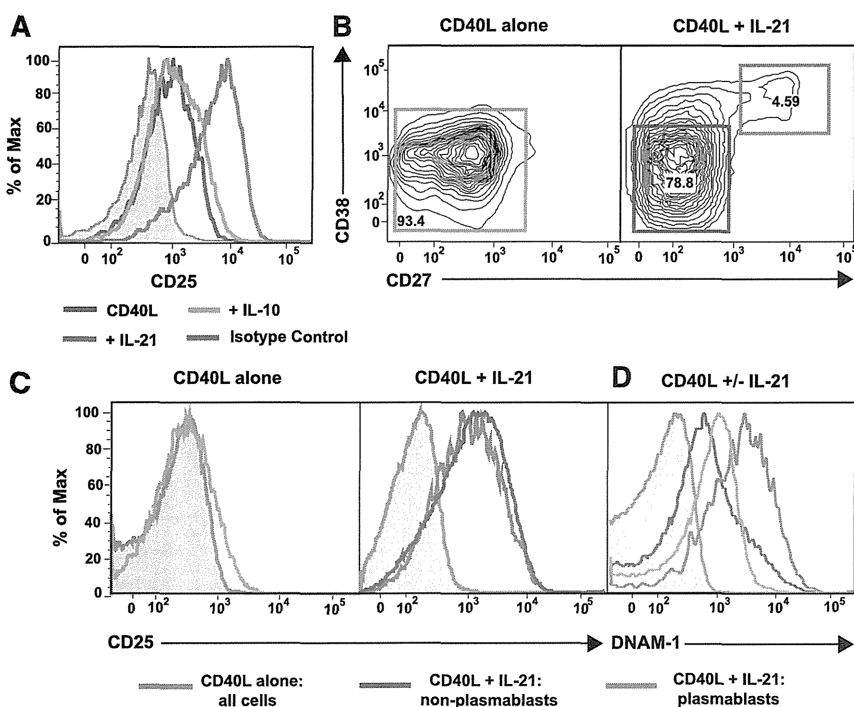

Figure 3. IL-21, but not IL-10, induces CD25 expression on all activated naïve B cells. (A) Naïve B cells from healthy donors were cultured for 5 days with CD40L alone (blue histogram) or in the presence of IL-10 (orange histogram) or IL-21 (red histogram). Expression of CD25 was determined after 5 days. (B-D) Normal naïve B cells were cultured for 5 days with CD40L alone or together with IL-21 for 5 days. (B) Differential expression of CD27 and CD38 delineates IL-21–induced plasmablasts ($CD38^{hi}CD27^{hi}$; red gate) and nonplasmablasts ($CD38^{lo}CD27^{lo}$; blue gate). Expression of CD25 (C) or DNAM-1 (D) was determined on all B cells in cultures stimulated with CD40L alone or on nonplasmablasts and plasmablasts present in cultures of CD40L/IL-21–stimulated B cells. Gray histograms represent isotype controls. Numbers represent percentage of live cells within each gate.

mechanism by which IL-21 induces STAT3-dependent CD25 expression.

The inability of IL-21 to induce CD25 on $STAT3_{MUT}$ naïve B cells is not due to preferential expression by in vitro-generated plasmablasts

Although microarrays detect changes in mRNA expression globally within a population of cells, if the population studied is heterogeneous, they cannot distinguish differing expression levels between subsets of these cells. Following in vitro culture with CD40L/IL-21, a subset of normal naïve B cells differentiates into plasmablasts.[12,13] $STAT3_{MUT}$ naïve B cells, however, do not undergo this differentiation program.[23] Thus, it was possible that impaired up-regulation of CD25 on IL-21–stimulated $STAT3_{MUT}$ naïve B cells reflected preferential expression on plasmablasts and was a consequence of an absence of such cells in these cultures. To address this, expression of CD25 was determined on plasmablasts ($CD38^{hi}CD27^{hi}$) and non-plasmablasts ($CD38^{lo}CD27^{-}$)[34] in cultures of CD40L/IL-21–stimulated normal naïve B cells (Figure 3B). CD25 was equally expressed on both of these populations of cells (Figure 3C). In contrast, *DNAM1* (CD226), which was found to be an IL-21–induced STAT3-dependent gene (Figure 1A), was preferentially induced on plasmablasts (Figure 3D). Thus, functional STAT3 is required for induction of CD25 on all IL-21–stimulated B cells, rather than being selectively acquired by a distinct subset.

IL-2 promotes IL-21–induced plasmablast generation and Ig secretion by naïve B cells

We next investigated the physiological significance of IL-21–induced CD25 expression on naïve B cells. Normal naïve B cells were cultured with CD40L alone or together with IL-2, IL-21, or both cytokines. CD40L alone or CD40L/IL-2 failed to induce plasmablasts ($CD38^{hi}CD27^{hi}$) from naïve cells (Figure 4A). However, a discrete plasmablast population was detected in cultures of CD40L/IL-21–stimulated naïve B cells, the frequency of which was increased by IL-2 (Figure 4A).

Induction of CD25 by IL-21 on CD40L-stimulated naïve B cells was dose dependent, and addition of IL-2 further enhanced CD25 expression above that induced by IL-21 (Figure 4B). Consistent with these dose-dependent changes in CD25 expression, IL-2 significantly augmented IL-21–induced plasmablast generation from, and IgM and IgG secretion by, naïve (Figure 4C-D) and memory (not shown) B cells at most IL-21 concentrations tested. As IL-21 induces isotype switching to IgG1 and IgG3,[11,12,16] we assessed the effect of IL-2 on IgG subclasses. The IL-2–mediated increase in total IgG secretion by CD40L/IL-21–stimulated naïve B cells predominantly resulted from enhanced IgG1 and IgG3 (Figure 4E). Thus, IL-2 amplifies the response induced by IL-21 rather than skewing IgG production to different subclasses. To further demonstrate the physiological significance of IL-2–mediated enhancement of IL-21–induced B-cell differentiation, we examined responses of B cells isolated from an individual with an *IL2RA* mutation.[32] Whereas IL-2 increased IgM and IgG production by IL-21–stimulated normal naïve and memory B cells by >2-fold, IL-2 failed to augment IL-21–induced differentiation of CD25-deficient B cells (Figure 4F). Thus, through a positive feedback whereby IL-21 induces CD25 expression on naïve B cells and IL-2 further amplifies its level of expression, IL-21–stimulated naïve B cells acquire responsiveness to the stimulatory effects of IL-2. Importantly, this requires expression of the high-affinity IL-2 receptor, as evidenced by unresponsiveness of B cells that lack CD25 but continue to express components of the low and intermediate affinity IL-2 receptor.[32]

IL-2 promotes proliferation of many cell types, including human B cells.[6] As various facets of lymphocyte differentiation are linked to cell division,[15,16,19] we established whether the effect of IL-2 on IL-21–stimulated B cells resulted from enhanced proliferation or differentiation. Carboxyfluorescein diacetate succinimidyl ester-labeled naïve B cells were cultured for 5 days with varying concentrations of IL-21 in the absence or presence of IL-2.

