STAT1-deficient CD4$^+$ T cells responded comparably to normal cells, that is, ~ 4- to 6-fold increase in IL-21 (Figure 2G) and ~ 3- to 6-fold induction in IFNγ expression (Figure 2I). Together these results indicate that IL-12–induced IL-21 and IFNγ in CD4$^+$ T cells is independent of STAT1 signaling.

Mutations in *STAT3* compromise the generation of CD4$^+$ memory and CXCR5$^+$ Tfh cells

To investigate further the signaling pathways involved in the differentiation of IL-21–expressing cells we used patients with autosomal-dominant hyper IgE syndrome resulting from heterozygous mutations in *STAT3*.[36,37] Patients heterozygous for these mutations display impaired, but not abolished, STAT3 function with ~ 25% residual signaling.[36,37] As detailed above, STAT3 is activated by many cytokines, including those that induce IL-21 expression in human CD4$^+$ T cells (ie, IL-6, IL-12, IL-21, IL-23, IL-27).[20,21,24,32-35] The frequency of total CD4$^+$ T cells was comparable between healthy donors and $STAT3_{MUT}$ patients (Figure 3C). Compared with healthy donors, $STAT3_{MUT}$ patients have a significant increase in the frequency of naive and a significant decrease in the frequency of memory CD4$^+$ T cells (Figure 3A-F; Table 3). Analysis of CXCR5$^+$ T cells within the CD45RA$^+$ and CD45RA$^-$ fractions revealed significant decreases in both of these compartments in $STAT3_{MUT}$ patients compared with healthy donors (Figure 3A-B,F; Table 3). Thus, mutations in *STAT3* cause a ~ 50% reduction in the frequency of circulating CXCR5$^+$CD45RA$^-$ and CD45RA$^+$ Tfh cells (Figure 3F; Table 3).

STAT3 mutations partially impairs IL-12–induced expression of IL-21 in naïve CD4$^+$ T cells

The observations that (1) IL-12 is the main driver of human Tfh cell differentiation in vitro,[11,22] (2) IL-12 is capable of activating STAT3,[21,32-35] and (3) $STAT3_{MUT}$ patients have a contracted population of circulating CD4$^+$ CXCR5$^+$CD45RA$^-$ and CD45RA$^+$ Tfh-like cells (Figure 3E) led us to investigate the consequences of *STAT3* mutations on the ability of IL-12 to induce IL-21 in human naive CD4$^+$ T cells. No differences were observed in the low frequencies of IL-21– and IFNγ–expressing cells detected in cultures of naive CD4$^+$ T cells from healthy donors or $STAT3_{MUT}$ patients after stimulation under neutral conditions (Figure 4A-D). However, induction of IL-21 expression by IL-12 was significantly reduced in $STAT3_{MUT}$ naive CD4$^+$ T cells compared with normal naive CD4$^+$ T cells (Figure 4A-C; normal, 20.9% ± 4.5%; $STAT3_{MUT}$, 5.2% ± 1.3%). In fact this defect was most pronounced when the frequency of IL-21$^+$IFNγ$^-$ cells were determined (Figure 4A-B). This analysis found a significant ($P < .01$) decrease in IL-21$^+$IFNγ$^-$ cells but not IL-21$^+$IFNγ$^+$ ($P > .05$) cells in $STAT3_{MUT}$ patients compared with healthy donors after Th1 polarization (IL-21$^+$IFNγ$^-$: normal, 16.7% ± 3.2%; $STAT3_{MUT}$, 2.3% ± 0.5%; IL-21$^+$IFNγ$^+$: normal, 4.8% ± 1.5%; $STAT3_{MUT}$, 3.3% ± 1%). In contrast to IL-21, expression of IFNγ in CD4$^+$ T cells in response to IL-12 was unaffected by *STAT3* mutations; in fact it was higher than normal CD4$^+$ T cells (Figure 4D). This increase in IFNγ production by $STAT3_{MUT}$ CD4$^+$ T cells, however, did not contribute to decreased IL-21 production because neutralizing IFNγ in these cultures had no effect on IL-21 expression, and there was a positive correlation between IL-21 and IFNγ expression in both normal and $STAT3_{MUT}$ naive CD4$^+$ T cells after Th1 polarization (data not shown). It was possible that induction of IL-21 by IL-12 resulted from not only a direct effect of IL-12 but also the effects of other cytokines induced by IL-12 that act in an autocrine manner to further promote IL-21 expression. To address this, we examined IL-21 and IFNγ expression in normal and $STAT3_{MUT}$ CD4$^+$ T cells by qPCR after 24 and 48 hours of

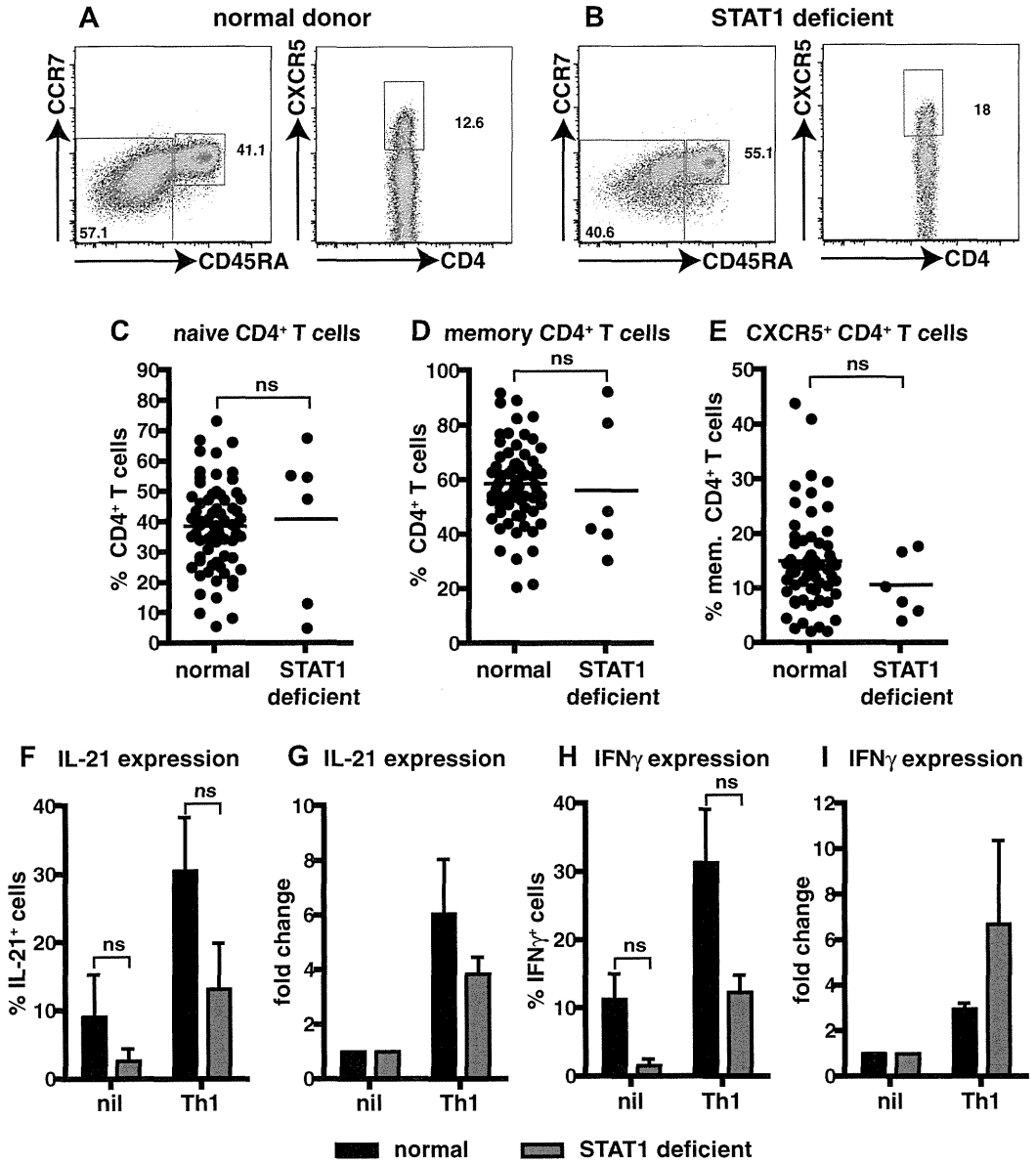

Figure 2. STAT1 is dispensable for IL-12–induced expression of IL-21 in human CD4+ T cells. (A-E) The frequency of naive ($CD45RA^+CCR7^+$), memory ($CD45RA^+CCR7^{-/+}$), and $CXCR5^+CD45RA^-$ $CD4^+$ T cells in PBMCs was determined for healthy donors and STAT1-deficient patients. (A-B) Representative dot plots from 1 donor and 1 STAT1-deficient patient. (C-E) The frequency of (C) naive ($CD45RA^+CCR7^+$), (D) memory ($CD45RA^+CCR7^{-/+}$), and (E) $CXCR5^+CD45RA^-$ $CD4^+$ T cells from all healthy donors (total $CD4^+$ T cells, n = 54; naive $CD4^+$ T cells, n = 70; memory $CD4^+$ T cells, n = 70; $CXCR5^+CD45RA^-$ $CD4^+$ T cells, n = 61) and STAT1-deficient patients (n = 6) was examined. (F-I) Total $CD4^+$ T cells isolated from healthy donors and STAT1-deficient patients (n = 3) were cultured for 5 days under neutral (nil) or Th1-polarizing (ie, IL-12) conditions, and expression of intracellular IL-21 (F-G) and IFNγ (H-I) was then determined. The graphs in panels F and H show the frequency of cytokine-positive cells; those in panels G and I depict cytokine expression after Th1 polarization as fold-increase relative to the nil culture in each experiment. The values represent the mean ± SEM (n = 3).

stimulation with or without IL-12. Expression of *IL21* was substantially increased by IL-12 in normal $CD4^+$ T cells within 24 hours of stimulation, compared with cells cultured under neutral (nil) conditions, and similar levels were detected after 48 hours (supplemental Figure 1A-B, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Consistent with the flow cytometric analysis, expression of *IL21* in IL-12–activated $STAT3_{MUT}$ naive $CD4^+$ T cells was reduced compared with normal controls (supplemental Figure 1A-B). In contrast to *IL21*, *IFNG* was abundantly expressed by IL-12–treated $STAT3_{MUT}$ naive $CD4^+$ T cells; in fact, consistent with the intracellular staining data, *IFNG* expression by these cells exceeded that of normal naive $CD4^+$ T cells at the 48-hour time point (supplemental Figure 1C-D). These data suggest that IL-12 acts directly to rapidly induce IL-21 expression in naive $CD4^+$ T cells.

