

Blood CD4⁺CD45RO⁺CXCR5⁺ T cells are decreased but partially functional in signal transducer and activator of transcription 3 deficiency

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Background: The generation of high-affinity antibodies requires the presence of a population of CD4⁺ T cells (follicular T_H [T_{FH}] cells) in the lymph node follicles. These cells differ from T_H1, T_H2, and T_H17 effector cells in that they strongly express activation markers and the chemokine receptor CXCR5 and secrete large amounts of IL-21 and CXCL13. Small numbers of nonactivated CD4⁺CD45RO⁺CXCR5⁺ T cells are also found in the blood.

Objective: We sought to obtain *in vitro* a population close to the T_{FH} cells and to study the presence of this cell population among patients with autosomal dominant hyper-IgE syndrome carrying heterozygous signal transducer and activator of transcription 3 (*STAT3*) mutations that impair the IL-21 signaling required for B-cell differentiation.

Methods: CD4⁺CD45RO⁺CXCR5⁺ T cells were isolated from blood and activated by CD3/T-cell receptor.

Results: We found that CD4⁺CD45RO⁺CXCR5⁺ activated T cells corresponding to circulating *bona fide* memory T_{FH} cells and that *STAT3*-deficient patients have abnormally low numbers of “T_{FH}-like” blood T cells. However, *STAT3*-deficient T_{FH} cells have much the same phenotypic and functional characteristics as T_{FH} cells from healthy control subjects. The ability of *STAT3*-deficient T_{FH} cells to produce IL-21 on CD28/T-cell receptor activation and to proliferate did not differ from that observed for control T_{FH} cells *in vitro*. Although the *STAT3*-deficient T_{FH} cells were also able to help control B cells to produce IgG and IgA, induction of IgG production by naive B cells was impaired.

Conclusion: Heterozygous mutations in *STAT3* lead to reduced numbers of circulating T_{FH}-like cells, a finding that might

account (at least in part) for the observed defect in antibody production. (J Allergy Clin Immunol 2013;■■■■:■■■-■■■.)

Key words: Human, T cells, signal transducer and activator of transcription 3 deficiency, cell activation, naive B cells

The provision of T-cell help to B cells is a fundamental feature of the immune system in mammals. The help enables the production of memory B cells and long-lived plasma cells that secrete high-affinity, antigen-specific immunoglobulins. The generation of antibody-forming cells occurs during a germinal center (GC) reaction within B-cell follicles in secondary lymphoid tissues. These follicles are essential for driving T cell-dependent antibody responses.¹ CD4⁺ T cells are essential for the formation of GC reactions and provide developmental signals for the differentiation of antigen-selected, high-affinity GC B cells into memory B cells or plasma cells.²⁻⁵ It has been shown that a subset of nonpolarized CD4⁺ follicular T_H (T_{FH}) cells constitutes the true helper cells in antibody responses.⁶⁻⁸ Discovery of CXCR5 has enabled the identification of this population in lymphoid tissues.⁹ Interaction between CXCR5 and its ligand (the chemokine CXCL13) is responsible for the migration of B and T cells in the follicular areas of lymphoid tissues.¹⁰⁻¹² T_{FH} cells express high levels of IL-21, a cytokine that promotes B-cell growth, differentiation, and class-switching^{13,14} but also acts in an autocrine manner on the T_{FH} cell population, with concomitant expression of the IL-21 receptor (IL-21R).¹⁵ T_{FH} cells strongly express surface molecules essential for helper functions, including CD40 ligand (CD40L) and inducible costimulator (ICOS).^{6,16} Studies have shown that tonsillar T_{FH} cells display distinct phenotypic and genetic profiles when compared with other canonical T_H cell subsets.^{12,15} T_H1 and T_H2 cells do not express CXCR5 but do express CCR7 and L-selectin (CD62L), which enables homing of circulating T cells to T-cell compartments within secondary lymphoid tissues.¹⁷ They do not express B-cell lymphoma 6 (Bcl6), which is necessary and sufficient for the development of T_{FH} cells *in vivo*.^{5,18} Recently, Morita et al⁸ and Chevalier et al¹⁹ have shown that after specific activation, human blood CXCR5⁺CD4⁺ T cells might correspond to a circulating pool of memory T_{FH} cells. This pool is distinct from the T_H1, T_H2, and T_H17 subsets and can prompt naive and memory B cells to differentiate into immunoglobulin-producing cells (mainly through IL-21- and ICOS-induced signals).^{8,19} Differentiation of naive T lymphocytes into effector cells is regulated by cytokine receptors and the subsequent activation of Janus kinase-signal transducer and activator of transcription (STAT) 3 signaling pathways.²⁰⁻²³

Heterozygous mutations in *STAT3* have been found to cause autosomal dominant hyper-IgE syndrome.²⁴ High serum IgE levels were detected in these patients, although with normal serum levels of IgM, IgG, and IgA. However, the patients exhibit poor antigen-specific

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Abbreviations used

Bcl6:	B-cell lymphoma 6
CD40L:	CD40 ligand
CD62L:	L-selectin
CSR:	Class-switch recombination
GC:	Germinal center
ICOS:	Inducible costimulator
ICOSL:	Inducible costimulator ligand
IL-21R:	IL-21 receptor
PD-1:	Programmed death 1
SEB:	Staphylococcal enterotoxin B
STAT3:	Signal transducer and activator of transcription 3
T _{FH} :	Follicular T _H

antibody responses after immunization with T cell-dependent antigens.²⁵ Analysis of the T cell-intrinsic actions of STAT3 revealed that the patients' characteristic susceptibility to infections at the epithelial surfaces is caused by a deficiency in T_H17 cells.²⁶⁻²⁸ Furthermore, studies of gene-modified murine and human B cells *in vitro* have revealed that IL-21 (one of the STAT3-activating cytokines)^{29,30} is a key molecule for establishing and maintaining antibody responses and regulating their quality.^{13,21,22,31-33} Recently, Avery et al³⁴ showed that STAT3 has a critical role in generating memory B cells and plasma cells from naive precursors *in vivo* and *in vitro* (mainly in response to IL-21). This group showed that this STAT3-activating cytokine is a key mediator of human B-cell effector function and humoral immune responses and suggested a mechanism to explain the functional antibody deficiency in STAT3-deficient patients. More recently, they further showed that IL-12 (which induces IL-21 expression in naive human CD4⁺CCR7⁺ T cells) was also important (in a predominantly STAT3-dependent manner) for obtaining functional T_{FH} cells.³⁵ Because IL-21 is also an autocrine cytokine for T_{FH} cell generation, we decided to determine whether STAT3-deficient patients lack circulating memory CD4⁺CD45RO⁺CXCR5⁺ T cells. As expected, percentages of cells were indeed low, but those T_{FH} cells present did not differ markedly from cells from healthy donors in terms of phenotype, except that their ability to provide help for class-switch recombination (CSR) to naive B cells was reduced.

METHODS**Antibodies and cytokines**

Conjugated antibodies against the following antigens were obtained: CD4, CD45RA, CD45RO, CD134/OX40, CD57, CD229/LY9, CD69, CD127/IL-7 receptor α , and CD62L from BD Biosciences (San Jose, Calif); CD278/ICOS and CD279/programmed death 1 (PD-1) from eBioscience (San Diego, Calif); CXCR5, CXCR4, CCR6, CCR7, CD23, CD24, CD25, CD27, CD200, and CD212/IL-12 receptor from R&D Systems (Minneapolis, Minn); CD154/CD40L from Abcyss SA (Paris, France); CD70 from BD Biosciences Pharmingen (San Diego, Calif); CD84 from Santa Cruz Biotechnology (Santa Cruz, Calif); and CD360/IL-21R from BioLegend (San Diego, Calif). MegaCD40L (soluble) was from Alexis Biochemicals (San Diego, Calif); the cytokines IL-10, IL-12 and IL-4 were from R&D Systems and IL-21 was from PeproTech (Neuilly-sur-Seine, France); CD3/CD28 Dynabeads were from Dynal Biotech (Compiègne, France); CpG/oligodeoxynucleotide 2006 was from InvivoGen (San Diego, Calif); Affinipure F(ab')₂ fragment goat anti-human IgM was from Jackson ImmunoResearch (West Grove, Pa); staphylococcal enterotoxin B (SEB) was from Sigma-Aldrich (St Louis, Mo); the human extracellular domains of CD40 or ICOS ligand fused to murine IgG_{2a}Fc

fusion protein (inducible costimulator ligand [ICOSL]–muIg or CD40–muIg) were from Ancell (Bayport, Minn); and recombinant human IL-21R/Fc chimera (the extracellular domain of human IL-21R fused to the Fc region of human IgG₁) and recombinant human IgG₁Fc were from R&D Systems.