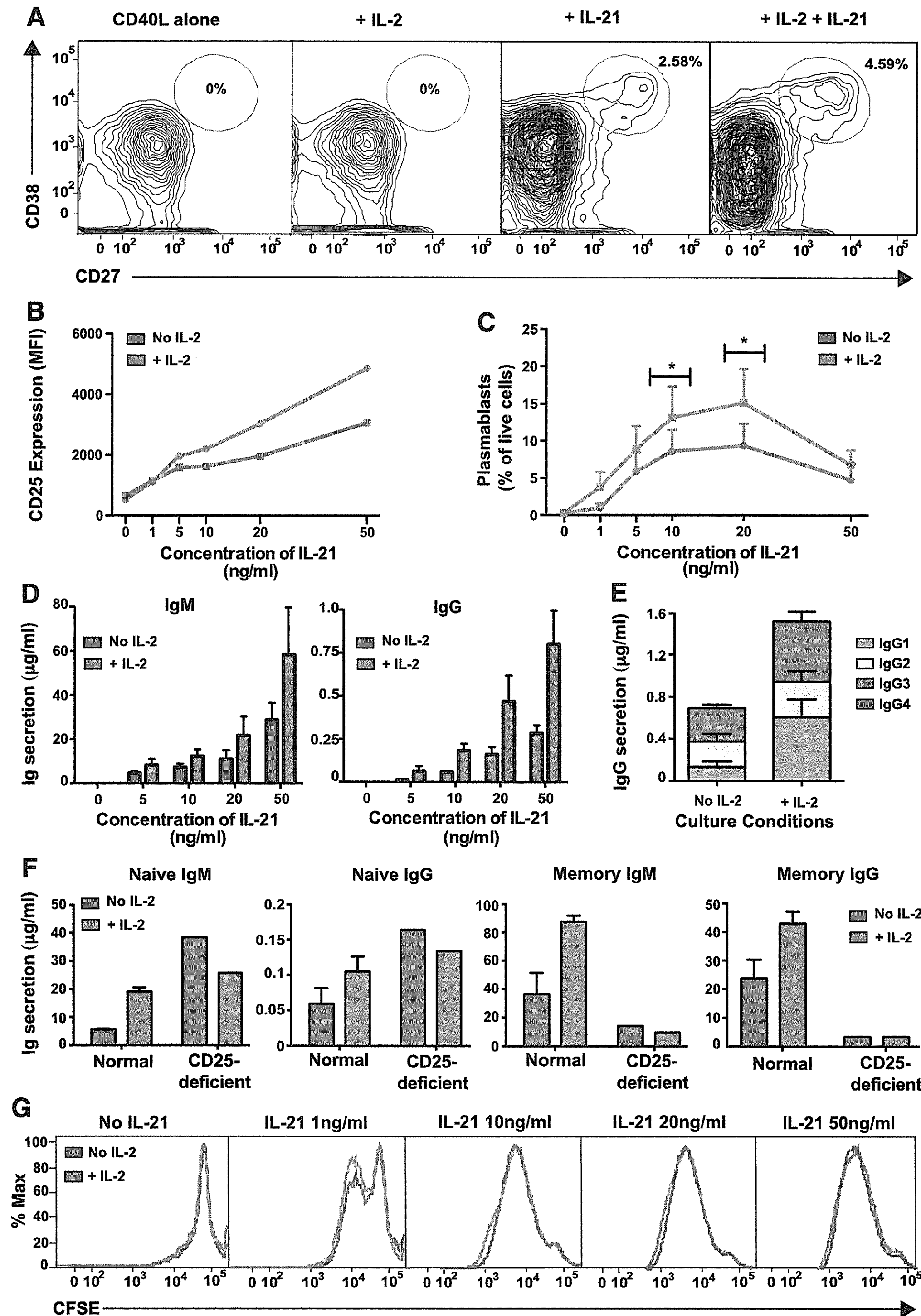

Figure 4. IL-21–induced CD25 expression enables naïve B cells to respond to IL-2 with enhanced plasmablast generation and Ig secretion. Naïve B cells were sorted from normal spleens and then cultured with CD40L alone in the presence or absence of IL-2 and/or IL-21. The proportion of plasmablasts ($CD27^{hi}CD38^{hi}$) (A,C) and expression of CD25 (B) were determined by flow cytometry after 5 days. The data depicted are representative of 4 (A) or 3 (B) separate experiments using naïve B cells isolated from different donor spleens. C represents the mean ± SEM from 4 separate experiments; **P* < .05, comparing cultures with and without IL-2. Secretion of IgM and IgG (D) or IgG subclasses (E) was determined after 10 to 12 days by enzyme-linked immunosorbent assay. Statistical analysis using 2-way ANOVA with Bonferroni post-test analysis confirmed a statistically significant difference between cultures with or without IL-2 (*P* < .005 for both IgM and IgG) and between different concentrations of IL-21 (*P* < .005 for IgM; *P* < .001 for IgG). Data in D show mean ± SEM from 3 independent experiments on 3 different donor spleens, each performed in triplicate. Data in E show mean ± SEM from a single experiment performed in triplicate but is representative of 2 independent experiments performed on different normal donor spleens. (F) Naïve and memory B cells were isolated from a normal donor and a CD25-deficient patient and then cultured with CD40L/IL-21 alone or together with IL-2. Secretion of IgM and IgG was determined after 10 days. Values represent the mean ± SEM of triplicate cultures for normal B cells and the mean of single cultures for CD25-deficient B cells. (G) Carboxyfluorescein diacetate succinimidyl ester profiles of splenic naïve B cells from normal donors cultured for 5 days with CD40L alone or with varying concentrations of IL-21 in the absence or presence of IL-2. Results are representative of 4 independent experiments using different normal donor spleens.

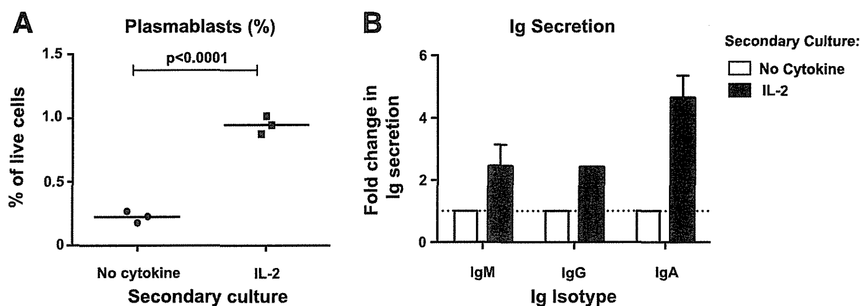

Figure 5. Initial upregulation of CD25 by IL-21 licenses IL-2 to maintain plasmablast generation in the absence of IL-21. Naive B cells from normal human spleens were initially cultured with CD40L together with IL-21. After 3 days, cells were harvested, washed, and recultured either in media alone or with IL-2. (A) After a further 3 days of culture, the proportion of plasmablasts ($CD27^{hi}CD38^{hi}$) was determined by flow cytometry. Each symbol corresponds to an individual experiment that used naïve B cells from a different normal donor spleen; the horizontal line represents the mean. (B) Secretion of IgM, IgG, and IgA was determined after 7 days of secondary culture in media alone or with IL-2 and Ig secretion was determined by enzyme-linked immunosorbent assay. Results are expressed as fold-change in Ig secretion relative to the "no cytokine" secondary culture (set to equal 1.0). The results for IgM represent mean ± SEM (n = 3); IgG secretion was detected in only 1 of 3 experiments; IgA was detected in 2 of 3 experiments.

IL-2 had no significant effect on proliferation of CD40L-stimulated B cells regardless of the IL-21 concentration (Figure 4G). Thus, IL-2 enhances plasmablast generation and Ig secretion by IL-21–stimulated naïve human B cells by increasing the rate of differentiation of these cells rather than promoting proliferation or survival.

IL-2 alone can promote plasmablast generation from IL-21–primed naïve B cells

To assess whether the effects of IL-2 required the continual presence of IL-21, naïve B cells were first cultured for 3 days with CD40L/IL-21, then washed and recultured in media alone or with IL-2. The proportion of plasmablasts was determined by flow cytometry after a further 3 days and Ig secretion after 7 days. The frequency of plasmablasts and amounts of secreted IgM, IgG, and IgA were increased 2- to 5-fold in cultures of IL-21–primed/IL-2–stimulated secondary cultures compared with cells primed with IL-21 but recultured without cytokines (Figure 5A-B). IL-2 failed to induce plasmablasts and Ig secretion by cells not primed with IL-21 (not shown), highlighting the dependence of IL-2 on the expression of its high-affinity receptor, induced by IL-21/STAT3 signaling, for these effects to occur. Thus, the initial up-regulation of CD25 by IL-21 enabled IL-2 to promote B-cell differentiation.