IL-12 is capable of activating the JAK family protein tyrosine kinase TYK2,[38] which subsequently phosphorylates STATs, including STAT3.[39] Mutations in *TYK2* have been reported in 2 patients who developed susceptibility to various pathogens, including mycobacteria/Bacille Calmette-Guérin and herpes viruses.[25,28] One of the contributing factors to disease pathogenesis is believed to be the unresponsiveness of their T cells to IL-12 with respect to induction of IFNγ expression.[25] To investigate this further, these 2 patients deficient for TYK2 were examined (supplemental Figure 2). $CXCR5^+CD45RA^-$ Tfh-like cells were detected in both patients at similar frequencies as healthy donors (supplemental Figure 2A-B,E; Table 3). When IL-21 expression in total

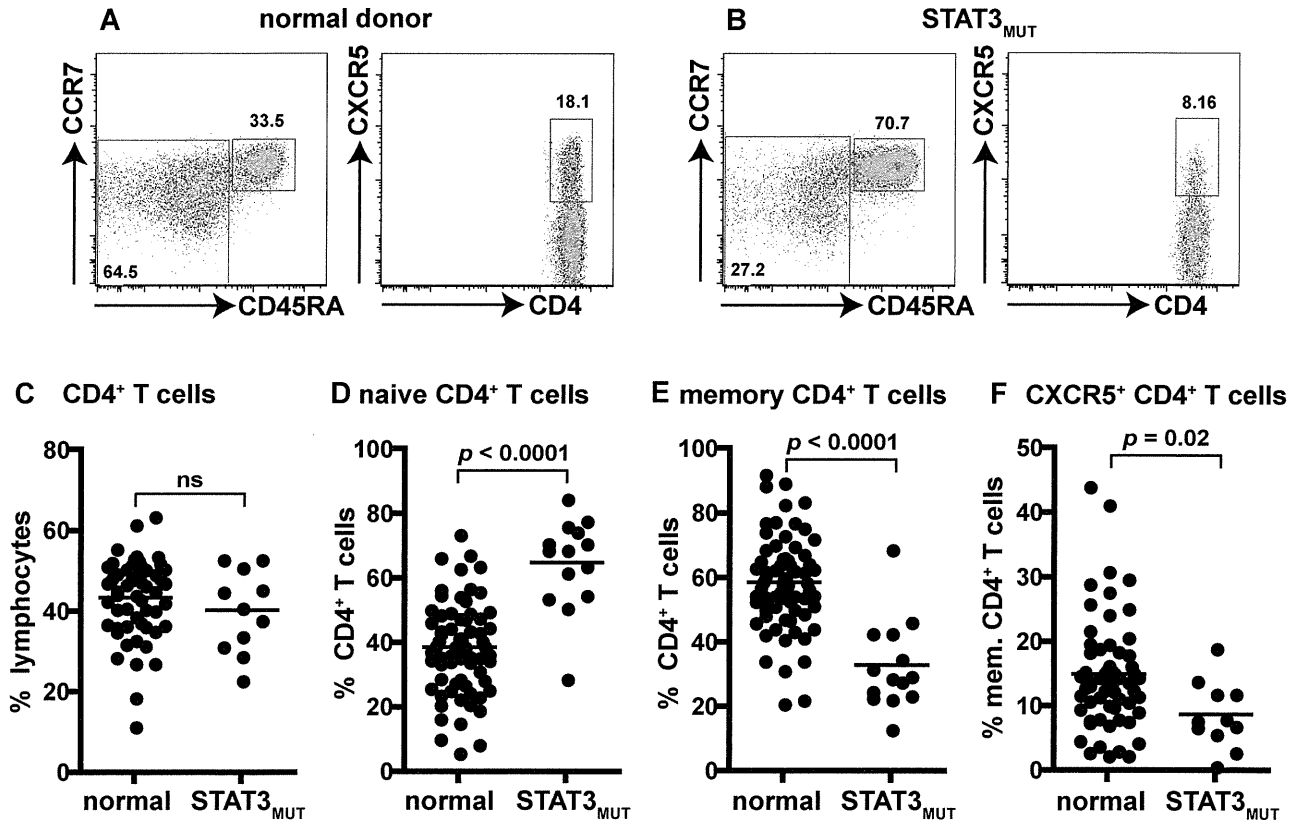

Figure 3. Mutations in *STAT3* compromise the generation of CD4+ memory and CXCR5+CD45RA− Tfh cells. (A-B) The frequency of naive (CD45RA+CCR7+), memory (CD45RA+CCR7−/+), and CXCR5+CD45RA− CD4+ T cells in PBMCs was determined for healthy donors and $STAT3_{MUT}$ patients. (A-B) Representative dot plots from 1 donor and 1 patient. (C-F) The frequency of (C) total CD4+ T cells, (D) naive (CD45RA+CCR7+), (E) memory (CD45RA+CCR7−/+), and (F) CXCR5+CD45RA− CD4+ T cells from all healthy donors (total CD4+ T cells, n = 54; naive CD4+ T cells, n = 70; memory CD4+ T cells, n = 70; CXCR5+CD45RA− CD4+ T cells, n = 61) and $STAT3_{MUT}$ patients (n = 14) was examined.

CD4+ T cells from 1 patient deficient for *TYK2* after culture with IL-12 was examined, the level of induction was ~ 2-fold less than that observed for normal CD4+ T cells (supplemental Figure 2F-G). We extended these studies by examining naive CD4+ T cells from the second patient deficient for TYK2. Compared with normal naive CD4+ T cells, induction of IL-21 expression by IL-12 in TYK2-deficient CD4+ T cells was severely reduced (supplemental Figure 2I). Induction of IFNγ was also dramatically compromised by *TYK2* mutations (supplemental Figure 2H,J). Taken together, these data suggest that mutations in *TYK2* and *STAT3*, which are both activated downstream of the IL-12R,[21,32-35,38,39] cause a significant impairment in the ability of naive CD4+ T cells to differentiate into IL-21–expressing cells in response to IL-12.

STAT3 mutations impede division-linked differentiation of CD4+ T cells into IL-21–expressing effector cells

Because IL-12 can promote proliferation of human activated T cells,[40] and differentiation of CD4+ T cells into cytokine-expressing cells is linked to cell division,[29,41] it was important to determine whether the reduction in expression of IL-21 in IL-12–stimulated $STAT3_{MUT}$ CD4+ T cells resulted from reduced cell division or reflected an intrinsic defect in the differentiation program of these cells.

To do this, we examined proliferation by labeling naive CD4+ T cells with CFSE and tracked their division after in vitro stimulation by monitoring CFSE dilution. There was no difference in cell division between normal or $STAT3_{MUT}$ naive CD4+ T cells activated under neutral conditions (Figure 4E). Similarly, IL-12 increased proliferation regardless of whether the naive CD4+ T cells were derived from healthy donors or $STAT3_{MUT}$ patients (Figure 4E). When IL-21 expression was examined on a per division basis, we found that it increased in normal CD4+ T cells after the first few divisions and then reached a maximum, being expressed in ~ 20%-30% of cells after 2-3 divisions (Figure 4F). Expression of IL-21 by $STAT3_{MUT}$ CD4+ T cells also modestly increased with division; however, the frequency of IL-21+ cells in each division never exceeded 10% and thus was dramatically reduced compared with normal CD4+ T cells (Figure 4F). These data establish that the defect in IL-12–induced IL-21 induction in the absence of functional STAT3 was not because of a difference in proliferation, but rather because of an inability to efficiently acquire IL-21 during Tfh cell differentiation. Notably, when IFNγ expression was also analyzed on a per division basis, the frequency of cytokine-positive cells continued to increase with each cell division (Figure 4G). Furthermore, the heightened frequency of IFNγ+ cells observed in $STAT3_{MUT}$ compared with normal CD4+ T cells at the population level (Figure 4D and supplemental Figure 1) was also detected for cells that had undergone different divisions (Figure 4G).

IL-12 can induce additional characteristics of Tfh cells in STAT3 mutant CD4+ T cells

IL-12 not only promotes IL-21 expression in human naive CD4+ T cells but also induces additional features of Tfh cells such as