Cell isolation

Purified PBMCs from healthy adult volunteers (n = 30; French Blood Transfusion Service, Paris, France) and STAT3-deficient patients (n = 15) were prepared by means of density gradient centrifugation on Lymphoprep (Abcys-SA). The age range was 20 to 50 years for healthy donors and 11 to 50 years from patients. CD4⁺ T cells (purity >98%) were isolated by depleting non-CD4⁺ T cells with a magnetic bead technique (Miltenyi Biotec, Bergisch Gladbach, Germany). Likewise, CD4⁺CD45RO⁺ cells (purity >98%) were isolated by depleting non-CD4⁺CD45RO⁺ T cells with a magnetic bead technique (STEMCELL Technologies, Grenoble, France). After staining with PE-CXCR5, CXCR5⁺ and CXCR5[−] T cells were separated with anti-PE beads according to the manufacturer's protocol (Miltenyi Biotec and STEMCELL Technologies, Vancouver, British Columbia, Canada). B cells were isolated from the low-density PBMC fraction. Naive CD19⁺CD27[−] B cells (purity >99%) were also isolated with a magnetic bead depletion method (Miltenyi Biotec). CD19⁺ B cells were purified with anti-CD19 beads, according to the manufacturer's protocol (Miltenyi Biotec). Fifty thousand events were collected for the flow cytometric analysis of PBMCs, and 5000 events were collected for flow cytometric analysis of isolated populations. The cell purity was greater than 98%. Expression of each molecule was assessed with FlowJo software (TreeStar, Ashland, Ore).

Lymphocyte cultures

Sorted CXCR5⁺ T cells or CXCR5[−]CD45RO⁺ T cells (30 × 10³) were incubated with anti-CD3/CD28 beads (according to the manufacturer's protocol) and cultured for 8 days in 96-well plates with CD19⁺CD27[−] B cells (25 × 10³) in the presence of anti-IgM (500 ng/mL), SEB (1 μg/mL), CpG (50 nmol/L), IL-12 (20 ng/mL), and IL-21 (5 ng/mL) to induce IgA and IgG secretion and anti-IgM (500 ng/mL) and IL-4 (200 U/mL) to induce IgE secretion. As positive controls, CD19⁺CD27[−] B cells were incubated with anti-IgM (500 ng/mL), MegaCD40L (300 ng/mL), and IL-21 (200 ng/mL) for IgA and IgG secretion and anti-IgM (500 ng/mL), MegaCD40L (500 ng/mL), and IL-4 (200 U/mL) for IgE secretion. For some experiments, CXCR5⁺ T cells (5 × 10³) purified from healthy volunteers (n = 11) and STAT3-deficient patients (n = 9) were activated with anti-CD3/CD28 beads and cultured for 8 days in 96-well plates with CD19⁺ B cells (50 × 10³) purified from healthy volunteers to induce IgA, IgG, and IgE secretion. For positive controls, CD19⁺ B cells were incubated with MegaCD40L (50 ng/mL) and IL-21 (100 ng/mL) for IgA and IgG production and with MegaCD40L (500 ng/mL) and IL-4 (300 U/mL) for IgE production. Supernatants were collected, and immunoglobulin levels were measured by using an ELISA. Plates were coated with 5 μg/mL anti-human IgA or IgE rat antibodies (Abcys-SA) in PBS overnight at 4°C. Samples or protein standards were incubated in the plates for 2 hours, followed by incubation with either biotinylated anti-IgA or anti-IgE antibodies (Abcys-SA) and then avidin peroxidase (Abcys-SA). Plates were developed with a 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid (Sigma-Aldrich) developing solution and read at OD₄₀₅. A kit from Mabtech AB (Stockholm, Sweden) was used to assay IgG.

For cytokine assays, CXCR5⁺ and CXCR5[−]CD45RO⁺ T cells (1 × 10⁵) were incubated with CD3/CD28 beads (according to the manufacturer's protocol), and the supernatants were collected and tested with an ELISA. Specific kits were used for IL-4, IFN-γ, IL-17 (A and F), CXCL13 (R&D Systems), IL-21 (PeproTech), and IL-10 (Diacione, Besançon, France). For the cell proliferation assay, cells were first labeled with carboxyfluorescein succinimidyl ester (Invitrogen, Cergy-Pontoise, France) and then cultured with CD3/CD28 beads in 96-well plates for 3 or 5 days in separate experiments. In some experiments, after 20 hours of incubation, CD3/CD28 beads were removed, and then cells were labeled with carboxyfluorescein succinimidyl ester and cultured for 5 more days. For flow cytometric analysis, 5000 events were collected for cells from healthy donors and patients.

Western blotting

Sorted $CD4^+CD45RO^+CXCR5^+$ and $CXCR5^-$ T cells were activated with anti-CD3/CD28 beads for 20 hours. Then, after washing, total proteins were extracted from 0.3×10^6 cells by using RIPA buffer (Sigma-Aldrich) supplemented with 1% protease inhibitor mixture (Sigma-Aldrich). The supernatants from spun cell lysates were loaded onto a 12% SDS-PAGE gel in loading buffer and transferred to polyvinylidene difluoride membranes (Invitrogen). Lysates from 0.1×10^6 DAUDI B cells were used as a positive control. Membranes were incubated with an anti-Bcl6 antibody (Santa Cruz Biotechnology) overnight and then with horseradish peroxidase-conjugated anti-mouse antibody (Amersham Biosciences, Arlington Heights, Ill). Proteins were visualized with a chemiluminescence detection system (ECL, Amersham Biosciences). Protein expression was quantified by means of densitometric scanning.

STAT3-deficient patients

We studied patients with various heterozygous *STAT3* mutations registered in the French national primary immunodeficiency database (CEREDIH). One patient had a heterozygous mutation in the transactivation domain (K709E), 9 had a heterozygous mutation in the DNA-binding domain (R382W, F384L, and delV463), and 5 had a heterozygous mutation in the SH2 domain (V637M, S668Y, and I665N).³⁶ Clinical description of this cohort of patients and data about their memory, naive, switched, and nonswitched B-cell subsets have been added in [Tables E1 and E2](#) in this article's Online Repository at www.jacionline.org. Only patient 3 showed a high percentage of $CD19^+CD27^+$ cells, but of note, he had global lymphopenia (0.8×10^9 lymphocytes/L). $CXCR5^+$ T cells were isolated from these patients, as described above. Informed consent was obtained from the patients (aged from 6-47 years) or, for minor patients, their parents. The study was performed in accordance with the precepts of the Declaration of Helsinki.

RESULTS

Phenotypic characteristics of resting and activated $CXCR5^+$ and $CXCR5^-CD4^+CD45RO^+$ T cells of healthy donors

In contrast to $CD4^+CXCR5^-$ cells, most of the $CD4^+CXCR5^+$ cells were $CD45RO^+$ cells ($90\% \pm 5\%$ [mean $\pm$ SD]; $n = 10$; range, 70% to 93%) and expressed CCR7 ($70\% \pm 7\%$ [mean $\pm$ SD]; $n = 10$; range, 59% to 83%; see [Fig E1, A](#), in this article's Online Repository at www.jacionline.org). After $CXCR5^+CD4^+$ T-cell activation through the CD3/CD28 pathway, most of the cells lost their CCR7 expression (in contrast to $CXCR5^-$ cells; see [Fig E1, B](#)). In a Western blot analysis, activated $CXCR5^+CD4^+$ T cells were found to express more Bcl6 than $CXCR5^-CD4^+$ cells (see [Fig E1, C](#)), as had been reported previously for tonsillar $CXCR5^+CD4^+$ cells.^{5,18} This expression was correlated with strong expression of the T_{FH} cell markers *CXCR5* and *PD-1* ([Fig 1](#)), thus confirming data reported by Baumjohann et al.³⁷ Other activation-associated molecules (eg, ICOS, which is involved in Bcl6 induction, and markers associated with T_{FH} cells, such as *CD40L*, *CD134/OX40*, *CD200*, *CD57*, and the SAP-associating transmembrane receptor *CD229/Ly9*) were also strongly expressed after CD3/CD28 activation ([Fig 1](#)). In contrast, expression of *CCR7*, *CD62L*, and *IL-7* receptor α was downregulated. Expression of *IL-21R* and the *IL-12* receptor was upregulated in activated $CXCR5^+CD4^+$ T cells; this contrasted with the situation in activated $CXCR5^-CD4^+$ T cells, despite even longer activation in the latter ([Fig 1](#) and see [Fig E2, A](#), in this article's Online Repository at www.jacionline.org). Taken as a whole, these data confirm previous results showing that circulating $CXCR5^+CD45RO^+CD4^+$

T cells have much the same phenotype as T_{FH} cells from lymph nodes and tonsils.^{8,9,12,19}

Cytokine production by $CXCR5^+CD45RO^+CD4^+$ T cells and the IL-21-dependent proliferation of $CXCR5^+CD4^+$ T cells of healthy donors

As with tonsillar T_{FH} cells,¹² 1 to 4 days of CD3/CD28 activation of $CXCR5^+CD45RO^+CD4^+$ T cells led to increased production of CXCL13 and IL-21 (with low amounts of IL-10 and IL-4), whereas $CXCR5^-CD45RO^+CD4^+$ cells barely secreted IL-21 and CXCL13 but did produce IFN- γ , IL-17, IL-4, and IL-10 (see [Fig E2, B](#)). In contrast to $CXCR5^-$ T cells, the number of dividing activated $CXCR5^+$ T cells decreased by half in the presence of a neutralizing IL-21R Fc chimeric molecule (see [Fig E2, C](#)), especially when activator CD3/CD28 beads were removed after 20 hours (see [Fig E2, D](#)). A control Fc chimeric protein had no effect. These results show that $CXCR5^+$ T cells (unlike $CXCR5^-$ T cells) demonstrated autocrine, IL-21-dependent proliferation in a similar manner to tonsillar T_{FH} cells.^{21,22}