IL-2 enhances IL-21–induced STAT3 phosphorylation in human B-cell lines

Binding of IL-21 to IL-21R on B cells activates STAT1, STAT3, and STAT5.[23,24] Correspondingly, IL-21 induced phosphorylation of STAT1, STAT3, and to a lesser extent STAT5 in normal LCLs, with a maximal response after 30 min and return to baseline by 120 min (Figure 6A). IL-2 predominantly signals through STAT5.[22] To determine whether the combination of IL-2 and IL-21 alters phospho-(p)STAT signaling, LCLs from normal donors or patients with mutations in *IL2RA*[32] or *IL21R*[33] (Figure 6B) were stimulated with IL-2 or IL-21 alone or together and intracellular pSTAT1, pSTAT3, and pSTAT5 were determined. The magnitude of STAT1 and STAT3 phosphorylation induced by IL-21, or of STAT5 induced by IL-2, was similar to that induced by the combination of both IL-2 and IL-21 (Figure 6C). Consistent with this, the frequency of cells expressing pSTAT3 and pSTAT5 was unchanged with IL-2/IL-21 stimulation compared with IL-2 or IL-21 alone (Figure 6D). However, in the presence of IL-2 and IL-21, a greater proportion of B cells coexpressed pSTAT3 and pSTAT5 than with either cytokine alone (Figure 6D). The effect of IL-21 was specific, as it did not induce STAT phosphorylation in $IL\text{-}21R_{MUT}$ LCLs, whereas the residual induction of pSTAT5 in $CD25_{MUT}$ LCLs most likely results from IL-2 signaling through the IL-2Rβ/γc complex (Figure 6C). Thus, IL-2 and IL-21 are likely to achieve their complementary effect on B-cell differentiation to plasmablasts by independent signaling pathways in a common subset of activated cells.

IL-2 and IL-21 produced by Tfh cells cooperate to induce Ig secretion by cocultured naïve B cells

B-cell differentiation into plasma cells and memory cells occurs predominantly in GCs.[1,2] Much of the IL-21 that stimulates B cells is derived from Tfh cells co-localizing with B cells in GCs. On the other hand, IL-2 is produced by most subsets of activated $CD4^+$ T cells.[35] For IL-2 to best promote the effects of IL-21 in vivo, both cytokines would need to be produced by the same cell type. To address this, naïve, $CXCR5^{lo}$, $CXCR5^{intermediate}$, and $CXCR5^{hi}$ Tfh cells were isolated from human tonsils,[35] stimulated for 5 days, and cytokine expression determined. While IL-2 was expressed by 50% to 80% of these $CD4^+$ T-cell subsets, expression of IL-21 was greatest for $CXCR5^{intermediate}$ and $CXCR5^{hi}$ Tfh cells[35] (Figure 7A). Importantly, 70% to 90% of IL-21–expressing $CD4^+$ T cells coexpressed IL-2 (Figure 7A).

Coexpression of these cytokines by Tfh cells could facilitate their combined effect on B-cell differentiation. To test this, naïve B cells and $CXCR5^+CD4^+$ T cells were co-cultured in media alone or with TAE beads in the absence or presence of neutralizing Ab against IL-2, IL-21R-Fc, or both. IgM secretion was measured after 9 days. Activated, but not resting, $CD4^+CXCR5^{intermediate}$ T cells and $CXCR5^{hi}$ Tfh cells induced substantial IgM secretion by co-cultured B cells, and this was unaffected by an isotype control mAb (Figure 7B). However, blocking endogenous IL-2 or IL-21 reduced IgM secretion by ~35-80% and ~70%, respectively. Importantly, there was a greater reduction in IgM secretion (>80%) when both IL-2 and IL-21 were concurrently blocked, confirming that IL-2 and IL-21 derived from $CD4^+CXCR5^+$ T cells cooperate to induce Ig secretion. Expression of IL-2, IL-21, and ICOS by $CXCR5^+CD4^+$ T cells was equivalent regardless of IL-2 or IL-21 neutralization (data not shown), suggesting that the reduction in IgM secretion was a direct effect of cytokine blockade on B-cell function rather than a consequence of impaired T-cell activation.

Discussion

Previous studies have suggested a role for IL-2 during human B-cell differentiation. However, its exact role in the complex network of cytokines influencing B cells has not been fully elucidated. IL-2 receptors have long been identified on normal and malignant human

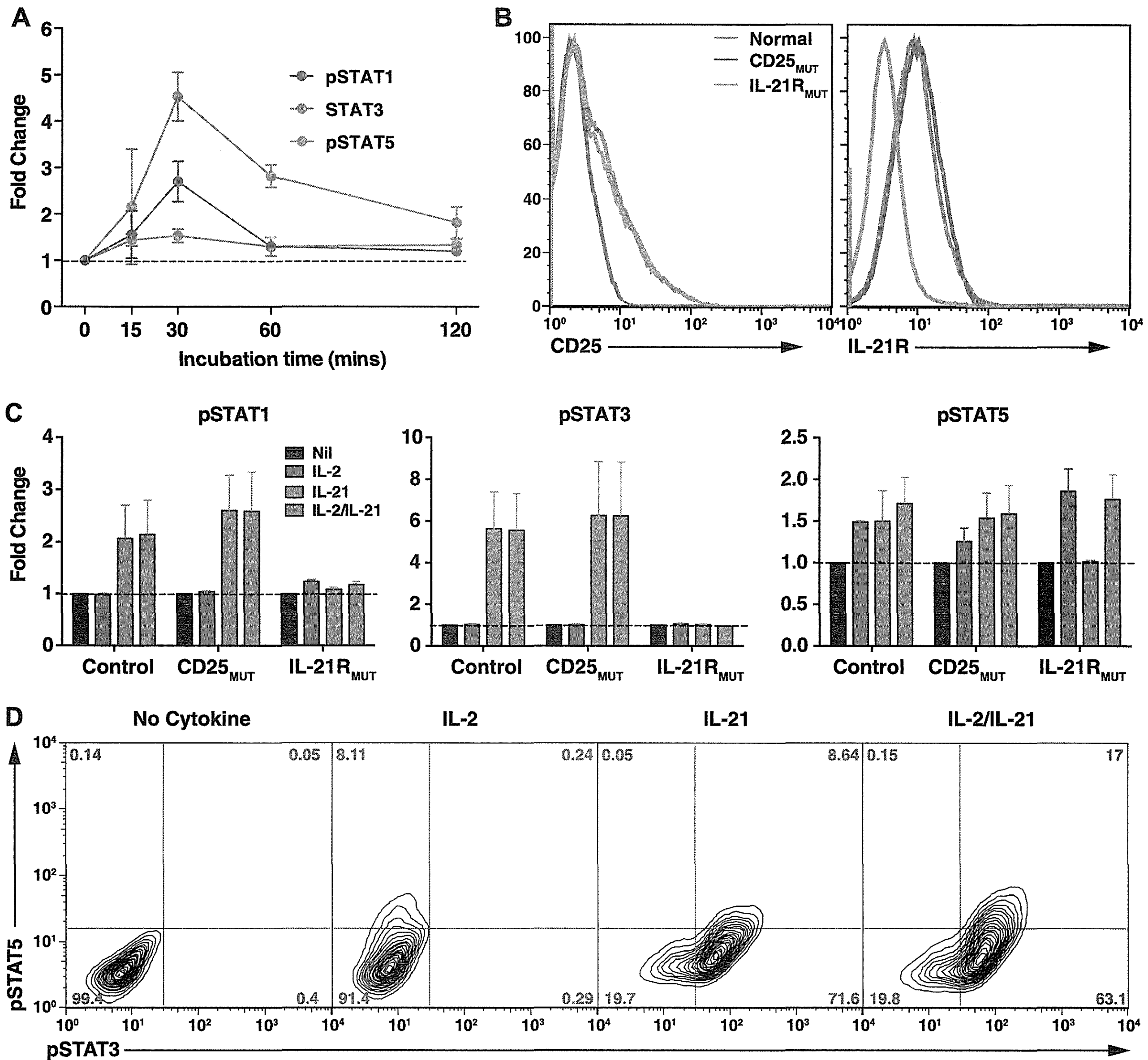

Figure 6. Effect of IL-2 and IL-21 on STAT phosphorylation in human B-cell lines. (A) LCLs from normal donors were incubated for varying times in the absence or presence of IL-21. Phosphorylation of STAT1, STAT3, and STAT5 was then determined by intracellular staining and flow cytometry. Data are depicted as fold change in mean fluorescence intensity of pSTAT in the presence of IL-21 over media alone. Results represent mean ± SEM of experiments using LCLs from 6 different donors. (B) Expression of CD25 and IL-21R was determined on LCLs derived from different normal donors (red histogram) or CD25-deficient (blue histogram) or IL21R-deficient (gray histogram) patients (n = 2/group). (C-D) Normal, CD25-deficient ($CD25_{MUT}$), and IL-21R–deficient ($IL\text{-}21R_{MUT}$) LCLs were incubated in the absence (Nil) or presence of IL-2, IL-21, or IL-2/IL-21. Expression of pSTAT1, pSTAT3, and pSTAT5 was determined after 30 minutes. Results in C are expressed as fold change above pSTAT expression in cells cultured with no cytokine and represent mean ± SEM of experiments using LCLs from 2 different donors or patients. D shows representative contour plots demonstrating coexpression of pSTAT3 and pSTAT5 in LCLs cultured in the absence (no cytokine) or presence of IL-2, IL-21, or IL-2/IL-21.