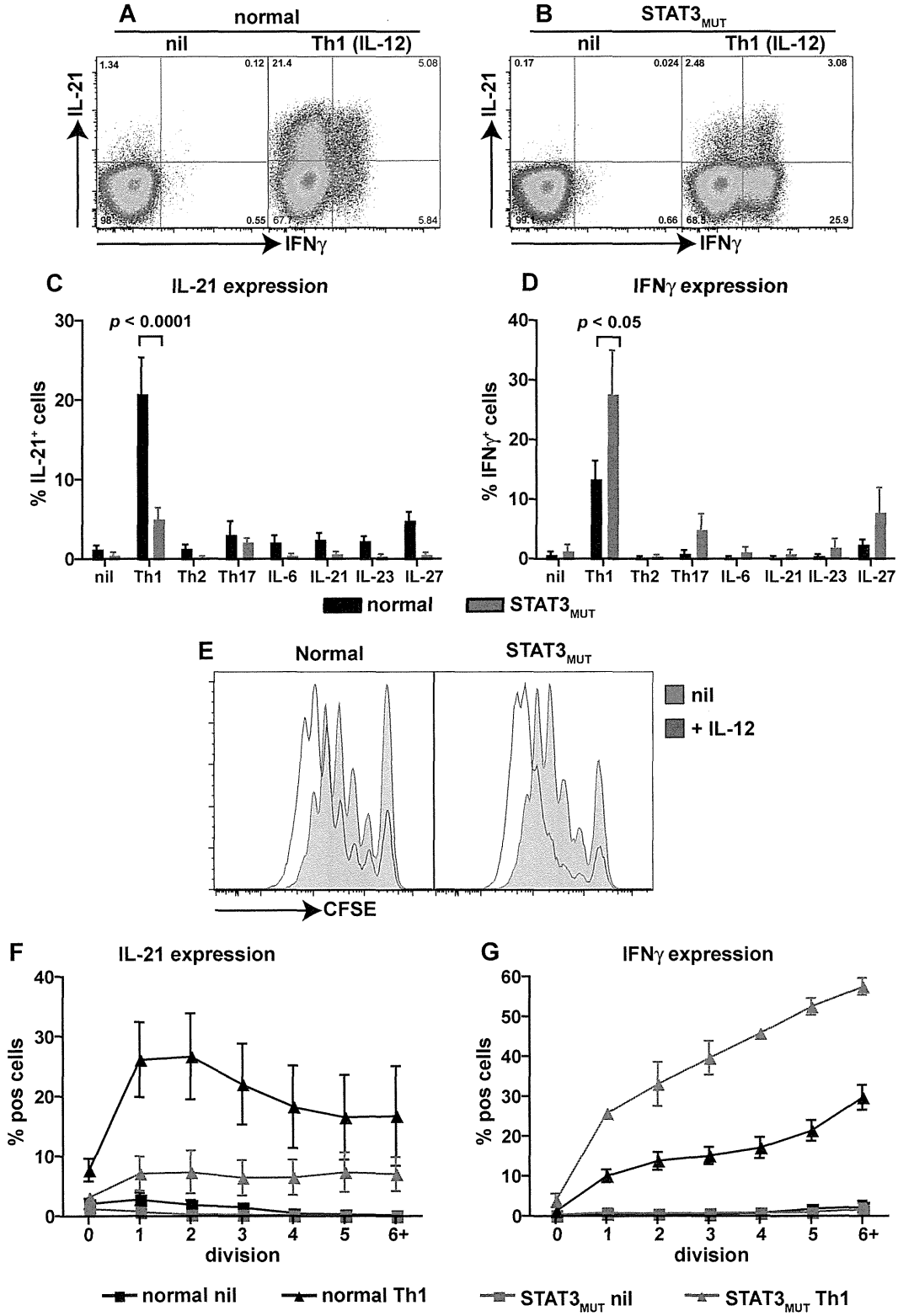

Figure 4. STAT3 mutations impair IL-12–induced expression of IL-21 in naive CD4+ T cells. (A-D) Naive CD4+ T cells isolated from healthy donors (n = 8) and $STAT3_{MUT}$ patients (n = 9) were cultured for 5 days under neutral conditions (nil); polarizing Th1, Th2, or Th17 conditions; or in the presence of IL-6, IL-21, IL-23, or IL-27, after which time expression of intracellular IL-21 and IFNγ was determined. (A-B) Representative dot plots of cytokine expression by activated naive CD4+ T cells from 1 donor and 1 patient. (C-D) Percentage of activated normal and $STAT3_{MUT}$ naive CD4+ T cells induced to express (C) IL-21 or (D) IFNγ in response to the indicated culture. The values represent the mean ± SEM. (E-F) Naive CD4+ T cells isolated from healthy donors and $STAT3_{MUT}$ patients were labeled with CFSE and cultured with anti-CD2/CD3/CD28 Abs in the absence (nil) or presence of Th1 polarizing conditions (+IL-12). After 5 days cells were harvested, and (E) proliferation and expression of (F) IL-21 and (G) IFNγ in cells that had undergone different divisions were then determined. (F-G) The values represent the mean ± SEM (n = 3).

sustained expression of CXCR5 and ICOS, and a modest induction of the Tfh lineage restricted transcription factor *BCL6*.[4,11] We therefore wanted to explore whether the effects of IL-12 on these aspects of CD4+ T-cell activation were also compromised by

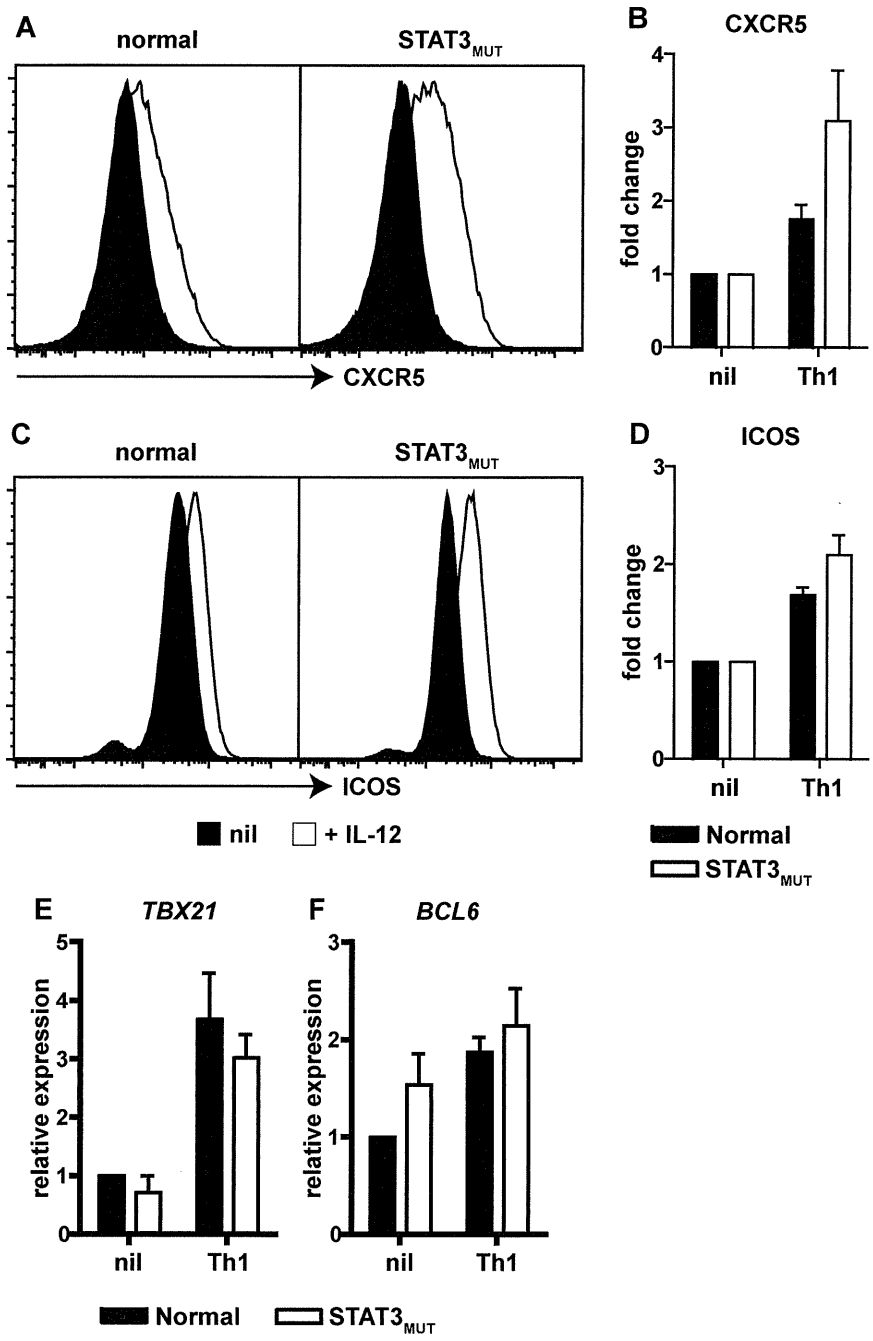

Figure 5. Induction of CXCR5, ICOS, and *BCL6* by IL-12 in CD4$^+$ T cells is independent of STAT3. (A-D) Naive CD4$^+$ T cells isolated from healthy donors and $STAT3_{MUT}$ patients were cultured with anti-CD2/CD3/CD28 Abs in the absence (nil) or presence of Th1-polarizing conditions (+IL-12). After 4 days the cells were harvested, and surface expression of CXCR5 and ICOS was determined by flow cytometry and of *TBX21* and *BCL6* by qPCR. (A,C) representative histogram plots from 1 healthy donor and 1 $STAT3_{MUT}$ patient. Expression of (B) CXCR5 (n = 3), (D) ICOS (n = 3), (E) *TBX21* (n = 4), and (F) *BCL6* (n = 5) after Th1 polarization is presented as fold-increase compared with the nil culture in each experiment. (B,D-F) The graphs represent the mean ± SEM of the indicated number of experiments.

mutations in *STAT3*. Compared with naive CD4$^+$ T cells activated under neutral conditions, elevated expression of the Tfh cell markers CXCR5 and ICOS was observed when either normal or $STAT3_{MUT}$ cells were activated in the presence of IL-12 (Figure 5 A,C). In fact, compared with cells cultured under neutral conditions, IL-12 up-regulated CXCR5 and ICOS expressions by 2- to 3-fold on both normal and $STAT3_{MUT}$ naive CD4$^+$ T cells (Figure 5B,D). IL-12 also up-regulated *TBX21* (encoding T-bet) and *BCL6*, the transcription factors required to generate Th1 and Tfh cells, respectively, to a comparable extent in normal and $STAT3_{MUT}$ naive CD4$^+$ T cells (Figure 5E,F). Thus, IL-12–mediated induction of CXCR5, ICOS, and *BCL6* in Tfh-like cells either only require the residual levels of functional STAT3 that are available in $STAT3_{MUT}$ CD4$^+$ T cells or are STAT3-independent resulting from IL-12–induced activation of STAT4.[20,21,34,38] This latter scenario would underlie the normal induction of *TBX21* and IFNγ in $STAT3_{MUT}$ Th1 cells.

Consistent with our previous findings,[11] most of the other culture conditions used in this study (ie, Th2- and Th17-polarizing conditions; exogenous IL-6, IL-21, IL-23) had no effect on expression of CXCR5 and ICOS on normal CD4$^+$ T cells above that observed for the nonpolarizing culture (supplemental Figure 3A-B). However, IL-27 did modestly enhance ICOS expression (supplemental Figure 3B) and induce *TBX21* in naive CD4$^+$ T cells (supplemental Figure 3C), and this was independent of STAT3. Interestingly, Th17 polarizing culture conditions induced the greatest levels of *BCL6* in naive CD4$^+$ T cells, and this was substantially reduced in $STAT3_{MUT}$ CD4$^+$ T cells (supplemental Figure 3D), reflecting the contribution of STAT3 to the combined signaling of IL-6, IL-21, and IL-23 through their respective receptors.