Specific ability of blood $CXCR5^+CD45RO^+CD4^+$ T cells of healthy donors to act as B-cell helpers in immunoglobulin production

We next examined the ability of both the $CXCR5^+$ and $CXCR5^-$ T-cell populations to help blood total $CD19^+$ B cells and naive $CD19^+CD27^-$ B cells to produce immunoglobulin. $CXCR5^+$ and $CXCR5^-CD4^+CD45RO^+$ T cells were activated with CD3/CD28 beads and cocultured with either nonactivated $CD19^+$ B cells ([Fig 2, A](#)) or naive $CD19^+CD27^-$ B cells activated with an anti-IgM and a Toll-like receptor 9 agonist and cross-linked to T cells with SEB ([Fig 2, B-D](#)). The medium was supplemented with low concentrations of IL-21 and the IL-21 inducer IL-12p70 to optimize B-cell activation.^{38,39} We found that both $CXCR5^+CD45RO^+CD4^+$ and $CXCR5^-CD4^+CD45RO^+$ T cells induced the secretion of different immunoglobulin classes. Indeed, $CXCR5^+CD4^+CD45RO^+$ T cells induced IgA and IgG secretion by both total and naive B-cell populations. In contrast, $CXCR5^-CD45RO^+CD4^+$ T cells had lower helper activity for IgA production, even when IL-21 was added ([Fig 2, A, B](#), and [D](#)). We also found that the IgG production by total B cells induced by $CXCR5^+CD45RO^+CD4^+$ T cells was less sensitive to IL-21 than IgG production by naive B cells. Furthermore, the IgG production induced by $CXCR5^-CD45RO^+CD4^+$ T cells differed from that induced by $CXCR5^+CD45RO^+CD4^+$ T cells because it was not increased by the addition of IL-21. This finding suggests another cytokine-dependent pathway ([Fig 2, A](#)). B-cell help was also found to be dependent on ICOS and CD40L,⁴⁰ as shown by the fact that the use of specific fusion proteins to block ICOSL/ICOS or CD40/CD40L interactions inhibited immunoglobulin secretion ([Fig 2, A, C](#), and [D](#)). Inhibition by ICOSL fusion protein was only observed in the presence of T cells, showing that this effect was T cell specific ([Fig 2, C](#)). Because IL-21 is known to strongly promote B-cell growth, differentiation,³³ and class-switching,¹³ we further determined the role of IL-21 secretion by $CXCR5^+CD45RO^+CD4^+$ T cells in the production of IgA in these cocultures. We observed that IgA production was inhibited by addition of a neutralizing IL-21R Fc chimeric protein ([Fig 2, A](#) and [C](#)). In contrast, a control Fc chimeric protein had no effect. Taken as a whole, these results demonstrate that

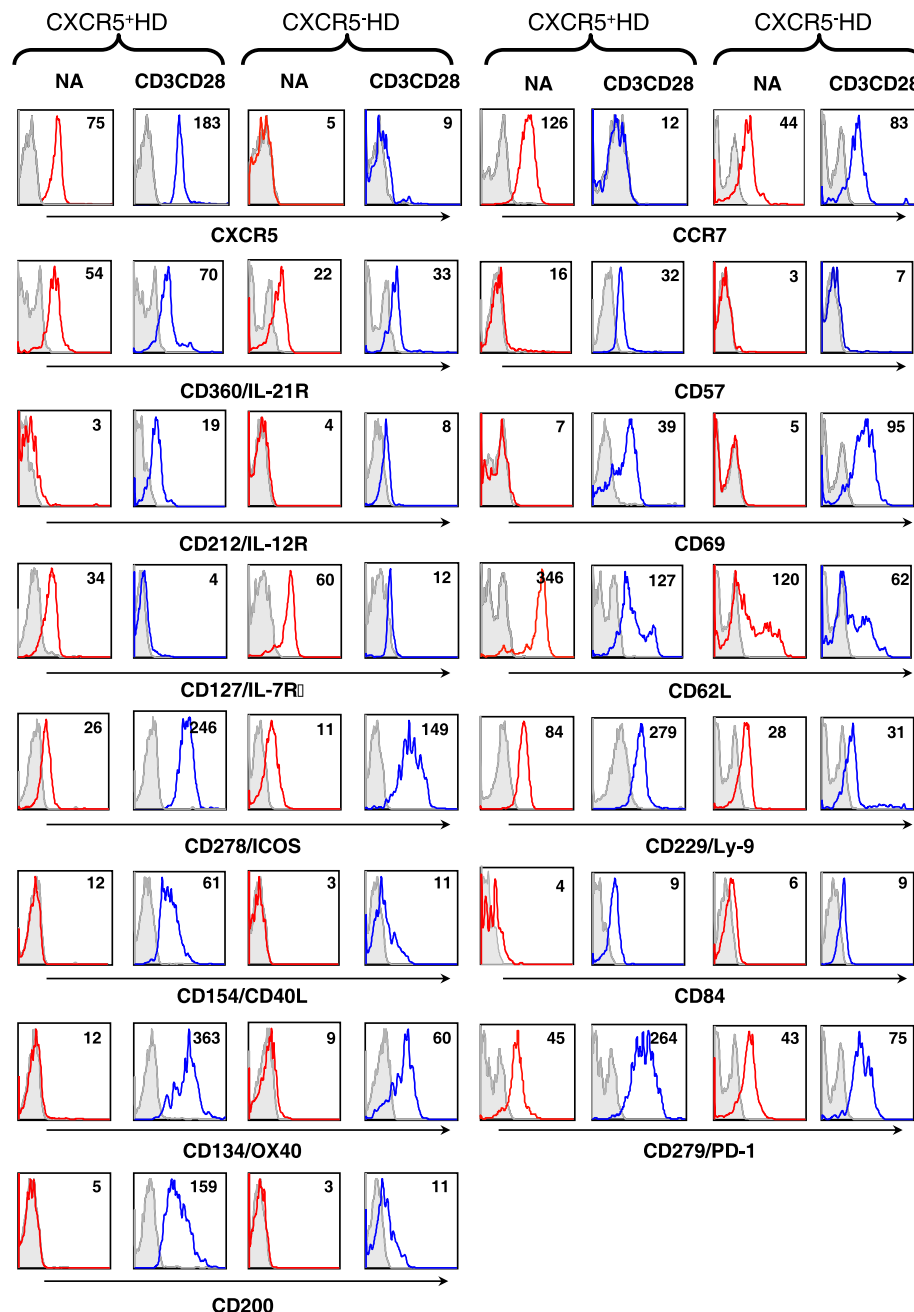


FIG 1. Phenotypic characteristics of CXCR5⁺ and CXCR5⁻ CD45RO⁺ CD4⁺ T cells from healthy donors. Membrane expression of chemokine receptors, cytokine receptors, and activation molecules on CXCR5⁺ T cells and RO⁺ CXCR5⁻ T cells from healthy donors (HD) after CD3/CD28 activation (CD3CD28) in comparison with nonactivated (NA) T cells from healthy donors is shown. The isotype control is also shown (gray shaded area). The mean fluorescence intensity (as an indicator of protein expression) is indicated on each histogram. This experiment is representative of 10 experiments performed with cells from different healthy donors.

CXCR5⁺CD45RO⁺CD4⁺ T cells help B cells in an IL-21-, ICOS-, and CD40L-dependent manner. We also showed, when IgE production was tested, that CXCR5⁻CD45RO⁺CD4⁺ T cells exerted much higher B helper activity (Fig 2, A and E). The lack of IgE production by naive B cells cocultured with CXCR5⁺CD45RO⁺CD4⁺ T cells could be rescued by the addition of IL-4, suggesting that impaired production of IL-4 by this subset accounted for its inability to provide help for IgE production (Fig 2, E).