B-cell subsets,[19,40] and CD25 can be induced by T-derived and T-independent stimuli, including CD40L,[41] IL-10,[19,42] BCR engagement,[41,43] and TLR ligands.[41] When combined with IL-2, most of these stimuli enable Ab secretion by activated B cells.[41,44,45] We have identified a novel role for IL-21 in regulating human B-cell differentiation. By inducing CD25, IL-21 sensitizes activated B cells to the differentiation-promoting effects of IL-2, thereby enabling cooperative interplay between IL-2 and IL-21 to amplify plasmablast generation and Ab secretion. Thus, IL-2 may play an adjunctive role in IL-21–induced B-cell differentiation.

By examining B cells from individuals with specific genetic mutations, we revealed the critical requirement for the IL-21R/γc complex as well as STAT3, but not STAT1, in this process. These findings identify an additional defect contributing to the humoral impairment in STAT3-deficient AD-HIES patients, who are predisposed to pyogenic infections with *Staphylococcus aureus* and encapsulated organisms (*Streptococcus pneumoniae*, *Haemophilus influenzae*), frequently culminating in parenchymal lung damage such as bronchiectasis and pneumatocoeles. This may reflect impaired Ag-specific Ab responses after immunization with T-dependent Ag.[29-31] Previous studies have suggested that the humoral impairment in AD-HIES arises predominantly from intrinsic B-cell abnormalities, thereby highlighting the dependence of normal B-cell function on STAT3. Thus, although serum levels of IgM, IgG, and IgA are normal in AD-HIES, STAT3-deficient individuals fail to generate normal levels of Ag-specific Ab or a normal pool of Ag-specific

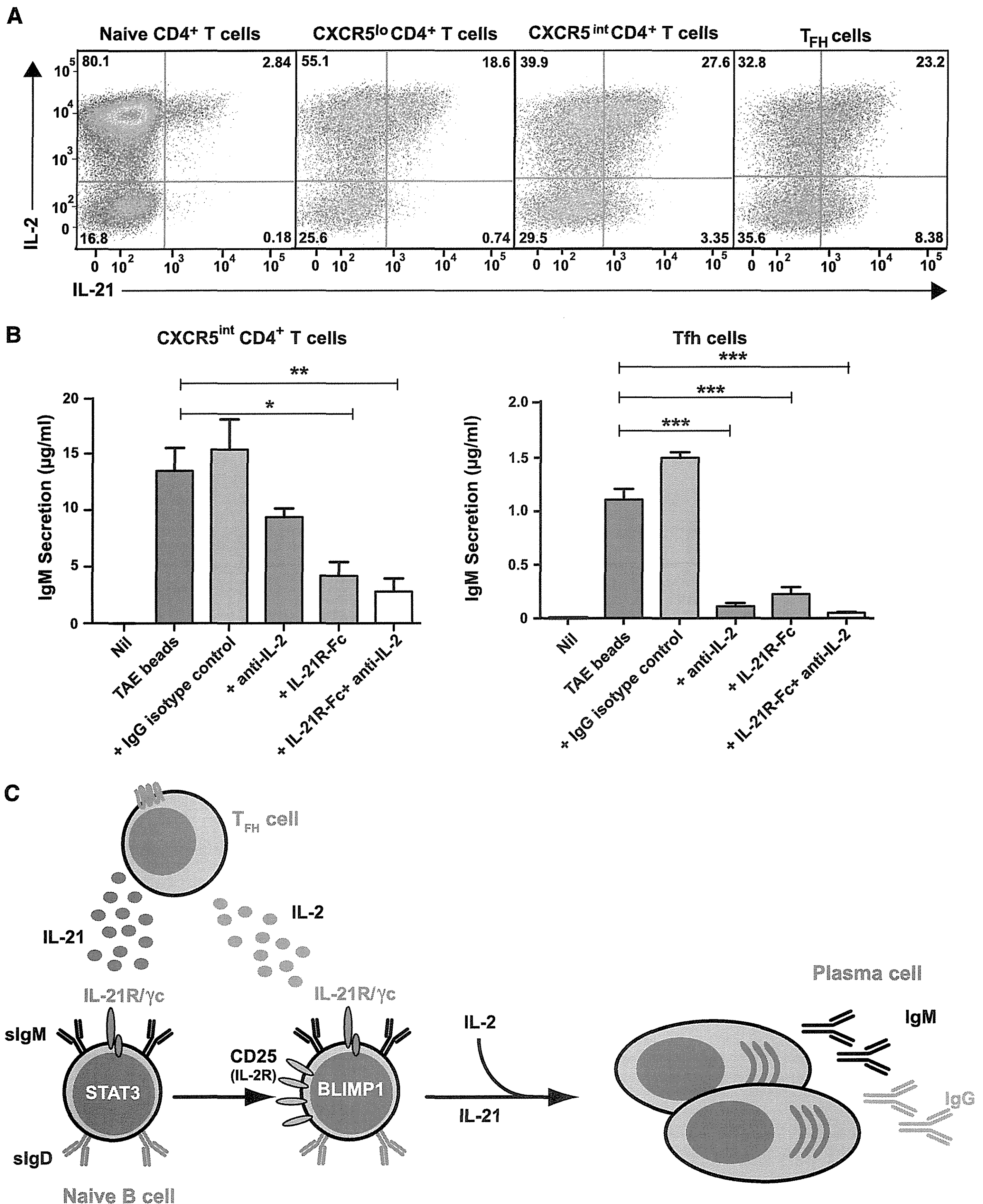

Figure 7. IL-2 and IL-21 produced by Tfh cells cooperate to induce Ig secretion by co-cultured naïve B cells. (A) Naïve, CXCR5^{lo}, CXCR5^{intermediate}, and CXCR5^{hi} Tfh CD4$^+$ T cells were sort-purified from human tonsils and then stimulated in vitro with TAE beads. After 5 days, expression of IL-2 and IL-21 in these different populations following restimulation with PMA/ionomycin was determined. The values represent the proportions of cytokine-expressing cells. (B) CD4$^+$CXCR5^{intermediate} T cells and CXCR5^{hi} Tfh cells were sorted from tonsil and co-cultured with autologous naïve B cells either in the absence (Nil) or presence of TAE beads (blue). Endogenous IL-2 and/or IL-21 were neutralized by the addition of anti-IL-2 mAb (red), IL-21R-Fc (pink), or anti-IL-2 mAb plus IL-21R-Fc (yellow), respectively. An isotype control mAb (green) was also included. IgM secretion was measured after 9 days. Results represent 2 independent experiments performed using cells from different donor tonsils. $^*P < .05$; $^{**}P < .01$; $^{***}P < .0001$. (C) IL-21, secreted from Tfh cells, promotes B-cell maturation by inducing Blimp-1. IL-21 also enhances CD25 expression on naïve B cells, sensitizing them to the effects of IL-2, which is also secreted by Tfh cells. IL-2 then enhances the effects of IL-21 on B cells. IL-21 and IL-2 thus work cooperatively to induce plasma cell development and Ig secretion.

memory B cells in vivo[23] and their naïve B cells fail to differentiate into Ab-secreting cells in response to IL-10 or IL-21 due to an inability to undergo the molecular changes required for commitment to the plasma cell lineage.[23] Our work extends these observations by demonstrating that $STAT3_{MUT}$ naïve B cells fail to up-regulate CD25 in response to IL-21, rendering them unable to respond to the stimulatory effects of IL-2. This secondary defect would exacerbate the limited response of $STAT3_{MUT}$ B cells to the direct effects of IL-21 on B-cell maturation.