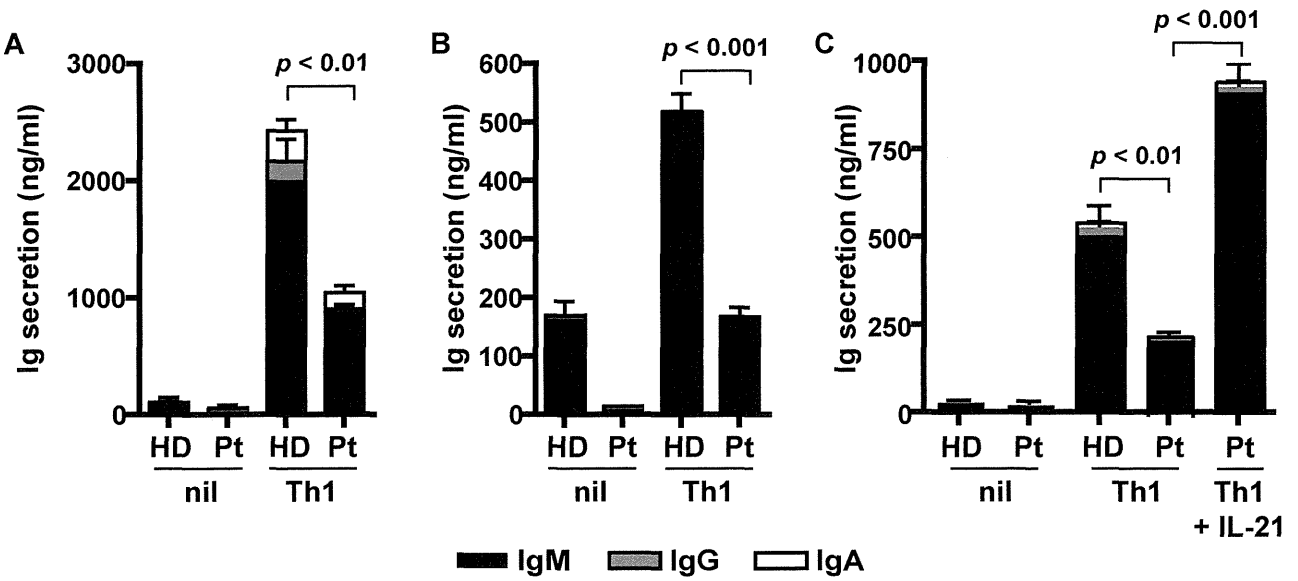

Figure 6. STAT3-deficient cells show impaired Tfh cell function in vitro. Naive CD4+ T cells isolated from healthy donors (HD) and $STAT3_{MUT}$ patients (Pt) were cultured under neutral (*nil*) or Th1 polarizing conditions. After 5 days, the cells were harvested and treated with mitomycin C before being cocultured with allogeneic naive B cells, in the absence or presence of exogenous IL-21, for an additional 7 days. After this time secretion of IgM, IgG, and IgA was determined. (A-B) The data were derived from experiments that used naive CD4+ T cells isolated from different $STAT3_{MUT}$ patients; (C) the data show the effect of exogenous IL-21 on the ability of $STAT3_{MUT}$ CD4+ T cells to provide B-cell help. Each graph represents the mean ± SEM of triplicate cultures; similar results were obtained in 5 (A-B) and 2 (C) experiments.

Defective IL-21 production by $STAT3_{MUT}$ CD4+ T cells results in impaired Tfh cell function in vitro

We next questioned whether a reduction in IL-21 expression in $STAT3_{MUT}$ CD4+ T cells would result in a detectable functional defect, such as a compromised ability of these cells to provide help to B cells. To investigate this, we established an in vitro B-cell helper assay in which naive CD4+ T cells were isolated from healthy donors and $STAT3_{MUT}$ patients and activated under neutral or Th1-polarizing conditions. After 5 days, the CD4+ T cells were harvested and cocultured with naive B cells for an additional 7 days, after which time Ig secretion was quantified. Normal naive CD4+ T cells activated under Th1 conditions provided significantly more help to support Ig production by cocultured B cells than when these T cells were cultured under neutral conditions (Figure 6A-C). Although priming under Th1 conditions also increased the helper function of $STAT3_{MUT}$ CD4+ T cells relative to those cultured under neutral conditions, it was significantly less than that of normal CD4+ T cells (Figure 6A-C). This reduction in B-cell help by IL-12–stimulated $STAT3_{MUT}$ CD4+ T cells approximated their reduction in expression of IL-21 under these culture conditions (ie, ~ 50%-70%; compare Figures 4C and 6A-C), thereby suggesting that the impaired ability of $STAT3_{MUT}$ CD4+ T cells to adequately promote B-cell differentiation resulted from insufficient production of IL-21. Indeed, the reduced ability of IL-12–primed $STAT3_{MUT}$ CD4+ T cells to provide help to B cells could be overcome when IL-21 was added to the cocultures (Figure 6C). Thus, functional STAT3 deficiency compromises the ability of IL-12 to induce IL-21, which is subsequently detrimental to the capacity of these CD4+ T cells to mediate the differentiation of B cells into Ig-secreting cells.

STAT3 mutations abolish IL-21 expression in naive CD4+ T cells induced by IL-6, IL-21, IL-23, and IL-27

As previously shown, other cytokines (IL-6, IL-21, IL-23, IL-27) can also give rise to IL-21–expressing cells, albeit to a much lesser extent than IL-12.[11,17,22] When $STAT3_{MUT}$ naive CD4+ T cells were exposed to these cytokines, they were unable to up-regulate IL-21 expression (Figure 4C). Thus, although mutations in *STAT3* substantially reduced the ability of IL-12 to induce IL-21 expression in CD4+ T cells, the ability of IL-6, IL-21, IL-23, and IL-27 to do this was completely dependent on STAT3. Consistent with this, $STAT3_{MUT}$ naive CD4+ T cells preactivated with IL-6, IL-21, or IL-23 were unable to support Ab production by cocultured B cells (supplemental Figure 3E-F).

Discussion

Lymphocyte differentiation is the outcome of the integration of signals from numerous external stimuli and the activation of specific transcription factors that regulate gene expression and ultimately cellular function. The differentiation of naive CD4+ T cells into Th1, Th2, Th17, and T-regulatory cells has been well-characterized for the roles of specific cytokines and transcription factors. The emergence of Tfh cells as the predominant subset of CD4+ T cells that mediate TD humoral immunity has been accompanied by the elucidation of the requirements for their generation and maintenance. Thus, engaging the TCR, ICOS, and the SLAM/SAP pathways by ligands present on APCs, together with signals mediated by STAT3 downstream of receptors for the cytokines IL-6, IL-21, and IL-27, coordinately induce expression of the transcription factors BCL6, IRF4, and c-MAF, which converge to yield Tfh cells.[8,12] Despite these generalized findings, much controversy remains over the relative contribution of these individual components to Tfh cell formation; this is most apparent from subsequent studies that have challenged the role of IL-6 and IL-21 in this process.[8,12,18,19] Furthermore, the molecular requirements for the generation of human Tfh cells remain incompletely defined.

We and others previously showed that IL-12 plays an important role in the differentiation of human Tfh cells, as evidenced by its ability to induce expression of IL-21 and to maintain expression of ICOS and CXCR5 on naive CD4+ T cells.[11,22] Similar to studies in mice,[7,14,17] IL-6, IL-21, and IL-27 also induce IL-21 expression in human naive CD4+ T cells, albeit to a much lesser extent than

IL-12.[11,17,22] We have now substantially extended these findings by investigating the in vivo and in vitro development of Tfh cells in patients with loss-of-function mutations in *IL-12RB1*, *STAT1*, *TYK2*, and *STAT3*; that is genes that compromise cytokine-mediated intracellular signaling pathways probably involved in regulating human Tfh cell formation.

The specific ability of IL-12 to induce IL-21 expression in human naive CD4$^+$ T cells was confirmed by showing that in the absence of a functional receptor (ie, in patients deficient for IL-12Rβ1) IL-12 was unable to give rise to IL-21–expressing cells (Figure 1I). Interestingly, IL-12–mediated IL-21 expression partially depended on intact STAT3 signaling, because heterozygous mutations in *STAT3* (which render most STAT3 dimers nonfunctional) reduced IL-21 expression by 50%-75%. Induction of IL-21 expression in CD4$^+$ T cells by IL-12 was also reduced in the absence of TYK2 but was unaffected by STAT1 deficiency. Further evidence that the generation of human Tfh cells is STAT1 independent was the finding that the frequency of circulating CXCR5$^+$CD45RA$^-$ T cells was unaffected by gain-of-function mutations in *STAT1* (22% ± 6.6%; n = 3). Although IL-12 is well-characterized for its ability to operate via STAT4-dependent pathways,[20] our finding of a requirement for STAT3 in IL-12 function is consistent with numerous studies that have documented STAT3 activation in human and murine T cells exposed to IL-12.[21,33,34,38,42] TYK2 is similarly phosphorylated in IL-12–treated T cells.[20,38,39] Despite the defect in IL-21 production, IL-12 could still induce $STAT3_{MUT}$ naive CD4$^+$ T cells to acquire other characteristics of Tfh cells such as increased expression of ICOS, CXCR5, and *BCL6* (Figure 5A-D). The intact induction of these phenotypic and molecular changes in Tfh-like cells, as well as residual expression of IL-21, in IL-12–treated CD4$^+$ T cells derived from $STAT3_{MUT}$ patients, are probably induced in a STAT4-dependent manner. Thus, IL-12 induces IL-21 expression predominantly through a TYK2/STAT3-dependent mechanism, with a minor contribution via STAT4 signaling. This is supported by the ability of both STAT3 and STAT4 to bind the promoters of the *IL21* and *BCL6* genes[43,44] and also by the recent finding that IL-12 induces IL-21 in murine CD4$^+$ T cells via STAT3- and STAT4-dependent pathways.[42] Importantly, the reduction in IL-12–induced expression of IL-21 in $STAT3_{MUT}$ CD4$^+$ T cells translated to a functional defect in TD B-cell differentiation in vitro (Figure 6). Interestingly, although IL-12 could still induce normal levels of ICOS on $STAT3_{MUT}$ CD4$^+$ T cells, this could not compensate for the deficiency in IL-21 production by these T cells, which is the primary source of help for B-cell differentiation. These findings are consistent with a model in which ICOS has a dual role in Tfh cells, first, in their generation from naive precursors, and, second, in enhancing IL-21 expression.[12,14,45,46]