Patients with STAT3 deficiency have a low proportion of CD4⁺CD45RO⁺CXCR5⁺ T cells

Given that the response to IL-21 is STAT3 dependent, we next determined whether STAT3-deficient patients, as described in Table E1, had CD4⁺CD45RO⁺CXCR5⁺ T cells *in vivo*. We found very low proportions of CD4⁺CD45RO⁺CXCR5⁺ cells (mean ± SEM, 3.4% ± 2.8%; range, 0.1% to 9.85%) in STAT3-deficient patients (n = 9) relative to healthy blood donors (n = 9; mean ± SEM, 11.4% ± 3.3%; range, 8.2% to 18.1%;

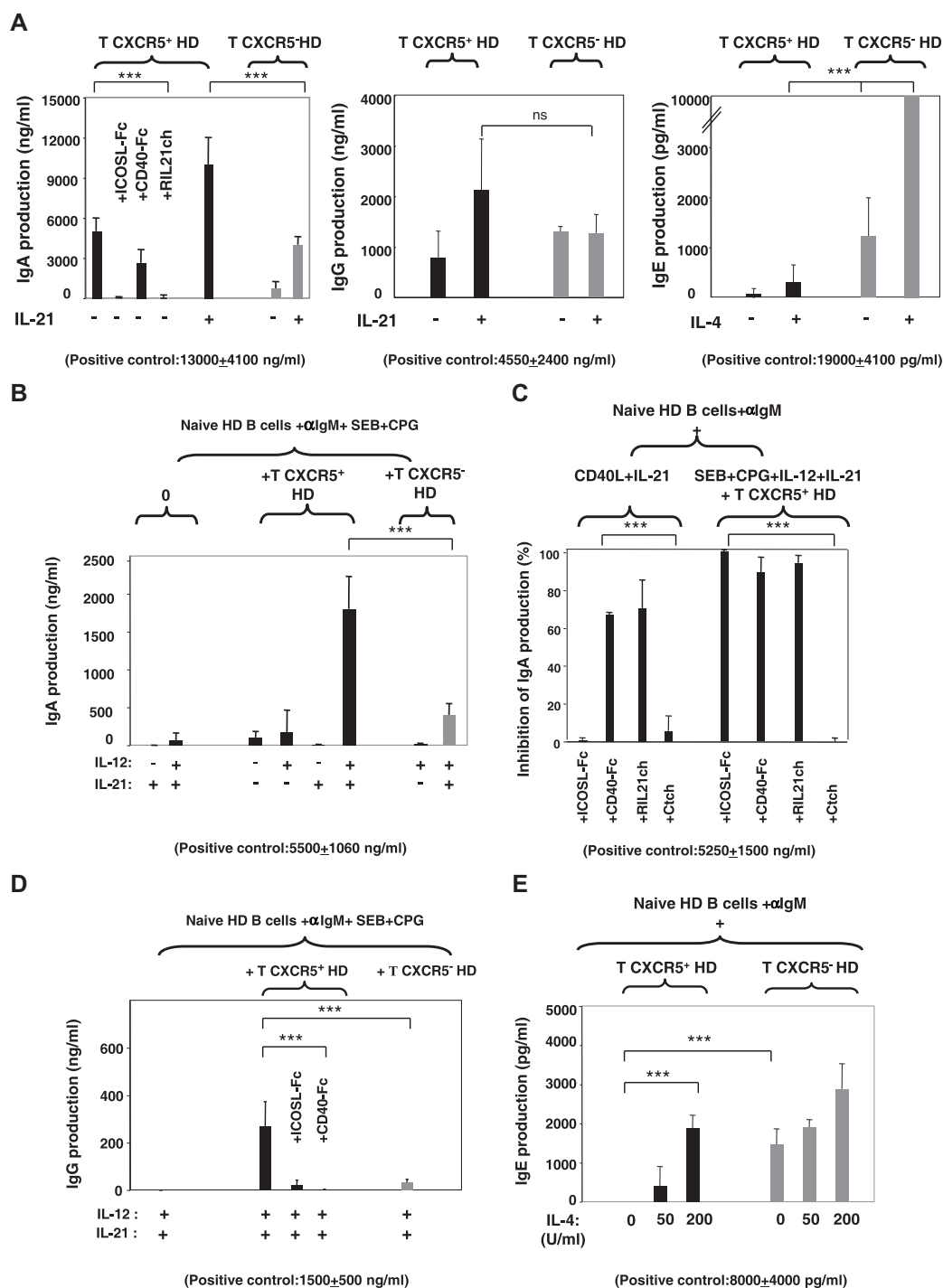


FIG 2. Helper activity of activated CXCR5⁺ and CXCR5⁻ CD45RO⁺ CD4⁺ T cells from healthy donors (HD) for IgA, IgG, and IgE production by autologous CD19⁺ B cells or naive CD19⁺CD27⁻ B cells. Immunoglobulin concentrations in the coculture of CD3/CD28-activated CXCR5⁺ T cells (black columns) or CXCR5⁻ T cells (gray columns) from healthy donors and autologous CD19⁺ B cells (A) or CD19⁺CD27⁻ B cells (B, D, and E) are shown. Human mulg-ICOS ligand (ICOSL) or mulg-CD40 (CD40) fusion proteins and recombinant human IL-12 receptor Fc chimera (RIL-21ch) were added at the start of the culture period in Fig 2, A (left panel), C, and D. Data from 10 (Fig 2, A and B), 5 (Fig 2, C and D), and 4 (Fig 2, E) independent experiments performed with cells from different healthy donors were pooled and reported as means $\pm$ SEMs. *** P < .001, Student t test.

P < .0001), as also recently reported by Ma et al.³⁵ Similarly, there was a low proportion of CXCR5⁺CCR7⁺ cells (mean $\pm$ SEM, 4.7% $\pm$ 1.7%; range, 1.39% to 7.4%) relative to that seen in

healthy blood donors (mean $\pm$ SEM, 11.3% $\pm$ 2.7%; range, 8.51% to 16.6%; P < .0001; Fig 3). It is noteworthy that after CD3/CD28 activation, the remaining cells showed increased

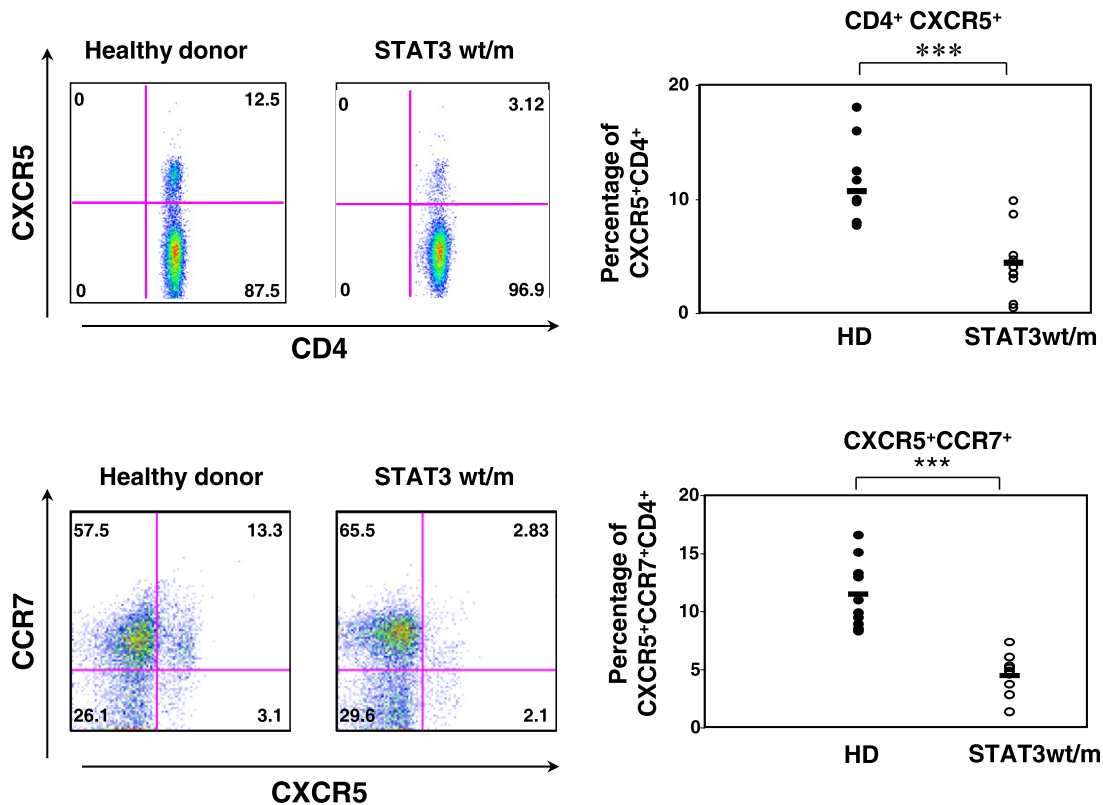


FIG 3. Frequency of CXCR5⁺CD4⁺ T cells in STAT3-deficient patients. CD4⁺CXCR5⁺ T cells as a percentage of total CD4⁺CD3⁺ T cells in whole blood from healthy donors (HD; n = 9) and STAT3-deficient patients (STAT3 wt/m; n = 9, top panels) and the CXCR5⁺CCR7⁺ T cells gated for CD4⁺CD3⁺ T cells (bottom panels) are shown. ***P < .0001, Student t test. Fluorescence-activated cell sorting analysis revealed the expression of CD4, CXCR5, and CCR7 in a healthy donor and an age-matched STAT3-deficient patient.