An inability to respond to IL-2 due to a lack of IL-21–induced priming of naïve B cells may also contribute to the humoral immunodeficiency in γc-deficient X-SCID patients, whose impaired response to IL-21 causes their profound Ab deficiency,[21] as well as in patients with loss-of-function mutations in *IL21R* who manifest a memory B-cell deficiency and an inability to elicit normal Ab responses following vaccination and infection.[33] Because the consequences of humoral immunodeficiencies are often severe, necessitating ongoing intravenous immunoglobulin therapy to prevent recurrent infections and bronchiectasis, an improved understanding of the molecular basis for their immune dysfunction may pave the way for more targeted therapies.

Our data revealed that IL-2 and IL-21 are coexpressed by a substantial proportion of Tfh cells, which would enable cooperation between these cytokines in promoting B-cell maturation in GCs (Figure 7C). The requirement for IL-21 or other exogenous stimuli such as BCR engagement[41] to prime B cells to the effects of IL-2 through enhanced CD25 expression ensures that the B-cell response to IL-2 is regulated to occur only "on demand," as B cells are sensitive to only IL-2 in the presence of specific Ag or Tfh-derived cytokines. Our data may also provide a mechanism for the positive correlation between the frequency of IL-2–producing Ag-specific memory $CD4^+$ T cells and IgG titers in vivo.[46] It is plausible that the IL-2^+ memory $CD4^+$ T cells examined[46] contained IL-21–producing Tfh cells.

IL-2, IL-21, and their receptors share several structural and functional similarities. Both are type I cytokines whose receptors not only require γc for signalling, but IL-21R exhibits substantial amino-acid homology to IL-2Rβ.[47] Both IL-2 and IL-21 are secreted by activated $CD4^+$ T cells[4,5,35,48] and act as T-cell growth factors.[22,48] BLIMP-1, which influences terminal differentiation of B and T lymphocytes,[49] is induced by IL-21 in B cells[1,2,23,24] and IL-2 in T cells.[50] Although this list is not exhaustive, these similarities support the evolution of a concurrent role for IL-2 in B-cell maturation, given the essential role of IL-21 in this process.

Overall, our study highlights the utility of examining B cells from patients with monogenic primary immunodeficiencies to not only clarify the roles of particular cytokines in the immune response of normal individuals but also identify mechanisms underlying the humoral defects characteristic of these conditions. In recent years, genetic mutations underlying many primary immunodeficiencies have been identified.[1,27,28,33,37] The phenotypic abnormalities in these "Experiments of Nature" demonstrate the nonredundant role for these genes. By examining B cells from patients with loss-of-function mutations in *STAT1*, *STAT3*, *IL2RG*, *IL21R*, and *IL2RA*, we have further elucidated the roles of γc cytokines, particularly IL-21 and IL-2, in human B-cell differentiation. The complete loss of IL-21–induced CD25 expression on γc-deficient and IL-21R–deficient B cells and substantial impairment on STAT3-deficient B cells confirms the nonredundant nature of the IL-21R/γc/STAT3 signaling pathway during B-cell differentiation. These patients with rare immunodeficiencies therefore provide a unique opportunity to study molecular requirements underlying human cytokine signaling pathways in health and disease and reveal potential targets for modulating B-cell responses in both immunodeficiency and autoimmunity.

Acknowledgments

The authors thank Rob Salomon, David Snowden, Nikki Alling, and Chris Brownlee for cell sorting, Drs. Warren Kaplan and Mark Cowley for bioinformatics advice, the Ramaciotti Centre (University of New South Wales) for GeneChip hybridization and scanning, Dr. Rene de Waal Malefyt (Merck, formerly DNAX Research Institute, Palo Alto, CA) for providing IL-10, the Australian Red Cross for providing donor spleens, Dr. Ron Bova for providing human tonsils, Lynn Corcoran for assistance with ChIP assays, Drs. Talil Chatilla and Luigi Notarangelo for facilitating access to CD25-deficient patients, and all the patients and their families for participating in this project.

This work was supported by research grants and fellowships awarded by the National Health and Medical Research Council of Australia (L.J.B., C.S.M., E.K.D., M.C.C., S.G.T.) and a Postdoctoral Fellowship from Research Foundation-Flanders (FWO), Belgium (L.M).

Authorship

Contribution: L.J.B. designed the research, performed experiments, analyzed and interpreted results, and wrote the manuscript; D.T.A., C.S.M., and L.M. performed experiments; C.S.M. and E.K.D. assisted with experimental design; S.B.D., J.B., M.W., S.A., P.D.A., R.B., L.B., H.D., C.M.R., D.A.F., J.B.Z., J.M.S., M.K., C.P., A.D., M.C.C., J.L.C., and G.U. provided patient samples or B-cell lines and clinical details; G.U. provided input into data interpretation; and S.G.T. designed the research, analyzed and interpreted results, and wrote the manuscript; all authors commented on the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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References

1. Tangye SG, Tarlinton DM. Memory B cells: effectors of long-lived immune responses. *Eur J Immunol.* 2009;39(8):2065-2075.
2. Goodnow CC, Vinuesa CG, Randall KL, Mackay F, Brink R. Control systems and decision making for antibody production. *Nat Immunol.* 2010;11(8): 681-688.
3. Victora GD, Nussenzweig MC. Germinal centers. *Annu Rev Immunol.* 2012;30:429-457.
4. Crotty S. Follicular helper CD4 T cells (TFH). *Annu Rev Immunol.* 2011;29:621-663.
5. Ma CS, Deenick EK, Batten M, Tangye SG. The origins, function, and regulation of T follicular helper cells. *J Exp Med.* 2012;209(7): 1241-1253.
6. Banchereau J, Rousset F. Human B lymphocytes: phenotype, proliferation, and differentiation. *Adv Immunol.* 1992;52:125-262.
7. Defrance T, Vanbervliet B, Brière F, Durand I, Rousset F, Banchereau J. Interleukin 10 and transforming growth factor beta cooperate to induce anti-CD40-activated naive human B cells to secrete immunoglobulin A. *J Exp Med.* 1992;175(3):671-682.
8. Dubois B, Massacrier C, Vanbervliet B, et al. Critical role of IL-12 in dendritic cell-induced differentiation of naive B lymphocytes. *J Immunol.* 1998;161(5):2223-2231.