While the ability of IL-12 to induce IL-21 in naive CD4$^+$ T cells was predominantly STAT3-dependent, induction of IL-21 by IL-6, IL-21, and IL-27; and the ability of CD4$^+$ T cells primed with these cytokines to help B cells, were completely abrogated by mutations in *STAT3*. Thus, the ability of all cytokines currently identified to induce IL-21 in human CD4$^+$ T cells, and subsequent B cell-helper function would be dramatically affected by *STAT3* mutations, that is, either strongly reduced or completely abolished. The importance of intact STAT3 signaling in generating human Tfh cells is reinforced by the significant reduction in the frequencies of circulating CD4$^+$ CXCR5$^+$ T cells in $STAT3_{MUT}$ patients. By contrast, the frequency of these CXCR5$^+$ Tfh-like cells was normal in patients deficient for IL-12Rβ1. This suggests that, although IL-12 induces the greatest frequency of IL-21–expressing CD4$^+$ T cells, there is sufficient redundancy among cytokine signaling pathways involved in generating Tfh cells to overcome the inability of CD4$^+$ T cells to give rise to Tfh cells in the absence of the IL-12 signaling. In other words, IL-6, IL-21, and IL-27 signaling through STAT3 will still give rise to Tfh cells from *IL12RB1*-mutant CD4$^+$ T cells. This is supported by the observation that humoral immune responses are intact in patients deficient for IL-12Rβ1.[26,30,31] Given the critical role of Tfh cells in humoral immune responses, it makes teleologic sense that this level of redundancy evolved to protect against the detrimental effects of Tfh cell deficiency. In contrast, use of STAT3 by several cytokines in the generation of human Tfh cells provides an explanation for why $STAT3_{MUT}$ patients exhibit defects in humoral immune responses (including reductions in circulating CD4$^+$ CXCR5$^+$CD45RA$^-$ and CD45RA$^+$ Tfh-like cells, memory B cells and an inability to mount protective Ab responses after vaccination or natural infection[24,47,48]) that cannot be compensated entirely by IL-12–dependent STAT4 signaling. These clinical, cellular, and serologic features of STAT3 deficiency are reminiscent of patients with mutations in *ICOS* and *CD40LG*,[49,50] which largely result from an absence of B-cell help by Tfh cells.[49,50] Thus, although an intrinsic defect resulting from the inability of B cells to respond to cytokines such as IL-6, IL-10, and IL-21 would contribute to the functional Ab deficiency in $STAT3_{MUT}$ patients,[24] this defect would be compounded further by the compromised generation and function of Tfh cells.

Overall, our findings have shed substantial light on the molecular requirements for generating human Tfh cells and have identified a signaling pathway that could be targeted to enhance Tfh cell generation in immunodeficient conditions. The corollary is that because dysregulated activation and/or generation of Tfh cells has been associated with autoimmunity in humans and mice, inhibiting this pathway may represent a novel approach to treating autoAb-mediated conditions.

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Authorship

Contribution: C.S.M. designed the research, performed experiments, analyzed and interpreted results, and wrote the manuscript; D.T.A., A.C., and E.K.D performed experiments; J.B., S.B.-D., P.D.A., A.Y.K, D.A., D.E., K.M., S.S.K., Y.M., S.N., M.A.F., S.C., J.M.S., J.P., M.W., P.G., M.C.C., D.A.F., and J.-L.C. provided patient samples and clinical details; M.C.C. also genotyped STAT3-mutant patients; M.B. provided intellectual input; and S.G.T designed the research, interpreted results, and wrote the manuscript. All authors commented on the manuscript.

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ORIGINAL ARTICLE

Endocrine complications in primary immunodeficiency diseases in Japan

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Summary

Background In spite of the accumulating evidence on the interaction between the immune and endocrine systems based on the recent progress in molecular genetics, there have been few epidemiological studies focused on the endocrine complications associated with primary immunodeficiency diseases (PID).

Objective To investigate the prevalence and clinical features of endocrine complications in patients with PID in a large-scale study.

Design and participants This survey was conducted on patients with PID who were alive on 1 December 2008 and those who were newly diagnosed and died between 1 December 2007 and 30 November 2008 in Japan. We investigated the prevalence and the clinical data of the endocrine complications in 923 patients with PID registered in the secondary survey.

Results Among 923 PID patients, 49 (5·3%) had endocrine disorders. The prevalence of the endocrine diseases was much higher in patients with PID than in the general population in the young age group, even after excluding patients with immune dysregulation.

Conclusions Endocrine disorders are important complications of PID. Analysis of the endocrine manifestations in patients with PID in a large-scale study may provide further insights into the relationship between the immune and endocrine systems.

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Introduction

A wide variety of clinical complications have been described in primary immunodeficiency diseases (PID).[1,2] PID have been reported to be associated with an increased risk of cancer, in particular non-Hodgkin lymphoma,[2] and the contribution of immune dysfunction in PID to cancer risk is receiving much attention. It is also well known that patients with PID often have complications such as autoimmune and allergic disorders.[1,3] Recently, the interaction between the immune and endocrine systems has been getting increasing attention.[4,5] However, there have so far been no reports focusing on the endocrine complications associated with PID in a large-scale survey.

Many endocrine disorders in patients with PID are thought to be due to the development of the autoimmunity, which is closely related to the pathophysiology of PID.[6] However, it is not known how the immunological and molecular defects in individual PID contribute to the development of various autoimmune endocrine disorders. In addition, the genetic defects in some PID can lead to these complications directly or indirectly via nonimmunological mechanisms.[6]

We analysed the endocrine complications in PID from the information obtained from the nationwide PID survey in Japan conducted in 2008. This is the first large-scale survey focusing on the endocrine complications in PID.

Materials and methods

This survey was performed according to the nationwide epidemiological survey manual of patients with intractable diseases (2nd edition 2006, Ministry of Health, Labour and Welfare of Japan) as described previously.[7] PID classification was based on the criteria of the International Union of Immunological Societies Primary Immunodeficiency Diseases Classification Committee in 2007.[8] The survey was conducted on patients with PID who were alive on 1 December 2008 and those who were newly diagnosed and died between 1 December 2007 and 30 November 2008 in Japan. The initial survey covered 1224 paediatric departments and 1670 internal medicine departments, which were randomly selected according to the number of beds among the 2291 paediatric departments and 8026 internal medicine departments in Japan. Primary questionnaires regarding the number of patients and the disease names based on the PID classification

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were sent to the selected hospitals. The initial survey was conducted to investigate the prevalence of the respective PID. The secondary survey was performed to study the detailed clinical features of individual patients with PID. Secondary questionnaires regarding age, gender, clinical manifestations and complications other than those related to haematopoietic stem cell transplantation of individual patients with PID were sent to the respondents who answered that they observed at least one PID patient with characteristics listed in the primary questionnaires. The details of the methods of the questionnaire investigation, the response rates and the breakdown of the number of patients in both paediatric and internal medicine departments were described elsewhere.[9] The questionnaires were designed to elucidate the clinical characteristics including the manifestations and laboratory data of the patients. In this study, all endocrine manifestations in patients with PID were included as complications of PID, even if they were well known major symptoms of PID.

Results

Detailed clinical information was available from 923 (secondary survey) out of 1240 patients with PID (initial survey).[9] Among the 923 patients with PID, 49 (5·3%) had endocrine disorders. As shown in Table 1, more than two thirds of the patients with PID were <20 years old and the prevalence of endocrine diseases was much higher in the young population of patients with PID than that in the general young population,[7,10–14] even after excluding patients with immune dysregulation (PID category IV). As expected, hypoparathyroidism was the most common endocrine disorder, because it is very frequently observed in patients with DiGeorge syndrome. Endocrine manifestations were also common in patients with diseases of immune dysregulation, such as immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome and autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED). Although the number of patients with defects in innate immunity was small, endocrine complications seemed to be more common than expected. Interestingly, endocrine disorders were not observed in patients with complement deficiencies. In addition, Graves' disease and Addison's disease were not observed in any of the patients with PID in this study.

Type 1 diabetes mellitus (T1D) was observed in six patients with PID (Tables 1 and 2) including four with type 1A (autoimmune) and two with type 1B (autoantibody-negative, idiopathic). Type 1A diabetes mellitus occurred frequently in patients with IPEX or IPEX-like syndrome (two of six patients, 33·3%) (Table 1). One patient of unknown aetiology in PID category IV showed type 1A diabetes and Hashimoto's thyroiditis along with recurrent viral infections (Tables 1, 2 and S1). In the cases of type 1A diabetes mellitus, anti-glutamic acid decarboxylase (GAD) autoantibodies and anti-insulin autoantibodies (IAA) were positive in all patients and in two of four patients, respectively (Table 2). The patients with IPEX and IPEX-like syndrome had a history of diabetic ketoacidosis with poor glycaemic control, and they developed T1D at a younger age than the other patients with PID. The first case of warts, hypogammaglobulinaemia, infections, and myelokathexis (WHIM) syndrome with T1D and hypothyroidism was included (Tables 2 and S2).[15] With regard to type 1B diabetes mellitus, the patient with hypogammaglobulinaemia of unknown aetiology had diabetic ketoacidosis (Table 2). On the other hand, type 2 diabetes mellitus (T2D) was observed in two patients with PID (Table 1).

Hashimoto's thyroiditis was observed in five patients with PID (Tables 1 and S1). The onset was very early in the patient with IPEX syndrome (at birth). All patients had at least 1 autoantibody among the anti-thyroid peroxidase (TPO), anti-thyroglobulin (Tg) and thyroid stimulating hormone receptor autoantibodies (TRAb).