CXCR5 and IL-21R expression (to a similar extent as in healthy donor cells) and did not express CCR7 (Fig 4). The phenotypic characteristics of the subset isolated from STAT3-deficient patients were similar to those observed in cells from healthy donors, except for a smaller increase in CD69 expression after CD3/CD28 activation (Fig 4). We next tested the cell population's functional characteristics. After culture for 5 days in the presence of CD3/CD28 beads, IL-12, and IL-21, the number of proliferative CXCR5⁺ T cells isolated from STAT3-deficient patients was decreased compared with that seen in healthy donors (Fig 5, A). When cells had been activated with CD3/CD28 beads for 20 hours and then cultured for 5 days in the absence of cytokines or in the presence of either IL-12p70 or IL-21, the proliferation of CXCR5⁺ T cells from STAT3-deficient patients was slightly lower than that seen in healthy donors (Fig 5, B). We then looked at whether the cells were able to induce the expression of chemoreceptors and activation markers on naive B cells preactivated with an anti-IgM antibody. Representative data from 3 independent experiments performed with CXCR5⁺ T cells isolated from different healthy donors and STAT3-deficient patients showed that there are no differences *in vitro* (Fig 5, C). Activated CXCR5⁺ T cells isolated from STAT3-deficient patients were able to produce IL-21 to much the same extent as those from healthy donors. There is no statistical difference between healthy donors and STAT3-deficient patients (Fig 6, A). Production of IL-21 was increased in the presence of IL-12 p70 (data not shown) and after coculture with activated naive B cells under the

conditions required for IgA production (Fig 6, B). There is no statistical difference between healthy donors and STAT3-deficient patients. Furthermore, CXCR5⁺ T cells isolated from STAT3-deficient patients provided help for IgA and IgG production by control total CD19⁺ B cells in both the presence and absence of IL-21 (Fig 6, C). There is no statistical difference between the helping capacity of T CXCR5⁺ cells from healthy donors or STAT3-deficient patients. The same results were obtained for IgM production (data not shown). However, CXCR5⁺ T cells isolated from STAT3-deficient patients provide very little help to CD19⁺CD27[−] naive B cells for secretion of IgG (Fig 6, D), despite normal activation of naive B cells (Fig 5, C). Thus STAT3 deficiency is associated with a small pool of CXCR5⁺CD4⁺ T cells that can nevertheless produce IL-21 normally, proliferate, and provide help to total B cells but not naive B cells.

DISCUSSION

In this report we confirm and extend in the first part of our results the recent reports by Morita et al⁸ and Chevalier et al¹⁹ indicating that a subset of human memory T_{FH} cells can recirculate in the blood. Indeed, we found that on activation, the CXCR5⁺ subset of circulating memory CD4⁺ T cells has phenotypic and functional characteristics that are typical of the T_{FH} cells found in lymphoid GCs¹⁶ when compared with the CXCR5[−] memory CD4⁺ T-cell pool. These characteristics include expression of Bcl6; strong expression of CD40L, OX40, ICOS, CD200,

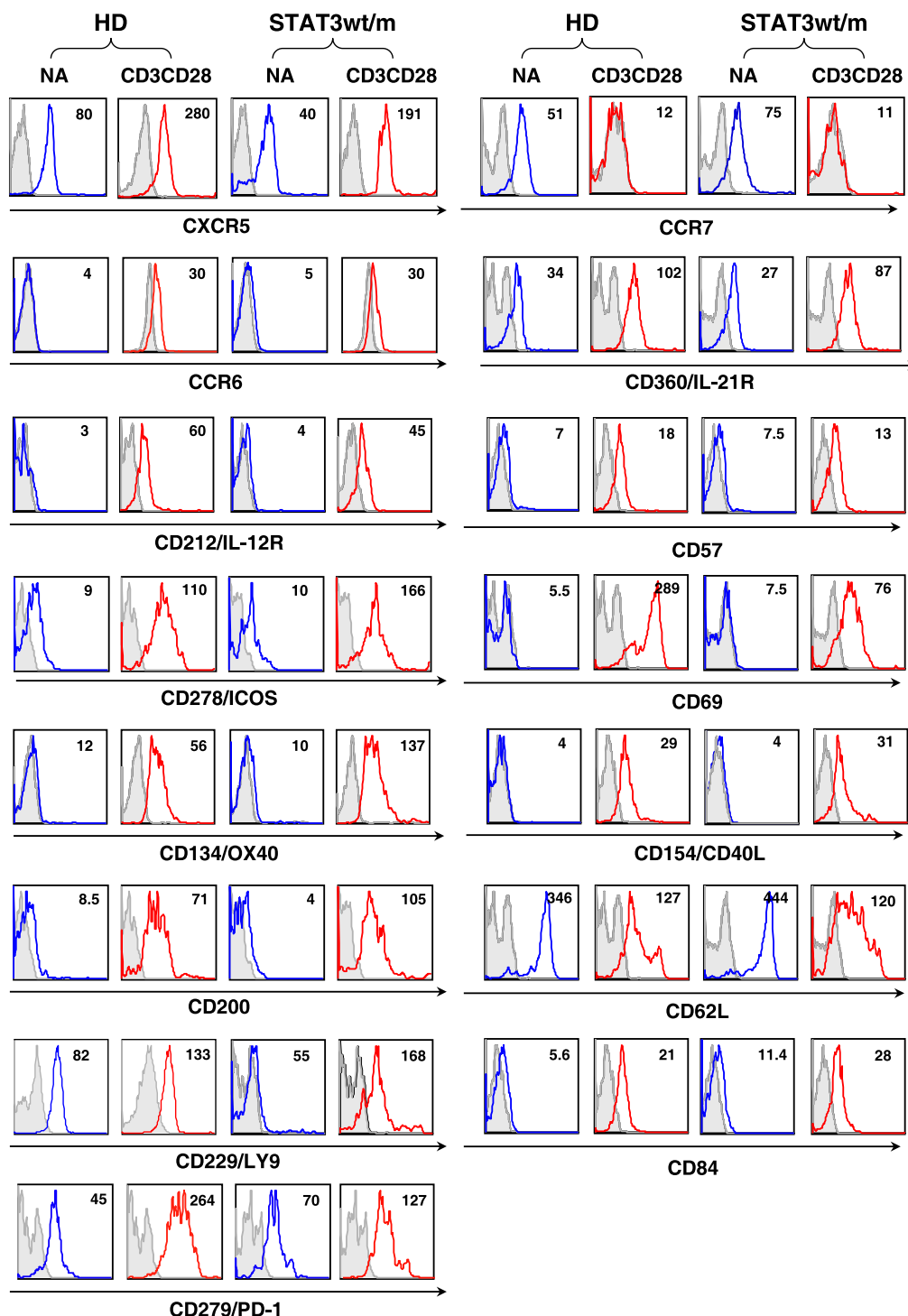


FIG 4. Phenotypic characteristics of CXCR5⁺CD45RO⁺CD4⁺ T cells from STAT3-deficient patients and healthy donors (HD). Membrane expression of chemokine and cytokine receptors and activation proteins by CXCR5⁺ T cells from healthy donors or STAT3-deficient patients (*STAT3wt/m*) after CD3/CD28 activation (*CD3CD28*) and compared with that seen in nonactivated (*NA*) T cells is shown. The isotype control is also shown (gray shaded areas). The mean intensity fluorescence (as an indicator of protein expression) is indicated on each histogram. Representative data from 3 independent experiments are shown.

CD57, PD-1, IL-21R (all of which increased further on activation), IL-21,⁶ and CXCL13¹²; and the abilities to proliferate through an IL-21-dependent pathway and to help B cells produce IgA and IgG (but not IgE) in a cognate manner. However, the IgG

production by total B cells was less IL-21 responsive than that of naive B cells, suggesting that total B cells required other cytokines (eg, IL-10 or IL-4⁴¹) secreted by CXCR5⁺ and CXCR5⁻ T cells to produce IgG. The triggering of IgA and IgG production