9. Punnonen J, Aversa G, Cocks BG, et al. Interleukin 13 induces interleukin 4-independent IgG4 and IgE synthesis and CD23 expression by human B cells. *Proc Natl Acad Sci USA.* 1993; 90(8):3730-3734.
10. Armitage RJ, Macduff BM, Eisenman J, Paxton R, Grabstein KH. IL-15 has stimulatory activity for the induction of B cell proliferation and differentiation. *J Immunol.* 1995;154(2):483-490.
11. Pène J, Gauchat JF, Lécart S, et al. Cutting edge: IL-21 is a switch factor for the production of IgG1 and IgG3 by human B cells. *J Immunol.* 2004; 172(9):5154-5157.
12. Ettinger R, Sims GP, Fairhurst AM, et al. IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells. *J Immunol.* 2005;175(12):7867-7879.
13. Bryant VL, Ma CS, Avery DT, et al. Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. *J Immunol.* 2007;179(12):8180-8190.
14. Tangye SG, Ferguson A, Avery DT, Ma CS, Hodgkin PD. Isotype switching by human B cells is division-associated and regulated by cytokines. *J Immunol.* 2002;169(8):4298-4306.
15. Tangye SG, Avery DT, Hodgkin PD. A division-linked mechanism for the rapid generation of Ig-secreting cells from human memory B cells. *J Immunol.* 2003;170(1):261-269.
16. Avery DT, Bryant VL, Ma CS, de Waal Malefyt R, Tangye SG. IL-21-induced isotype switching to IgG and IgA by human naive B cells is differentially regulated by IL-4. *J Immunol.* 2008; 181(3):1767-1779.
17. Brière F, Servet-Delprat C, Bridon JM, Saint-Remy JM, Banchereau J. Human interleukin 10 induces naive surface immunoglobulin D+ (sIgD+) B cells to secrete IgG1 and IgG3. *J Exp Med.* 1994;179(2):757-762.
18. Avery DT, Ma CS, Bryant VL, et al. STAT3 is required for IL-21-induced secretion of IgE from human naive B cells. *Blood.* 2008;112(5): 1784-1793.
19. Tangye SG, Avery DT, Deenick EK, Hodgkin PD. Intrinsic differences in the proliferation of naive and memory human B cells as a mechanism for enhanced secondary immune responses. *J Immunol.* 2003;170(2):686-694.
20. Arpin C, Banchereau J, Liu YJ. Memory B cells are biased towards terminal differentiation: a strategy that may prevent repertoire freezing. *J Exp Med.* 1997;186(6):931-940.
21. Recher M, Berglund LJ, Avery DT, et al. IL-21 is the primary common γ chain-binding cytokine required for human B-cell differentiation in vivo. *Blood.* 2011;118(26):6824-6835.
22. Leonard WJ. Cytokines and immunodeficiency diseases. *Nat Rev Immunol.* 2001;1(3):200-208.
23. Avery DT, Deenick EK, Ma CS, et al. B cell-intrinsic signaling through IL-21 receptor and STAT3 is required for establishing long-lived antibody responses in humans. *J Exp Med.* 2010; 207(1):155-171.
24. Diehl SA, Schmidlin H, Nagasawa M, et al. STAT3-mediated up-regulation of BLIMP1 is coordinated with BCL6 down-regulation to control human plasma cell differentiation. *J Immunol.* 2008;180(7):4805-4815.
25. Ozaki K, Spolski R, Ettinger R, et al. Regulation of B cell differentiation and plasma cell generation by IL-21, a novel inducer of Blimp-1 and Bcl-6. *J Immunol.* 2004;173(9):5361-5371.
26. Nutt SL, Taubenheim N, Hasbold J, Corcoran LM, Hodgkin PD. The genetic network controlling plasma cell differentiation. *Semin Immunol.* 2011; 23(5):341-349.
27. Minegishi Y, Saito M, Tsuchiya S, et al. Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome. *Nature.* 2007; 448(7157):1058-1062.
28. Holland SM, DeLeo FR, Elloumi HZ, et al. STAT3 mutations in the hyper-IgE syndrome. *N Engl J Med.* 2007;357(16):1608-1619.
29. Dreskin SC, Goldsmith PK, Gallin JI. Immunoglobulins in the hyperimmunoglobulin E and recurrent infection (Job's) syndrome. Deficiency of anti-Staphylococcus aureus immunoglobulin A. *J Clin Invest.* 1985;75(1): 26-34.
30. Leung DY, Ambrosino DM, Arbeit RD, Newton JL, Geha RS. Impaired antibody responses in the hyperimmunoglobulin E syndrome. *J Allergy Clin Immunol.* 1988;81(6):1082-1087.
31. Sheerin KA, Buckley RH. Antibody responses to protein, polysaccharide, and phi X174 antigens in the hyperimmunoglobulinemia E (hyper-IgE) syndrome. *J Allergy Clin Immunol.* 1991;87(4): 803-811.
32. Goudy K, Aydin D, Barzaghi F, et al. Human IL2RA null mutation mediates immunodeficiency with lymphoproliferation and autoimmunity. *Clin Immunol.* 2013;146(3):248-261.
33. Kotlarz D, Zietara N, Uzel G, et al. Loss-of-function mutations in the IL-21 receptor gene cause a primary immunodeficiency syndrome. *J Exp Med.* 2013;210(3):433-443.
34. Avery DT, Ellyard JI, Mackay F, Corcoran LM, Hodgkin PD, Tangye SG. Increased expression of CD27 on activated human memory B cells correlates with their commitment to the plasma cell lineage. *J Immunol.* 2005;174(7):4034-4042.
35. Ma CS, Suryani S, Avery DT, et al. Early commitment of naïve human CD4(+) T cells to the T follicular helper (T(FH)) cell lineage is induced by IL-12. *Immunol Cell Biol.* 2009;87(8): 590-600.
36. Shimizu Y, Meunier L, Hendershot LM. pERp1 is significantly up-regulated during plasma cell differentiation and contributes to the oxidative folding of immunoglobulin. *Proc Natl Acad Sci USA.* 2009;106(40):17013-17018.
37. Boisson-Dupuis S, Kong XF, Okada S, et al. Inborn errors of human STAT1: allelic heterogeneity governs the diversity of immunological and infectious phenotypes. *Curr Opin Immunol.* 2012;24(4):364-378.
38. Butch AW, Chung GH, Hoffmann JW, Nahm MH. Cytokine expression by germinal center cells. *J Immunol.* 1993;150(1):39-47.
39. Matthes T, Werner-Favre C, Tang H, Zhang X, Kindler V, Zubler RH. Cytokine mRNA expression during an in vitro response of human B lymphocytes: kinetics of B cell tumor necrosis factor alpha, interleukin (IL)6, IL-10, and transforming growth factor beta 1 mRNAs. *J Exp Med.* 1993;178(2):521-528.
40. Waldmann TA, Goldman CK, Robb RJ, et al. Expression of interleukin 2 receptors on activated human B cells. *J Exp Med.* 1984;160(5): 1450-1466.
41. Le Gallou S, Caron G, Delaloy C, Rossille D, Tarte K, Fest T. IL-2 requirement for human plasma cell generation: coupling differentiation and proliferation by enhancing MAPK-ERK signaling. *J Immunol.* 2012;189(1):161-173.
42. Fluckiger AC, Garrone P, Durand I, Galizzi JP, Banchereau J. Interleukin 10 (IL-10) upregulates functional high affinity IL-2 receptors on normal and leukemic B lymphocytes. *J Exp Med.* 1993; 178(5):1473-1481.
43. Mittler R, Rao P, Olini G, et al. Activated human B cells display a functional IL 2 receptor. *J Immunol.* 1985;134(4):2393-2399.
44. Teranishi T, Hirano T, Lin BH, Onoue K. Demonstration of the involvement of interleukin 2 in the differentiation of Staphylococcus aureus Cowan I-stimulated B cells. *J Immunol.* 1984; 133(6):3062-3067.
45. Depper JM, Leonard WJ, Robb RJ, Waldmann TA, Greene WC. Blockade of the interleukin-2 receptor by anti-Tac antibody: inhibition of human lymphocyte activation. *J Immunol.* 1983; 131(2):690-696.
46. Litjens NH, Huisman M, Hijdra D, Lambrecht BM, Stittelaar KJ, Betjes MG. IL-2 producing memory CD4+ T lymphocytes are closely associated with the generation of IgG-secreting plasma cells. *J Immunol.* 2008;181(5):3665-3673.
47. Ozaki K, Kikly K, Michalovich D, Young PR, Leonard WJ. Cloning of a type I cytokine receptor most related to the IL-2 receptor beta chain. *Proc Natl Acad Sci USA.* 2000;97(21):11439-11444.
48. Boyman O, Sprent J. The role of interleukin-2 during homeostasis and activation of the immune system. *Nat Rev Immunol.* 2012;12(3):180-190.
49. Kallies A, Nutt SL. Terminal differentiation of lymphocytes depends on Blimp-1. *Curr Opin Immunol.* 2007;19(2):156-162.
50. Gong D, Malek TR. Cytokine-dependent Blimp-1 expression in activated T cells inhibits IL-2 production. *J Immunol.* 2007;178(1):242-252.

Signal transducer and activator of transcription 3 (*STAT3*) mutations underlying autosomal dominant hyper-IgE syndrome impair human CD8$^+$ T-cell memory formation and function

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Background: The capacity of CD8$^+$ T cells to control infections and mediate antitumor immunity requires the development and survival of effector and memory cells. IL-21 has emerged as a potent inducer of CD8$^+$ T-cell effector function and memory development in mouse models of infectious disease. However, the role of IL-21 and associated signaling pathways in protective CD8$^+$ T-cell immunity in human subjects is unknown.

Objective: We sought to determine which signaling pathways mediate the effects of IL-21 on human CD8$^+$ T cells and whether defects in these pathways contribute to disease pathogenesis in patients with primary immunodeficiencies caused by mutations in components of the IL-21 signaling cascade.

Methods: Human primary immunodeficiencies resulting from monogenic mutations provide a unique opportunity to assess the requirement for particular molecules in regulating human lymphocyte function. Lymphocytes from patients with loss-of-function mutations in signal transducer and activator of transcription 1 *(STAT1)*, *STAT3*, or IL-21 receptor *(IL21R)* were used to assess the respective roles of these genes in human CD8$^+$ T-cell differentiation *in vivo* and *in vitro*.