Nonautoimmune hypothyroidism was reported in seven patients with PID (Tables 1 and S2). Anti-thyroid autoantibodies were all negative when measured. Among these, three patients with X-linked agammaglobulinaemia (XLA), IgG subclass deficiency or WHIM syndrome had primary (congenital) hypothyroidism detected by newborn mass screening. Hypothyroidism in the other four patients with normal TSH levels was considered to be due to central hypothyroidism, a disorder of the pituitary, hypothalamus or hypothalamic-pituitary portal circulation. Two patients with severe combined immunodeficiency (SCID) developed hypothyroidism before they received haematopoietic stem cell transplantation.

Growth hormone deficiency (GHD) was observed in six patients with PID (Tables 1 and S3), whose heights at the diagnosis of GHD ranged from −11·3 SD to −2·5 SD. Five patients were treated with growth hormone. One patient with SCID received cord blood transplantation when she was 20 months old, without conditioning chemotherapy or radiation.

Hypogonadism was observed in three patients with PID (Tables 1 and S4). Among them, two had hypergonadotrophic (primary) hypogonadism, whereas the other had hypogonadotrophic (central) hypogonadism. None of the patients received haematopoietic stem cell transplantation.

One common variable immunodeficiency disease (CVID) patient had isolated ACTH deficiency (Table 1). The other endocrine complications included hypophosphataemia, pseudohypoaldosteronism, adrenal crisis, hypoglycaemia and hypophosphataemic rickets as shown in Table 1.

Discussion

This is the first nationwide survey focusing on the endocrine complications of PID. Among these, hypoparathyroidism was the most common, observed in patients with DiGeorge syndrome and APECED.[16,17] In APECED, the calcium-sensing receptor has been reported to be the autoantigen responsible for hypoparathyroidism.[18] Although it has been reported that 79% of patients with APECED have hypocalcaemia due to hypoparathyroidism,[17] only 1 (25%) among four patients with APECED developed hypoparathyroidism in this study, which might be one of the clinical characteristics of patients with APECED in Japan.

The prevalence (33·3%) of T1D in patients with IPEX syndrome in this study seemed to be lower than that (>70%) of the previous reports.[19,20] The low prevalence of T1D might be due to

Table 1. Endocrine complications in PID patients

PID category	Hypopara-thyroidism	Diabetes mellitus			Thyroid disease		GHD	Hypogonadism	Isolated ACTH deficiency	Others	The number of PID patients			
		T1D			Autoimmune hypothyroidism (Hashimoto's thyroiditis)	Non-autoimmune hypothyroidism					*n*	0–19 years	Total	Percent in total
		1A	1B	T2D										
I. Combined T and B cell immunodeficiencies											**4**	**67**	**75**	**5·3**
RAG1 deficiency						1					1	6	6	16·7
CD4 deficiency					1						1	2	2	50·0
Undetermined											2	10	10	20·0
T-B-SCID						1	1				2	4	4	50·0
II. Predominantly antibody deficiencies											**13**	**231**	**378**	**3·4**
X-linked agammaglobulinaemia						1				2*	3	93	138	2·2
Common variable immunodeficiency disorders			1		1††		1		1	2†	6	29	93	6·5
IgG subclass deficiency						2					2	45	50	4·0
Undetermined			1				1**	1**			2	9	9	22·2
III. Other well-defined immunodeficiency syndromes											**20**	**126**	**165**	**12·1**
Hyper-IgE syndrome							1	1		1‡	3	31	46	6·5
DiGeorge syndrome	14										14	29	32	43·8
Ataxia telangiectasia				1							1	8	13	7·7
Chronic mucocutaneous candidiasis					1††						1	9	13	7·7
ICF syndrome								1			1	0	1	100·0
IV. Diseases of immune dysregulation											**6**	**31**	**38**	**15·8**
IPEX syndrome		2			1					1§	4	5	6	66·7
APECED	1										1	3	4	25·0
Undetermined		1**			1**						1	2	2	50·0
V. Congenital defects of phagocyte number, function or both											**3**	**106**	**153**	**2·0**
Chronic granulomatous disease						1	1				2	54	87	2·3

Table 1. *(continued)*

PID category	Hypopara-thyroidism	Diabetes mellitus: T1D 1A	T1D 1B	Diabetes mellitus: T2D	Thyroid disease: Autoimmune hypothyroidism (Hashimoto's thyroiditis)	Thyroid disease: Non-autoimmune hypothyroidism	GHD	Hypogonadism	Isolated ACTH deficiency	Others	The number of PID patients: *n*	0–19 years	Total	Percent in total
Shwachman–Diamond syndrome							1				1	2	2	50·0
VI. Defects in innate immunity											**2**	**9**	**12**	**16·7**
NEMO deficiency										1¶	1	7	7	14·3
WHIM syndrome		1**				1**					1	2	3	33·3
VII. Autoinflammatory disorders											**1**	**54**	**74**	**1·4**
Familial Mediterranean fever				1††							1	23	36	2·8
VIII. Complement deficiencies											**0**	**18**	**23**	**0**
IX. Undetermined											**0**	**3**	**5**	**0**
Total	15	6		2	5	7	6	3	1	7	49	645	923	5·3
Estimated prevalence per 10 000 in the young population (0–19 years) of PID patients (95% CI)	232·6 (141·4–380·1)	93·0 (42·7–201·5)		15·5 (2·7–87·3)	46·5 (15·8–135·9)	108·5 (52·7–222·3)	93·0 (42·7–201·5)	46·5 (15·8–135·9)	15·5 (2·7–87·3)					
Prevalence per 10 000 in the general young Japanese population	0·072‡‡	1·19		0·461§§	30·0§§	13·5¶¶	1·47	ND	0·035					
References	[7]	[10]		[10]	[11]	[12]	[13]	ND	[14]					

SCID, severe combined immunodeficiency; ICF, immunodeficiency with centromeric instability and facial anomalies; IPEX, immune dysregulation, polyendocrinopathy, enteropathy, X-linked; APECED, autoimmune polyendocrinopathy with candidiasis and ectodermal dystrophy; NEMO, NF-κB essential modulator; WHIM, warts, hypogammaglobulinaemia, infections, and myelokathexis; T1D, type 1 diabetes; T2D, type 2 diabetes; GHD, growth hormone deficiency.
*Hypophosphatemia 1, Obesity 1.
†Obesity 2.
‡Pseudohypoaldosteronism 1.
§Adrenal crisis, Hypoglycaemia 1.
¶Hypophosphatemic rickets 1.
**Two endocrine disorders were observed in the same patient.
††the case whose onset age of a endocrine complication is 20 years or older, *n*: number of PID patients who had endocrine disorders, CI: confidence interval.
‡‡prevalence in all age groups.
§§incidence data.
¶¶prevalence in the United States, ND: no data available.

Table 2. Clinical data of T1D patients

Case		1	2	3	4	5	6
Disease		IPEX syndrome	IPEX-like syndrome	Immune dysregulation (undetermined)	WHIM syndrome	CVID	Hypogammaglobulinaemia (unknown aetiology)
Genetic mutations (gene name)		+ (*FOXP3*)	Unknown	Unknown	+ (*CXCR4*)	Unknown	NT
HSCT		–	–	–	–	–	–
Sex		M	M	F	F	F	M
Present age		8 years 5 months	14 years 5 months	21 years 8 months	18 years 9 months	19 years 1 month	25 years 3 months
Onset age of T1D		3 months	10 months	7 years 9 months	5 years 7 months	7 years 9 months	6 years 5 months
Type of T1D		1A	1A	1A	1A	1B	1B
Clinical symptoms		Polydipsia, polyuria	Polydipsia, weight loss	ND	Polydipsia, polyuria	None	None
Diabetic ketoacidosis		+ (pH 7·112)	+ (pH 7·012)	–	–	–	+ (urine ketone body (4+))
Laboratory data	Normal range						
Fasting blood glucose (mmol/l)	3·9–6·1	31·7	29·1	6·1*	7·6	8·3	7·7
HbA1c (%)	4·3–5·8	7·9	8·3	8·7*	8·9	5·6	9·1
Plasma CPR (nmol/l)	0·33–0·93	ND	0·27	0·10*	ND	0·27	ND
Urinary CPR (μg/day)	20–100	ND	ND	2·5*	15	NT	ND
Anti-GAD Ab							
Result		+	+	+*	+	None	None
Value (U/ml)	<1·5	69·1	4860	9·3*	92	ND	ND
Anti-IAA Ab							
Result		–	ND	+*	+	ND	ND
Value (nIU/ml)	<125	2·8		ND	ND		
Treatment							
Age at the start		3 months	10 months	7 years 9 months	5 years 7 months	8 years 1 month	6 years 5 months
Content		Insulin	Insulin	Insulin	Insulin	Insulin	Insulin

NT, not tested; ND, no data available; FOXP3, forkhead box P3; CXCR4, CXC chemokine receptor 4; HSCT, haematopoietic stem cell transplantation; CPR, C-peptide immunoreactivity; GAD, glutamic acid decarboxylase; IAA, insulin autoantibody.
*Post-treatment data.

some genetic factor, because the Japanese have been have reported to be one of the races with the lowest incidence of T1D.[21] With regard to the patient with WHIM, Takaya *et al.*[15] have reported that mutations of *CXCR4*, the gene responsible for WHIM syndrome, might be closely related to the development of T1D, because recent findings have suggested that impaired CXCR4 signalling is involved in the pathogenesis of T1D. The prevalence of T1D in patients with CVID was 1·1% (one in 93 patients) in our study, which was almost equal to that in the previous report.[3]

The development of T2D was observed in only one of 13 patients with ataxia telangiectasia (AT) (7·7%) in contrast to the high prevalence of T2D in the previous report (five of eight patients),[22] suggesting the unique clinical characteristics of patients with AT in Japan.