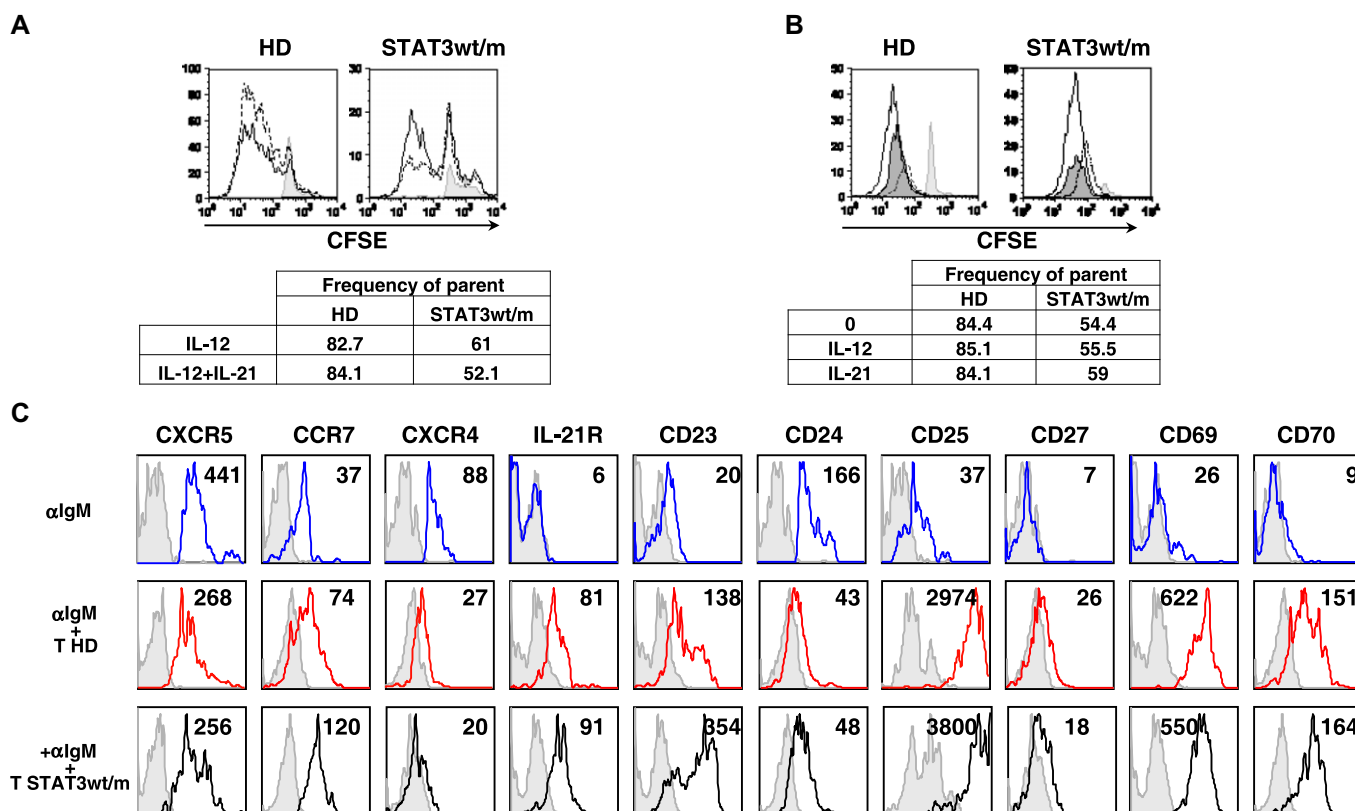


FIG 5. Proliferation and naive B-cell activation ability of CXCR5⁺CD45RO⁺CD4⁺ T cells from STAT3-deficient patients. **A** and **B**, Proliferation of activated carboxyfluorescein succinimidyl ester (CFSE)-labeled CXCR5⁺ T cells from healthy donors (HD) or STAT3-deficient patients (STAT3wt/m) cultured with IL-12 (solid line), IL-21 and IL-12 (dashed line), or IL-21 (tinted line; gray shaded area). Fig 5, B, CD3CD28 beads were removed as described in the Methods section. Results for nonactivated cells are shown (gray shaded areas). Representative data from 3 independent experiments are shown. The tables represent the frequency of cells with diminished intensity compared with the beginning of the culture for each condition. **C**, Membrane expression of various surface molecules on naive CD19⁺CD27⁻ B cells from a healthy donor after incubation with anti-IgM (αIgM) or with CXCR5⁺ T cells from a healthy donor (αIgM + T HD) or a STAT3-deficient patient (αIgM + T STAT3wt/m). The isotype control is shown (gray shaded area). The mean fluorescence intensity (as an indicator of protein expression) is indicated.

was dependent on CD40L, ICOS, and IL-21. The inability of blood CXCR5⁺ T cells to provide help for IgE synthesis might be due to impaired IL-4 production or inhibition by IL-21.^{42,43} Indeed, it has been shown that the *in vivo* CSR to IgE occurs in a GC microenvironment with high IL-4 and low IL-21 levels.⁴⁴ Similarly, previous observations indicated that IgE⁺ cells are detected outside the GC areas and that lymph node IgE⁺ B cells do not express CXCR5 or Bcl6.⁴⁴ It is also known that the T_{FH} cell deficiency induced by Bcl6 inactivation in the mouse leads to high levels of IgE production in the absence of GCs.^{5,18,45} This setting is reminiscent of that observed in STAT3-deficient patients with high serum IgE concentrations and partially defective antibody production.^{24,34,36} It is known that the inability of STAT3-deficient B cells to respond to the effects of IL-21 is related to heterozygous STAT3 mutations with a transdominant negative effect because IL-21-induced survival and proliferation are compromised in STAT3-mutated naive B cells.^{41,46} As reported by Avery et al³⁴ and Speckmann et al,⁴⁷ these patients have few switched B cells in their blood, as shown in Table E2. Here we showed, in the second part of our results, that defective IL-21 signaling probably also results in low numbers of circulating CXCR5⁺ T cells in the blood; indeed, Ma et al³⁵ recently reported on low

CD4⁺CD45RO⁺CXCR5⁺ T-cell counts and increased CD4⁺CD45RA⁺CCR7⁺ counts in STAT3-deficient patients. However, we found that the remaining CD4⁺CD45RO⁺CXCR5⁺ T-cell population is partially functional in the absence of IL-12 because it (1) expresses activation markers to a similar extent as in healthy donors, (2) produces equivalent amounts of IL-21 in the absence or presence of activated naive B cells, (3) can increase the expression of activation markers on naive B cells, and (4) helps CD19⁺ B cells from healthy donors to produce IgA and IgG. These findings could explain the normal serum IgG and IgA levels detected *in vivo* in STAT3-deficient patients.³⁵ However, in the absence of any cytokines or in the presence of IL-21 or IL-12, this memory population proliferates poorly *in vitro* compared with that seen in healthy donors, suggesting reduced activation of IL-21R after IL-21 binding. This result is likely explained by the presence of low STAT3 activity in the patients' cells. We have also observed weak IgG production by naive B cells from healthy donors cocultured with activated CXCR5⁺ T cells from STAT3-deficient patients. Because T_{FH} cells from STAT3-deficient patients were able to induce IgM production by naive B cells from healthy donors (data not shown), these results suggest either that naive B cell did not switch from IgM