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Supported by project and program grants from the National Health and Medical Research Council (NHMRC) of Australia (to E.K.D., S.G.T., C.S.M., D.A.F., and M.C.C.), Cancer Council NSW (to S.G.T. and U.P.), and Rockefeller University Center for Clinical and Translational science (5UL1RR024143, to J.-L.C.). C.S.M. is a recipient of a Career Development Fellowship and S.G.T. is a recipient of a Principal Research Fellowship from the NHMRC of Australia.

Disclosure of potential conflict of interest: C. S. Ma has been supported by one or more grants from the National Health and Medical Research Council of Australia (NHMRC). J. Peake has received one or more payments for lecturing on allergy topics and has been reimbursed for travel/accommodations/meeting expenses. M. A. French has received one or more payments for lecturing from or is on the speakers' bureau for ViiV Australia, MSD Australia, and Janssen Australia and has received one or more payments for travel/accommodations/meeting expenses from MSD Australia and ViiV Australia. J.-L. Casanova has been supported by one or more grants from the National Institutes of Health (grant no. 8UL1TR000043); has consultancy arrangements with Regeneron, GlaxoSmithKline, NovImmune, BiogenIdec, Merck, and Sanofi-Aventis; and has received one or more grants from or has one or more grants pending with Merck. S. G. Tangye has been supported by one or more grants from the NHMRC of Australia; has received support for travel from ESID, Keystone Symposia, and the Jeffrey Modell Foundation; and has received one or more fees for serving as an expert witness in a patent dispute. E. K. Deenick has been supported by one or more grants from NHMRC, has received one or more payments for travel/accommodations/meeting expenses from the Japanese Society for Immunology, and has received one or more paid honoraria as an Editor for *Immunology and Cell Biology*. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication January 22, 2013; revised May 16, 2013; accepted for publication May 16, 2013.

Available online July 10, 2013.

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0091-6749

http://dx.doi.org/10.1016/j.jaci.2013.05.029

Results: Mutations in *STAT3* and *IL21R*, but not *STAT1*, led to a decrease in multiple memory CD8$^+$ T-cell subsets *in vivo*, indicating that STAT3 signaling, possibly downstream of IL-21R, regulates the memory cell pool. Furthermore, STAT3 was important for inducing the lytic machinery in IL-21–stimulated naive CD8$^+$ T cells. However, this defect was overcome by T-cell receptor engagement.

Conclusion: The IL-21R/STAT3 pathway is required for many aspects of human CD8$^+$ T-cell behavior but in some cases can be compensated by other signals. This helps explain the relatively mild susceptibility to viral disease observed in STAT3- and IL-21R–deficient subjects. (J Allergy Clin Immunol 2013;132:400-11.)

Key words: *Autosomal dominant hyper-IgE syndrome, STAT3, STAT1, IL-21, human CD8$^+$ T cells, memory, differentiation*

Abbreviations used

AD-HIES: Autosomal dominant hyper-IgE syndrome
BCL: B-cell lymphoma
CTV: CellTrace Violet
EOMES: Eomesodermin
IL-21R: IL-21 receptor
LCMV: Lymphocytic choriomeningitis virus
PB: Peripheral blood
PID: Primary immunodeficiency
STAT: Signal transducer and activator of transcription
T_{CM}: Central memory T
TCR: T-cell receptor
T_{EM}: Effector memory T
T_{EMRA}: Effector memory T cells expressing CD45RA

CD8$^+$ T-cell responses are essential for the control of viruses and protection against some tumors. Common γ-chain family cytokines are important regulators of CD8$^+$ T-cell behavior. Thus IL-7 and IL-15 control lymphocyte homeostasis,[1-3] whereas IL-2 regulates differentiation of naive cells into effector or memory populations.[4,5] IL-21 has also been reported to control CD8$^+$ T-cell function. *In vitro* IL-21 increases survival and proliferation of mouse[6-9] and human[10-12] CD8$^+$ T cells and induces effector molecules, such as IFN-γ, granzyme B, and perforin,[6,13-15] and the transcription factors B-cell lymphoma 6 (BCL6) and eomesodermin (EOMES), which control their differentiation into effector and memory populations.[16]

IL-21 is also implicated in controlling immune responses *in vivo*. Treatment of patients with cancer with IL-21 resulted in upregulation of cytotoxic molecules, such as granzyme B, perforin, and IFN-γ, in their CD8$^+$ T cells and natural killer cells.[17] In mice IL-21 enhanced memory CD8$^+$ T-cell responses during vaccinia infection[9,18] and was required for CD8$^+$ T cell–mediated control of chronic lymphocytic choriomeningitis virus (LCMV) infection.[19-21] IL-21, either alone or together with IL-15, also increased the efficacy of antitumor responses by CD8$^+$ T cells.[8,9,22-25] Thus IL-21 is a potent inducer of CD8$^+$ T-cell effector function and memory, with clinical relevance in both antiviral and antitumor immunity.

IL-21 mediates its effects by activating Janus kinases 1 and 3,[15,26,27] leading to phosphorylation of signal transducer and activator of transcription (STAT) 1, STAT3, and STAT5.[6,15,26] IL-21 can also activate mitogen-activated protein kinase and Akt.[6] However, it is not clear which of these pathways mediates the stimulatory effects of IL-21 on human CD8$^+$ T cells. Primary immunodeficiencies (PIDs) resulting from mutations in single genes provide a unique opportunity to address the role of individual molecules in regulating immune responses. Autosomal dominant hyper-IgE syndrome (AD-HIES) is a PID characterized by chronic eczema, increased serum IgE levels, and recurrent infections of the skin, mucosa, and lungs.[28,29] Notably, some patients with AD-HIES have impaired control of reactivation of infection with herpes viruses (HSV and varicella zoster virus)[29,30] and are predisposed to non-Hodgkin B-cell lymphoma.[29-32] The molecular lesion in patients with AD-HIES is a heterozygous mutation in *STAT3*, with mutant alleles working in a dominant negative manner.[33,34] Mutations in *STAT1* also result in infectious susceptibility to particular pathogens. Thus monoallelic or biallelic loss-of-function *STAT1* mutations severely compromise responses to IFN-γ. However, responses to IFN-α/β and IFN-λ are either intact (autosomal dominant STAT1 deficiency; heterozygous mutations) or impaired (autosomal recessive STAT1 deficiency; biallelic mutations). Consequently, these mutations result in clinical disease caused by weakly virulent mycobacteria and occasionally nonlethal viral infection.[35,36] On the other hand, biallelic null mutations abolish STAT1-dependent cellular responses to IFN-γ, IFN-α/β, and IFN-λ, thereby predisposing affected subjects to fatal infection with herpes viruses and mycobacteria.[35,36] The importance of IL-21 signaling in human subjects was recently highlighted by the identification of 4 patients with *IL21R* mutations who have recurrent respiratory tract and gastrointestinal infections, particularly with cryptosporidia, resulting in chronic liver disease.[37] Two of these patients also exhibited ongoing infection with norovirus and rhinovirus, but immunity against herpes viruses and other pathogens that are commonly problematic for patients with combined immunodeficiencies (eg, cytomegalovirus and EBV) appeared to be intact.[37]

Here we used STAT3 mutant (STAT3^{MUT}), STAT1^{MUT}, and IL21R^{MUT} patients to determine the requirement for STAT1 and STAT3 in regulating human CD8$^+$ T-cell responses. IL-21 in combination with IL-15 induced proliferation of and granzyme expression in naive CD8$^+$ T cells. Loss of STAT3 function impaired IL-21–induced granzyme B expression but did not affect its ability to induce proliferation. However, strong T-cell receptor (TCR)/costimulatory signals could rescue granzyme expression in STAT3^{MUT} T cells. Loss of STAT1 function did not affect proliferation or granzyme B production. We also found that STAT3, but not STAT1, controlled the formation/maintenance of effector and memory CD8$^+$ T-cell subsets *in vivo*, as evidenced by reduced frequencies of differentiated memory cell populations. We also observed some memory deficiencies in patients with *IL21R* mutations, implicating IL-21 as a potential STAT3-activating cytokine required for CD8$^+$ memory T-cell homeostasis. These findings provide insight into some of the clinical features of AD-HIES and IL-21 receptor (IL-21R) deficiency, including impaired control of viral infection and susceptibility to B-cell lymphoma.