Hashimoto's thyroiditis is a relatively common endocrine manifestation in patients with IPEX syndrome.[19,20] The prevalence of Hashimoto's thyroiditis in patients with CVID in our study was 1·1% (one in 93 patients), which was similar to that of the previous report.[23] There have been only a few reports of Hashimoto's thyroiditis in patients with (S) CID.[24,25] Interestingly, this was the first report of Hashimoto's thyroiditis in a patient with CD4 deficiency, while autoimmune cytopenia is frequently associated with this disease (19%).[26] The patient with a patient with CD4 deficiency and Hashimoto's thyroiditis did not receive stem cell transplantation, suggesting that this complication was caused by autoimmunity based on the combined immunodeficiency. Nagpala *et al.*[25] reported an infant with autoimmune thyroiditis and hypothyroidism with SCID due to adenosine deaminase deficiency despite an extremely low number of T cells and a low level of IgG, which suggested that the leaky SCID phenotype permitted the survival of a few T cells with autoimmune potential.[27]

Central hypothyroidism (no TSH elevation) was observed in two patients with SCID before they received haematopoietic stem cell transplantation (Table S2), also suggesting the possibility that this complication was related to the combined immunodeficiency itself. In addition, this was the first report of primary hypothyroidism (elevated TSH levels at birth) in patients with

XLA or IgG subclass deficiency, although the aetiologies remain to be determined.

Of note, the prevalence of GHD in patients with PID seemed much higher than that in the general population (Table 1). Until now, GHD has been reported in patients with several diseases in PID including SCID, CVID and Shwachman–Diamond syndrome, as shown in our study.[28–30] However, to the best of our knowledge, this was the first report of GHD in patients with hyper-IgE syndrome (HIES) and chronic granulomatous disease (CGD). Some SCID patients with GHD have been reported to have *STAT5b* gene mutations.[31] However, the gene was not investigated in our patient with SCID. With respect to the mechanism underlying the development of GHD in patients with CVID, common impairment in the IGF-1 and IgG pathways has been suggested as a cause of the growth retardation in some patients with CVID.[32] In addition, anti-pituitary antibodies have been detected in some of these patients.[33] The patient with congenital agammaglobulinaemia had various other complications in addition to GHD (Table S3), suggesting that this patient might have had a novel primary immunodeficiency.

Hypogonadism in patients with immunodeficiency with centromeric instability and facial anomalies (ICF) syndrome has been reported previously[34], although the mechanism is unclear. On the other hand, this was the first report of hypogonadism in patients with congenital agammaglobulinaemia and HIES. It is possible that hypogonadism has not been a major concern in PID for clinicians.

Isolated ACTH deficiency usually occurs during adult life, and only a few cases have been reported in childhood.[35] However, the development of isolated ACTH deficiency in a 14-year-old girl with CVID has been reported[35], in addition to the present case (Table 1). Therefore, a common pathological background is suspected in some of the patients with CVID.

Several limitations of this study should be considered. First, there were only a small number of adult patients with PID reported in this study, from which we could not estimate the accurate prevalence of endocrine manifestations in adults. Second, not all of the patients with PID were given sufficient examinations by endocrinologists and different examination methods were used at the respective hospitals.

There has been growing evidence of the interaction between the immune and endocrine systems.[4,5] In this study, we have found an increased prevalence of endocrine complications in patients with PID, which appear to be caused by immune dysregulation or by the underlying genetic disorders of the respective PID. Although various endocrine abnormalities have been reported to occur after stem cell transplantation,[36] therapy-related endocrine abnormalities were not included in the present study. A large-scale study such as a nationwide survey, focusing on the endocrine diseases, may have the potential to provide further insights into the mechanisms or pathophysiology of endocrine disorders in non-PID as well as patients with PID.

Conflicts of interest/financial disclosure

We declare that we have no conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Clinical data of patients with Hashimoto's thyroiditis.

Table S2. Clinical data of patients with nonautoimmune hypothyroidism.

Table S3. Clinical data of patients with GHD.

Table S4. Clinical data of patients with hypogonadism.

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Letter to the Editor

Common variable immunodeficiency classification by quantifying T-cell receptor and immunoglobulin κ-deleting recombination excision circles

To the Editor:

Common variable immunodeficiency (CVID) is the most frequent primary immunodeficiency associated with hypogammaglobulinemia and other various clinical manifestations. CVID was originally reported to be a disease primarily caused by defective B-cell function, with defective terminal B-cell differentiation rendering B cells unable to produce immunoglobulin. However, combined immunodeficiency (CID) involving both defective B and T cells is often misdiagnosed as CVID.[1] Indeed, one study reported that $CD4^+$ T-cell numbers were decreased in 29% of 473 patients with CVID[2]; similarly, another study found that naive T-cell numbers were markedly reduced in 44% (11/25) of patients with CVID.[3] These observations indicated that a subgroup of patients with clinically diagnosed CVID is T-cell deficient. Consistently, some patients with CVID have complications that might be related to T-cell deficiency, including opportunistic infections, autoimmune diseases, and malignancies, which is similar to that observed in patients with CID.[1,4] Therefore identifying novel markers to better classify CVID and distinguish CID from CVID will be required to best manage medical treatment for CVID.

We recently performed real-time PCR-based quantification of T-cell receptor excision circles (TREC) and signal joint immunoglobulin κ-deleting recombination excision circles (KREC) for mass screening of severe combined immunodeficiency (SCID)[5] and B-lymphocyte deficiency[6] in neonates. TREC and KREC are associated with T-cell and B-cell neogenesis, respectively.[7] Here we retrospectively report that TREC and KREC are useful for classifying patients with clinically diagnosed CVID.

Hypogammaglobulinemic patients (n = 113) were referred to our hospital for immunodeficiency from 2005-2011, and the following patients were excluded from the CVID pool by estimating their SCID genes based on clinical manifestations and lymphocyte subset analysis: 18 patients with SCID diagnoses; 14 patients less than 2 years of age (transient infantile hypogammaglobulinemia); 10 patients with IgM levels of greater than 100 mg/dL (hyper-IgM syndrome); 26 patients with diseases other than CVID caused by known gene alterations (10 with X-linked agammaglobulinemia and 11 with hyper-IgM syndrome [*CD40L* or *AICDA* mutated]), (2 with DiGeorge syndrome, and 3 with *FOXP3*, *IKBKG*, or *6p* deletions); and 5 patients with drug-induced hypogammaglobulinemia. The remaining 40 patients with decreased IgG (≥2 SDs below the mean for age), IgM, and/or IgA levels, as well as absent isohemagglutinins, poor response to vaccines, or both were included in this study as patients with CVID and analyzed for TREC/KREC levels, retrospectively.

Ages of patients with CVID ranged from 2 to 52 years (median age, 15.5 years). The sex ratio of the patients was 21 male/19 female patients. Serum IgG, IgA, and IgM levels were 370 ± 33 mg/dL (0-716 mg/dL), 30 ± 7 mg/dL (1-196 mg/dL), and 40 ± 6 mg/dL (2-213 mg/dL), respectively. TREC and KREC quantification was performed by using DNA samples extracted from peripheral blood, as reported previously.[5,6] Clinical symptoms were then assessed retrospectively. The study protocol was approved by the National Defense Medical College Institutional Review Board, and written informed consent was obtained from adult patients or parents of minor patients in accordance with the Declaration of Helsinki.

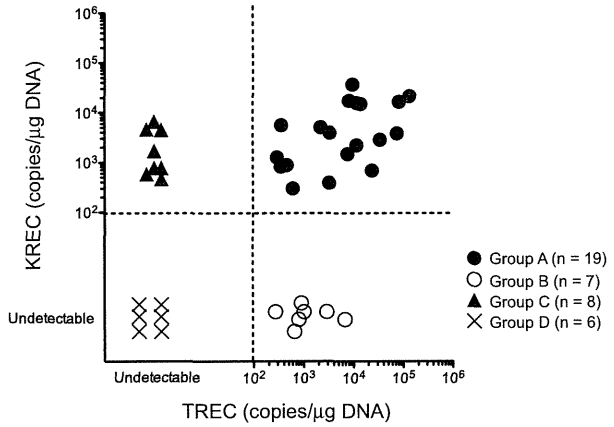

FIG 1. Quantifying TREC and KREC classifies patients with CVID into 4 groups. Patients with CVID were classified as follows: TREC(+)/KREC(+), group A (19 patients); TREC(+)/KREC(−), group B (7 patients); TREC(−)/KREC(+), group C (8 patients); and TREC(−)/KREC(−), group D (6 patients). *Undetectable,* Less than 100 copies/μg DNA.

Based on TREC and KREC copy numbers, the 40 patients with CVID were classified into 4 groups (groups A, B, C, and D; Fig 1). Comparing lymphocyte subsets, $CD3^+$ T-cell numbers were similar among groups A, B, and D but were significantly lower in group C ($P < .05$; group A, 1806 ± 204 cells/μL; group B, 1665 ± 430 cells/μL; group C, 517 ± 124 cells/μL; and group D, 1425 ± 724 cells/μL; $P = .0019$, Tukey multiple comparison test based on 1-way ANOVA). $CD3^+CD4^+CD45RO^+$ memory T-lymphocyte percentages in groups B, C, and D were significantly higher than those in group A ($P < .0001$; group A, 37% ± 16%; group B, 67% ± 13% [$P = .0006$]; group C, 92% ± 8.2% [$P < .0001$]; and group D: 83% ± 14% [$P < .0001$]; see Fig E1 in this article's Online Repository at www.jacionline.org); additionally, the percentages of these cells in groups C and D were higher than in group B ($P = .0115$). These results indicate that group C and D patients have markedly decreased $CD4^+CD45RA^+$ naive T-cell counts than group A patients and that counts in group B are also significantly decreased, although less so than in groups C or D, which is consistent with a report showing lower TREC copy numbers in $CD4^+CD45RO^+$ cells. Some patients in groups B, C, and D exhibited normal $CD4^+CD45RO^+$ percentages, although TREC levels, KREC levels, or both decreased. This discrepancy indicates that TREC/KREC levels could be independent markers to determine the patient's immunologic status in addition to $CD4^+CD45RA^+$; the reasons underlying the discrepancy between $CD4^+CD45RA^+$ and TREC/KREC levels remain unsolved.