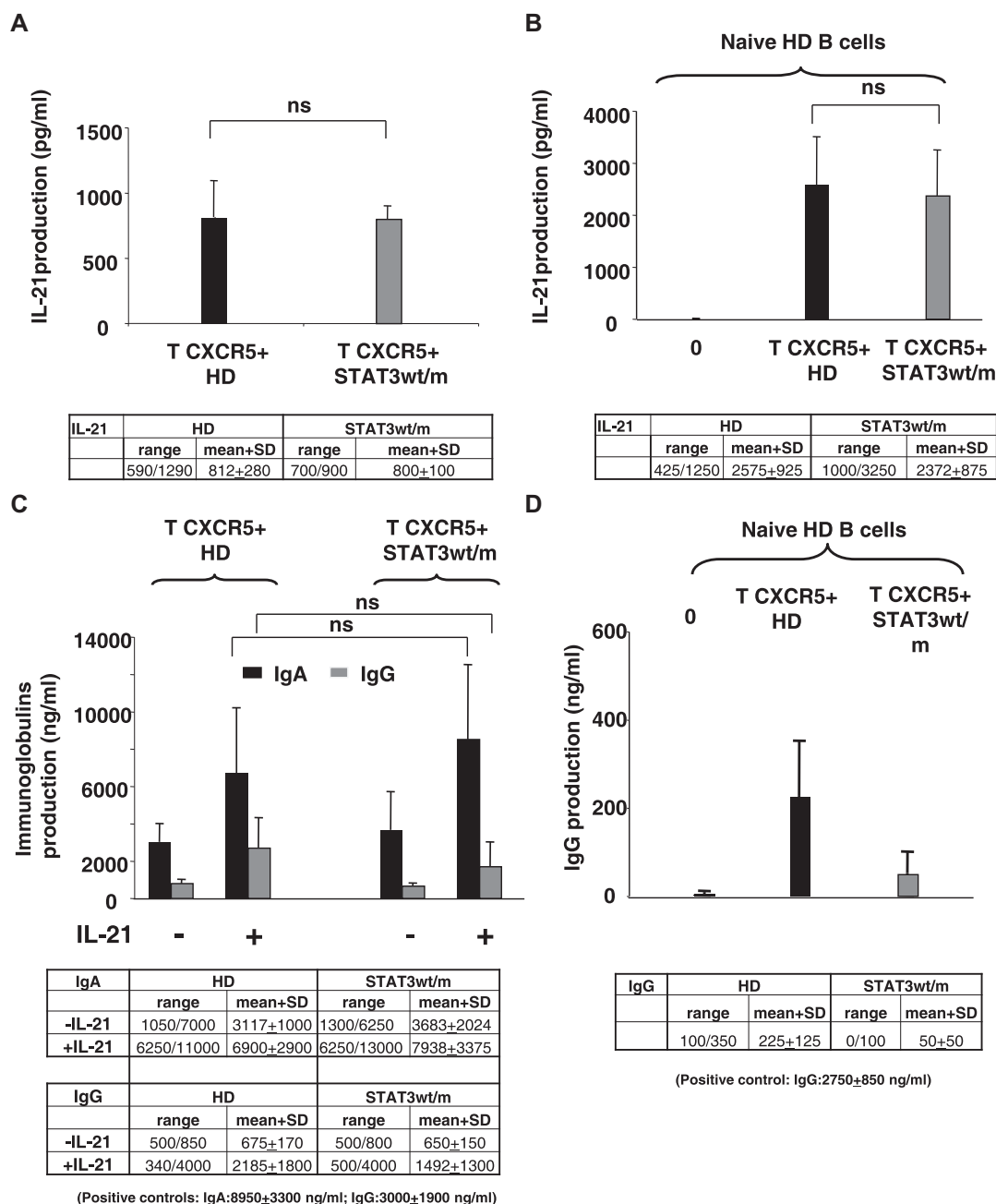


FIG 6. Functional characteristics of CXCR5⁺CD45RO⁺CD4⁺ T cells from STAT3⁻ deficient patients. **A** and **B**, IL-21 production by CXCR5⁺CD45RO⁺CD4⁺ T cells from healthy donors (*T CXCR5⁺ HD*; black columns; *n* = 4) or from STAT3-deficient patients (*T CXCR5⁺ STAT3wt/m*; gray columns; *n* = 2) incubated with CD3/CD28 beads and cultured for 4 days (Fig 6, **A**) or cocultured with naive CD19⁺CD27⁻ B cells from healthy donors (Fig 6, **B**). **C** and **D**, Immunoglobulin concentrations in the coculture of CD3/CD28-activated *T CXCR5⁺ HD* (black columns; *n* = 4) or *T CXCR5⁺ STAT3wt/m* (gray columns; *n* = 4) and CD19⁺ B cells from healthy donors (Fig 6, **C**) and naive CD19⁺CD27⁻ B cells from healthy donors (*n* = 2; Fig 6, **D**). *ns*, Nonstatistically significant difference.

production (as in the case of CSR defects) or that they switched directly from IgM production to IgA or IgE production⁴⁸ (accounting for the hyper-IgE phenotype) after help by STAT3-deficient T_{FH} cells. A defect in CSR is more likely because our results are in agreement with the report by Ma et al,³⁵ who found a defect in the help provided by IL-12-activated, STAT3-deficient naive CD4⁺ T cells to tonsillar naive B cells. However, the weak help provided by activated CD4⁺CD45RO⁺CXCR5⁺ T cells

from STAT3-deficient patients to naive peripheral blood B cells in our present experiments was not related to a poor activation of B-cell molecules or low IL-21 expression by STAT3-deficient T_{FH} cells. It could reflect an inability of STAT3-deficient T_{FH} cells to induce immunoglobulin CSR toward IgG and IgA. The reduced number of circulating CD4⁺CD45RO⁺CXCR5⁺ T cells could also reflect an impaired migration of these cells in the follicular areas of lymphoid tissues.

However, no difference of migration in response to CXCL13 was detected between activated CD4⁺CD45RO⁺ T cells from STAT3-deficient patients and from healthy donors (n = 2, data not shown).

Our results reinforce the putative role of STAT3 in the generation of different T-cell populations. Indeed, Siegel et al⁴⁹ recently reported on a cell-intrinsic defect in the number of central memory CD4⁺ and CD8⁺ T cells that was partly due to an intrinsic lack of ability to proliferate and differentiate from naive T precursors. Whatever the mechanism underlying the defective helper activity of STAT3-deficient T_{FH} cells for induction of CSR in naive B cells, this finding might account, at least in part, for the defective antibody response observed in patients with STAT3 deficiency.²⁵

Our results further indicate that this CXCR5⁺ T-cell subset represents an easily accessible window on the immune system and should be a valuable tool for studying human T_H cell function under various conditions, such as those leading to the lack of this population in patients with ICOS or CD40L deficiencies⁵⁰ or a STAT3 deficiency (this study and Ma et al³⁵) or, conversely, abnormally high counts in patients with dermatomyositis.⁸

We thank members of the Institut National de la Santé et de la Recherche Médicale (INSERM) Unit 768 for helpful discussions. We thank Monique Forveille, Nathalie Lambert, Virginie Grandin, Corinne Jacques, Chantal Harre, Stéphanie N'daga, and Aminata Diabate for excellent technical assistance and Alexandra Arnold and Olivier Pellé for FACS analysis.

Clinical implications: Heterozygous mutations in STAT3 lead to reduced numbers of circulating T_{FH}-like cells, a finding that might account (at least in part) for the observed defect in antibody production.

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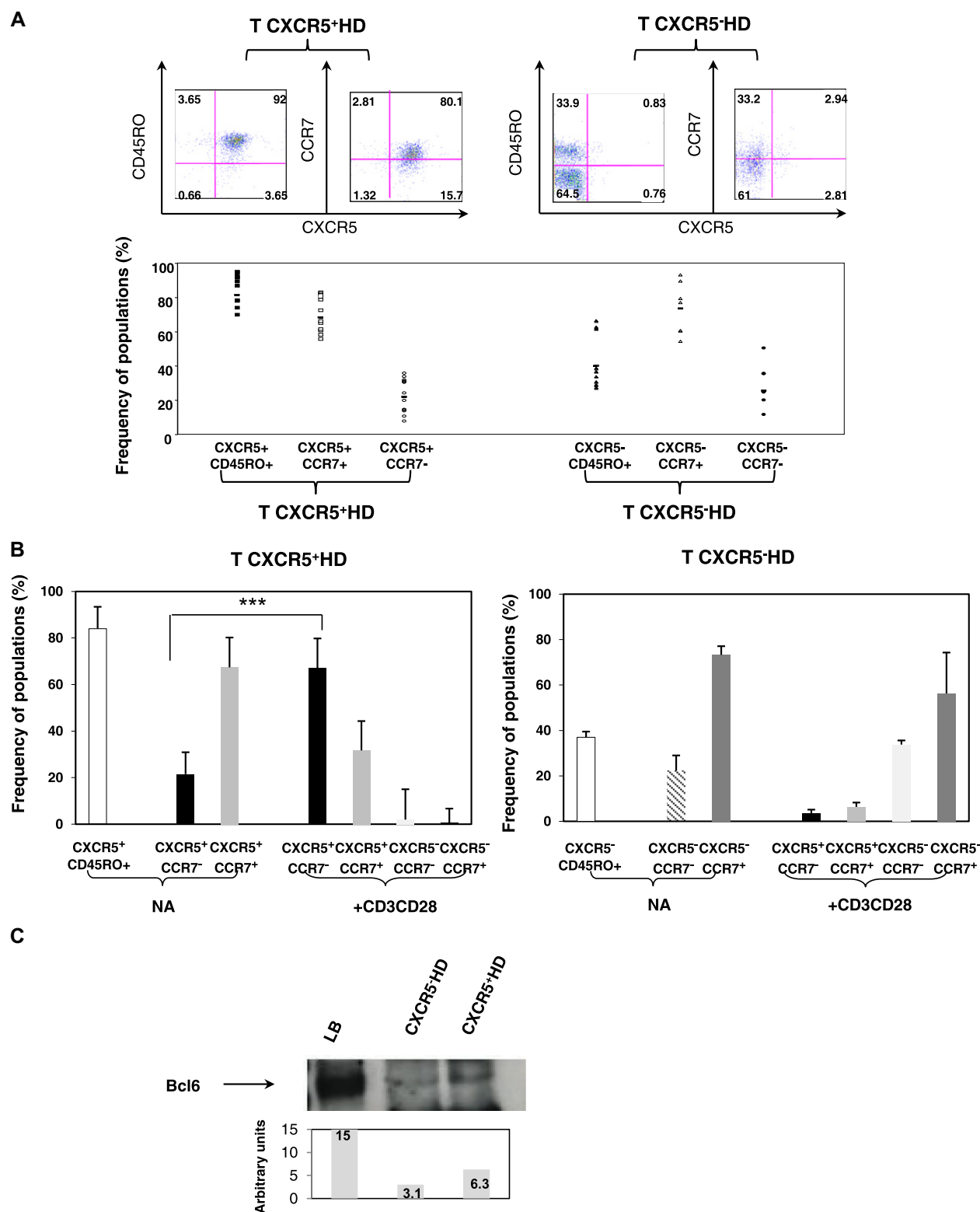


FIG E1. Differential expression of CXCR5, CCR7, and Bcl6 on activated CXCR5⁺ and CXCR5⁻CD4⁺CD45RO⁺ T cells from a healthy donor. **A**, Expression of CXCR5, CCR7, and CD45RO by purified CXCR5⁺ T cells, CXCR5⁻ T cells, RO⁺CXCR5⁻ T cells, and RA⁺CXCR5⁻ T cells after sorting of human CD4⁺CXCR5⁺ T cells and CD4⁺CXCR5⁻ T cells from healthy donors (HD). **B**, Expression of CXCR5 and CCR7 on CXCR5⁺ and CXCR5⁻CD45RO⁺ T cells from healthy donors. The graphs show the mean frequency of the various subpopulations detected after 20 hours of CD3/CD28 activation or with no activation (NA) for separate CXCR5⁺ and CXCR5⁻CD45RO⁺ T cells (n = 10 donors). ***P < .0001, Student t test. **C**, Detection of Bcl6 in CXCR5⁺ and CXCR5⁻ T cells by using Western blot analysis. LB, B cell line, DAUDI.

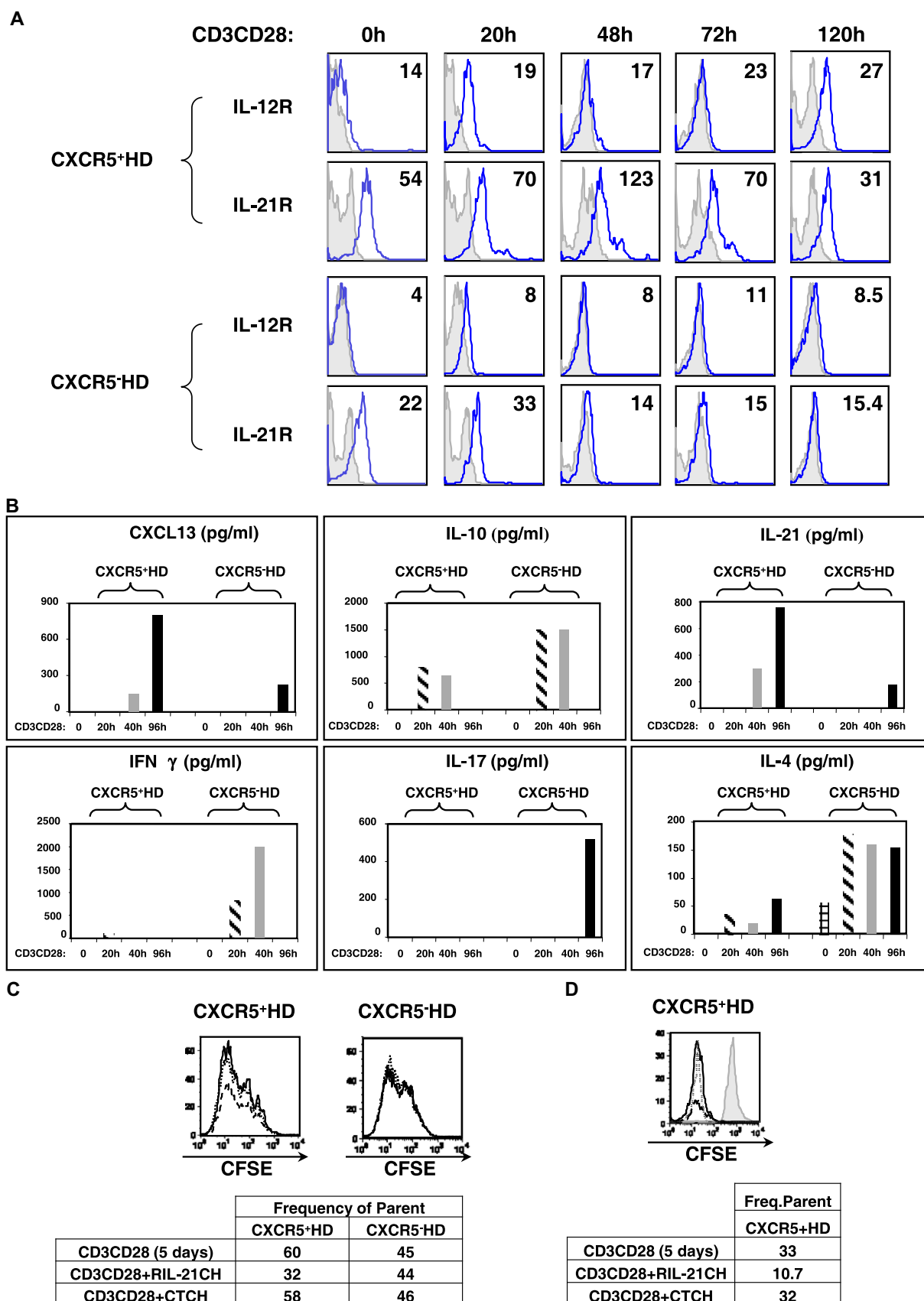
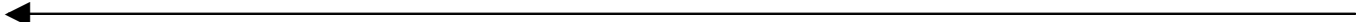


FIG E2. Time course of expression of the IL-12 and IL-2 receptors, cytokine production, and IL-21-dependent CXCR5⁺CD4⁺ cell proliferation. Expression of IL-12R and IL-21R (A), secretion of cytokines (B), and



proliferation (**C** and **D**) were studied on CXCR5⁺ or CXCR5⁻ CD4⁺ T cells from a healthy donor (*HD*) after CD3/CD28 activation during the time indicated (*solid line* in Fig E2, *C* and *D*) in the presence of 20 μ g/mL recombinant human IL-21R/Fc chimera (*RIL-21CH*; *dashed line*) or recombinant human IgG1Fc (*CTCH*; *dot-dotted line*) in Fig E2, *C* and *D*. In Fig E2, *D*, CD3/CD28 beads were removed as described in the Methods section. Tables depict the frequency of cells with diminished intensity compared with data at onset of the culture for each condition. In Fig E2, *B*, for each cytokine, the experiment shown was representative of independent experiments performed with cells from 3 different healthy donors.

TABLE E1. Clinical and genetic features of 15 STAT3-deficient patients*

Patient no.	Sex	Country of origin	Status	Age (y)†	STAT3 mutation	Domain	NIH score	Age at first sign (y)	Infections							Connective tissue signs					
									Skin	Lung	Severe‡ bacterial	ENT	CMC	Aspergillus	Dermatitis	Dysmorphia	Teeth	Osteopenia	Fracture	Hyperextensibility	Aneurysms
1	F	France	Alive	17	V637M	SH2	57	1	+	+		+	+	+	+	+	+				
3	M	France	Dead	36	F384L	DNA	72	<1	+	+		+	+	+	+	+	+	+		+	+
4	F	France	Alive	6	F384L	DNA	56	1	+	+		+	+		+	+			+	+	
11	M	Algeria	Alive	20	R382W	DNA	74	<1	+	+	+	+	+	+	+	+	+	+	+		
21	F	West Indies	Alive	22	S668Y	SH2	74	<1	+	+	+	+			+	+	+		+		
28	M	France	Alive	31	R382Q	DNA	67	<1	+	+			+		+	+					
32	M	Algeria	Alive	17	R382W	DNA	54	<1	+	+		+	+	+	+	+				+	
33	F	France	Alive	28	V637M	SH2	73	<1	+	+		+		+	+	+	+	+	+	+	
34	M	France	Alive	20	V637M	SH2	64	<1	+	+		+	+		+	+		+	+		
42	F	France	Alive	14	R382Q	DNA	59	<1	+	+		+			+	+	+	+		+	
43	F	Pakistan	Alive	41	Vdel463	DNA	79	<1	+	+	+	+	+		+	+		+	+	+	
44	M	Pakistan	Alive	13	Vdel463	DNA	55	<1	+	+	+	+	+		+	+	+				
46	M	France	Alive	21	N472D	DNA	55	<1	+	+	+		+		+	+				+	
48	F	France	Alive	47	I665N	SH2	65	<1	+	+	+	+	+		+	+					
50	M	France	Alive	21	K709E	TA	77	<1	+	+	+	+	+		+	+	+	+			

CMC, Chronic mucocutaneous candidiasis; ENT, ear, nose, and throat infections; F, female; M, male; NIH, National Institute of Health; Teeth, retention of deciduous teeth.

*All the STAT3-deficient patients reported here have been described in Chandesaris et al.³⁶

†Age at the time of the study.

‡Severe bacterial infections include septicemia, meningitis, deep inner abscess, arthritis, and osteomyelitis.

TABLE E2. B-cell subsets in STAT3-deficient patients

	Age (y)	B cells, CD19 ⁺ total (/μL of blood)	B cells, CD19 ⁺ CD27 ⁺ (%)	Naive B cells, CD19 ⁺ CD27 ⁻ IgD ⁺ (%)	Switched memory B cells, CD19 ⁺ CD27 ⁺ IgD ⁻ (%)
Patients					
1	17	260	6	ND	ND
3*	36	24	58	46.5	23.3
4	6	1080	2	96.5	0.8
11	20	660	4	96.2	1.4
21	22	208	9	94.1	2.3
28	31	132	2	ND	ND
32	17	304	5	ND	ND
33	28	720	3	ND	ND
34	20	396	4	96.6	1.5
42	14	330	4	ND	ND
43	41	308	6	94.9	2.6
44	13	700	4	97.3	0.9
46	21	204	6	91	3
48	47	143	10	ND	ND
50	21	312	8	ND	ND
Healthy donors					
n = 7	7-10	350 (range, 180-594)	21 (range, 17-24)	77 (range, 75-79); n = 3	11 (range, 4-18)
n = 5	13-25	220 (range, 153-308)	26.5 (range, 12-35)	83 (n = 1)	11.5 (range, 4.5-19)
n = 6	>26	147 (range, 100-243)	30 (range, 18-43)	65 (range, 53-79)	15.8 (range, 9.9-24)

Absolute number of total CD19⁺ B cells and B-cell subsets (per microliter of blood and as a percentage of all CD19⁺ B cells) are shown. *ND*, Not done.

*Patient 3 displayed global lymphopenia.