$CD19^+$ B-cell numbers in group A were significantly higher ($P < .05$) than those in groups B and D (group A, 269 ± 65 cells/μL; group B, 35 ± 16 cells/μL; group C, 60 ± 11 cells/μL; and group D, 29 ± 16 cells/μL; $P = .0001$). However, B-cell subpopulations, including $CD27^-$, IgD^+CD27^+, and

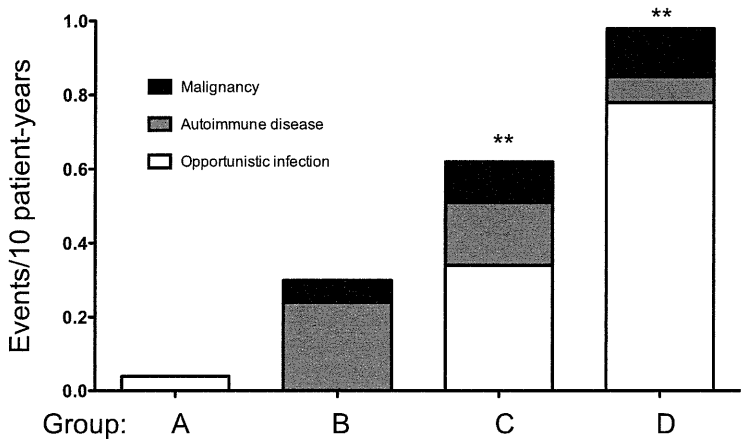

FIG 2. Cumulative incidence of complication events per 10 patient-years differs among groups. Opportunistic infections, autoimmune diseases, and malignancies were evaluated for each patient group. Complication incidences in group D (0.98 events/10 patient-years), group C (0.63 events/10 patient-years), and group B (0.30 events/10 patient-years) were higher than in group A (0.04 events/10 patient-years). Group A versus group D: $^{**}P = .0022$; group A versus C: $^{**}P = .0092$; group A vs group B: $P = .0692$.

IgD^-CD27^+ cells, were not significantly different among the groups. Standardizing KREC copy numbers for each patient by dividing their $CD19^+$ by their $CD27^+$ percentages revealed the same patient classification as that shown in Fig 1 (data not shown), indicating that the original classification was independent of $CD19^+$ B-cell or $CD27^+$ memory B-cell percentages.

Because TREC and KREC levels decrease with age (see Fig E2 in this article's Online Repository at www.jacionline.org)[5,6] and age distribution was wide in this study, we compared patients' ages among groups at the time of analysis to determine whether classification was associated with age. TREC/KREC-based classification was independent of both age and sex because age distribution was not significantly different among groups ($P > .05$; group A, 12.7 ± 2.3 years [2-30 years]; group B, 23.4 ± 4.2 years [6-39 years]; group C, 21.5 ± 6.1 years [4-52 years]; and group D, 25.5 ± 4.4 years [15-46 years]; data not shown) nor was male/female sex ratio (overall, 21/19; group A, 10/9; group B, 2/5; group C, 5/3; and group D, 4/2; $P = .4916$, χ^2 test; data not shown).

We next evaluated whether any correlation existed between TREC/KREC-based classification and clinical symptoms in each patient group. All patients in the study had been treated with intravenous immunoglobulin (IVIG) substitution at the time of analysis. We found that the cumulative events of complications (opportunistic infections, autoimmune diseases, and malignancies) per 10 patient-years were highest in group D (0.98 events/10 patient-years), followed by group C (0.63 events/10 patient-years), group B (0.30 events/10 patient-years), and group A (0.04 events/10 patient-years), where events in groups D and C were significantly higher than group A (group A vs group D, $P = .0022$; group A vs group C, $P = .0092$; group A vs group B, $P = .0692$; Fig 2). Furthermore, we found similar results when evaluating only patients 19 years old or older for group D (1.01 events/10 patient-years), group C (0.56 events/10 patient-years), group B (0.32 events/10 patient-years), and group A (0.06 events/10 patient-years; group A vs group D, $P = .0074$; group A vs group C, $P = .0407$; group A vs group B, $P = .1492$; data not shown). Categorizing patients by using several different previously reported CVID classifications (focused primarily on separating patients based on levels of circulating B-cell subsets), we found that no classification scheme showed any significant event increases in any particular group (see Fig E3 in this article's Online Repository at www.jacionline.org). Assessing longitudinal cumulative opportunistic infection incidence among the groups, group D and C values were significantly higher than in group A (see Fig E4, A, in this article's Online Repository at www.jacionline.org; $P = .0059$). Autoimmune and malignant diseases ($P = .5168$ and $P = .6900$, respectively) were observed in groups B and D but not in group A (see Fig E4, *B* and *C*). Cumulative events were significantly different between groups ($P = .0313$, log-rank test; group A, 5.3% and 5.3%; group B, 14.3% and 57.1%; group C, 27.1% and 63.5%; and group D, 33.3% and 83.3% at 10 and 30 years of age, respectively; see Fig E4, *D*). One patient in group D died of *Pneumocystis jirovecii* pneumonia, and 2 other patients in the same group received hematopoietic stem cell transplantation after complications caused by EBV-related lymphoproliferative disorder.

Assessing these data, TREC/KREC-based classification matches clinical outcomes. Because group D patients exhibited the most frequent complications (opportunistic infections, autoimmune diseases, and malignancies), they could receive a diagnosis of CID based on these symptoms. If they are indeed determined to have CID, then TREC/KREC analysis is helpful to distinguish between CID and CVID. Their TREC(−)/KREC(−) phenotype might relate to defective V(D)J recombination in T- and B-cell development[8] because patients with B-negative SCID (*RAG1*, *RAG2*, *Artemis*, and *LIG4*), as well as patients with ataxia-telangiectasia (AT) and Nijmegen breakage syndrome (NBS; see Fig E5 in this article's Online Repository at www.jacionline.org),[5,6] were also negative for both TREC and KREC; it is intriguing to speculate that an unknown V(D)J recombination gene or genes is responsible. As for treatment, hematopoietic stem cell transplantation should be considered the preferred treatment to "cure" group D patients, as reported in patients with severe CVID/CID, because event-free survival is poor.[9]

In contrast to group D patients, TREC(+)/KREC(+) group A patients treated with IVIG substitution therapy remained healthy. One possible explanation is that these patients harbor

defects only in terminal B-cell differentiation, but not in T cells, and represent typical patients with CVID, as originally reported.

Group C patients had a high frequency of both opportunistic infections and malignancies, suggesting that these TREC(−) patients have T-cell defects. Although group C patients had a similar TREC/KREC pattern to patients with SCID with B cells (*IL2RG* and *JAK3*; see Fig E5, *A*), they do not fulfill the European Society for Immunodeficiencies criteria for SCID, and no mutation was identified in the SCID genes estimated from clinical manifestation and lymphocyte subset analysis. However, from our data, they would likely benefit from undergoing similar treatment to patients with SCID or CID to prevent these complications.

Although opportunistic infections were rare in group B patients, autoimmune diseases were often observed. This is consistent with this group being TREC(+)/KREC(−) and the idea that balance between T and B cells is important to prevent autoimmune diseases in patients with CVID.[1] Intriguingly, a group of patients with AT and NBS were also TREC(+)/KREC(−) (see Fig E4, *B*), which is similar to group B patients. Additionally, $CD45RA^{+}CD4^{+}$ naive T-cell numbers were reduced in most group B patients, which is similar to the phenotype exhibited by patients with AT and NBS. This finding raises the possibility that although some group B patients are also T-cell deficient, as well as B-cell deficient, and should be treated similarly to patients with CID, other patients have only B-cell deficiency and are effectively treated with IVIG substitution therapy.

By analyzing a large CVID patient cohort, the overall survival rate of patients with more than 1 complication was worse than that for patients without other complications.[4] Our findings indicate that low TREC levels, KREC levels, or both are useful markers that correlate well with the overall survival rate in patients with CVID. Therefore we conclude that TREC and KREC are useful markers to assess the clinical severity and pathogenesis of each patient with CVID and to distinguish CID from CVID. Thus patient classification based on TREC/KREC levels would provide a helpful tool for deciding on an effective treatment plan for each patient with CVID.

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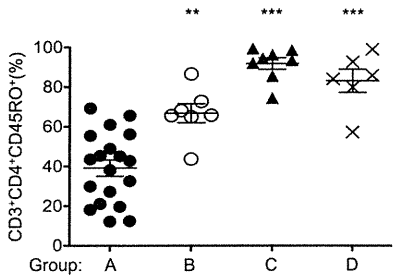

FIG E1. $CD45RO^+CD3^+CD4^+$ T-cell frequency within $CD4^+CD3^+$ lymphocytes was analyzed among groups. $CD45RO^+CD3^+CD4^+$ lymphocyte counts were significantly higher in groups B, C, and D compared with those in group A ($P < .0001$). Group A: 37% ± 16%; group B: 67% ± 13% (**$P < .01$); group C: 92% ± 8.2% (***$P < .001$); and group D: 83% ± 14% (***$P < .001$).

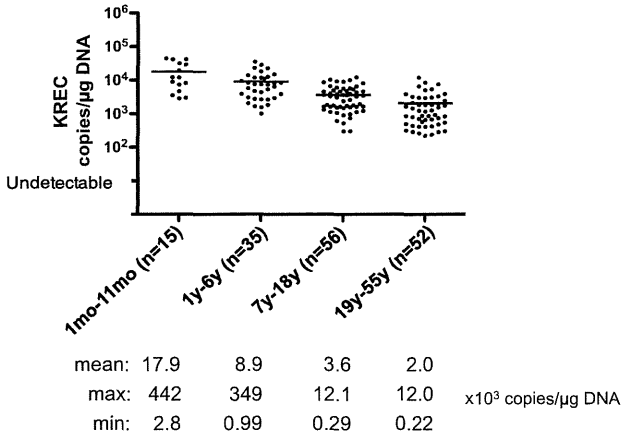

FIG E2. KREC levels were analyzed in genomic DNA samples extracted from peripheral blood of control subjects at different age groups (n = 158; age range, 1 month to 55 years). KREC levels were significantly higher in infants (17.9 ± 3.9 × 10^3 copies/μg DNA) compared with other children's age groups (8.9 ± 1.3 × 10^3 copies/μg DNA in the 1- to 6-year-old group and 3.6 ± 3.8 × 10^3 copies/μg DNA in the 7- to 18-year-old group) and adults (2.0 ± 3.3 × 10^3 copies/μg DNA; $P < .0001$).