METHODS

Human samples

Buffy coats from healthy donors were purchased from the Australian Red Cross Blood Service. Peripheral blood (PB) was collected from patients with mutations in *STAT3*, *STAT1*, or *IL21R* (see Table E1 in this article's Online

Repository at www.jacionline.org for patient details). All human experiments were approved by ethics committees in Canberra, Sydney, Melbourne, Brisbane, and Perth and the institutional review boards of Necker Medical School, Rockefeller University, and the National Institutes of Health.

T-cell phenotyping and isolation

PB $CD8^+$ T cells were stained with mAbs to CD4, CD8, CCR7, and CD45RA. Subsets were defined as naive ($CD8^+CD4^-CCR7^+CD45RA^+$) cells, central memory T (T_{CM}) cells ($CD8^+CD4^-CCR7^+CD45RA^-$), effector memory T (T_{EM}) cells ($CD8^+CD4^-CCR7^-CD45RA^-$), or effector memory T cells expressing CD45RA (T_{EMRA}; revertant CD45RA effector memory T cells; $CD8^+CD4^-CCR7^-CD45RA^+$). For experiments with $STAT3^{MUT}$ samples, naive cells were isolated from samples by using a Positive Isolation Dynal Kit (Invitrogen, Carlsbad, Calif), followed by sorting $CD8^+CCR7^+CD45RA^+$ cells (FACSAria; BD, Franklin Lakes, NJ). Because of limiting numbers of cells in $STAT1^{MUT}$ and $IL\text{-}21R^{MUT}$ samples, naive $CD8^+$ T cells were isolated directly by means of sorting. For phenotyping, cells were also stained for further cell-surface markers (see Table E2 in this article's Online Repository at www.jacionline.org for the mAbs used).

Expression of phospho-STATs

Normal naive $CD8^+$ T cells were cultured for 4 days with TAE beads (Miltenyi Biotech, Bergisch Gladbach, Germany), rested for 2 hours in OPTI-mem (Life Technologies, Carlsbad, Calif) plus Normicin (InvivoGen, San Diego, Calif), and then stimulated in the absence or presence of IL-2 (50 U/mL), IL-15 (50 ng/mL), and/or IL-21 (50 ng/mL) for 30 minutes. Cells were fixed with 2% paraformaldehyde, permeabilized with 90% methanol, and stained with anti–phospho-STAT1, STAT3, and STAT5 mAbs.

In vitro stimulation of naive $CD8^+$ T cells

Naive $CD8^+$ T cells were labeled with CellTrace Violet (CTV; Invitrogen) and then cultured (approximately 4×10^4 cells/200 μL/well) with or without TAE beads (1 bead/5 cells) for 4 or 10 days, respectively, either alone or together with 50 U/mL IL-2 (Millipore, Temecula, Calif), 50 ng/mL IL-15, or 50 ng/mL IL-21 (PeproTech, Rocky Hill, NJ). Cells were then harvested, permeabilized, and stained with anti-perforin and anti-granzyme B mAbs. Cell division and phenotype were determined by using FlowJo software (TreeStar, Ashland, Ore).

Quantitative PCR analysis

RNA was isolated immediately after *ex vivo* isolation or after 4 or 10 days of culture with the RNeasy kit (Qiagen, Hilden, Germany). For quantitative PCR, total RNA was reverse transcribed with oligo-dT. Expression of genes was determined by using real-time PCR with the LightCycler 480 Probe Master Mix and System (Roche, Mannheim, Germany). All primers (see Table E3 in this article's Online Repository at www.jacionline.org) were from Integrated DNA Technologies (Coralville, Iowa). All reactions were standardized to glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*.

RESULTS

IL-21 activates STAT1, STAT3, and STAT5 in human $CD8^+$ T cells

IL-21 activates numerous intracellular signaling pathways, including STAT1, STAT3, STAT5, mitogen-activated protein kinase, and Akt.[6,15,26] We assessed which pathways were activated by IL-21 in human naive $CD8^+$ T cells. IL-21 induced strong phosphorylation of STAT3 and a low level of STAT1 and STAT5 phosphorylation (Fig 1). We also analyzed STAT activation induced by 2 other γ-chain cytokines that are potent inducers of $CD8^+$ T-cell proliferation and differentiation, namely IL-2 and IL-15. In contrast to IL-21, IL-2 and IL-15 did not result in phosphorylation of STAT1 or STAT3 but did induce STAT5 phosphorylation (Fig 1). The combination of IL-15 and IL-21 did not alter the level of STAT phosphorylation above that observed with these cytokines alone (Fig 1). Therefore, of these cytokines, IL-21 uniquely activates STAT1 and STAT3 in human $CD8^+$ T cells.

STAT1 and *STAT3* mutations do not impair proliferation of naive $CD8^+$ T cells

IL-21 plays a pivotal role in inducing proliferation of $CD8^+$ T cells.[10-12,38] However, because IL-21 activates multiple signaling pathways, it is not clear which of these underlies this proliferative effect. To address this, we used naive $CD8^+$ T cells from patients with mutations in *STAT1* (n = 8), *STAT3* (n = 15), or *IL21R* (n = 3).

Homeostatic cytokines support survival of $CD8^+$ T cells and can induce proliferation and differentiation in the absence of extrinsic TCR stimulation. Thus IL-15 mediates homeostatic proliferation of memory cells and, combined with IL-21, drives naive cells to effector phenotypes.[9,12,38,39] *In vitro* culture of naive $CD8^+$ T cells with IL-2 or IL-15 for 10 days significantly increased the recovery of viable cells (Fig 2, *A*). In contrast, IL-21 alone did not increase survival above that seen with media alone. However, coculture with IL-15 plus IL-21 induced significant proliferation, as assessed by means of CTV dilution (Fig 2, *B*). $STAT3^{MUT}$ or $STAT1^{MUT}$ $CD8^+$ T cells stimulated with IL-15 and IL-21 showed comparable proliferation to control cells (Fig 2, *B-D*); however, proliferation of $IL\text{-}21R^{MUT}$ $CD8^+$ T cells was strongly reduced, with the residual proliferation likely being induced by IL-15 (Fig 2, *C* and *D*). Thus IL-21's involvement in the homeostatic turnover requires a functional IL-21R but is unaffected by loss-of-function mutations in *STAT3* or *STAT1*.

STAT3 is required for IL-21–induced expression of granzyme B

The capacity of $CD8^+$ T cells to produce the cytotoxic molecules granzyme B and perforin is important for their effector function.[40] IL-2, IL-15, and IL-21 can all induce expression of these molecules.[7-9,16,41-43] Therefore we assessed the effect of *STAT1*, *STAT3*, and *IL21R* mutations on the ability of these cytokines to induce granzyme B. Coculture with IL-21 plus IL-15 induced higher granzyme B expression in normal naive $CD8^+$ T cells than did IL-2, IL-15, or IL-21 alone (Fig 3, *A*). However, both $IL21R^{MUT}$ and $STAT3^{MUT}$ $CD8^+$ T cells cultured with IL-15 and IL-21 did not upregulate granzyme B to the same level as seen in control cells (Fig 3). In contrast, $STAT1^{MUT}$ $CD8^+$ T cells showed normal upregulation of granzyme B after stimulation with IL-15/IL-21 (Fig 3, *B* and *C*). Thus acquisition of the lytic machinery by naive $CD8^+$ T cells stimulated with IL-21 in combination with IL-15 was dependent on STAT3 signaling downstream of a functional IL-21R.

TCR/costimulation rescues defective IL-21 responses in $STAT3^{MUT}$ $CD8^+$ T cells

During an immune response, $CD8^+$ T cells also receive signals through the TCR and costimulatory molecules. Therefore we examined naive $CD8^+$ T-cell responses after culture with cytokines and anti-CD3/anti-CD28/anti-CD2 stimulus (provided by TAE beads). Addition of IL-15 or IL-21/IL-15 to cells from healthy donors resulted in the recovery of significantly more $CD8^+$ T cells than stimulation with TAE beads alone or TAE beads plus IL-2 (Fig 4, *A*). Proliferation analysis revealed that treatment of naive

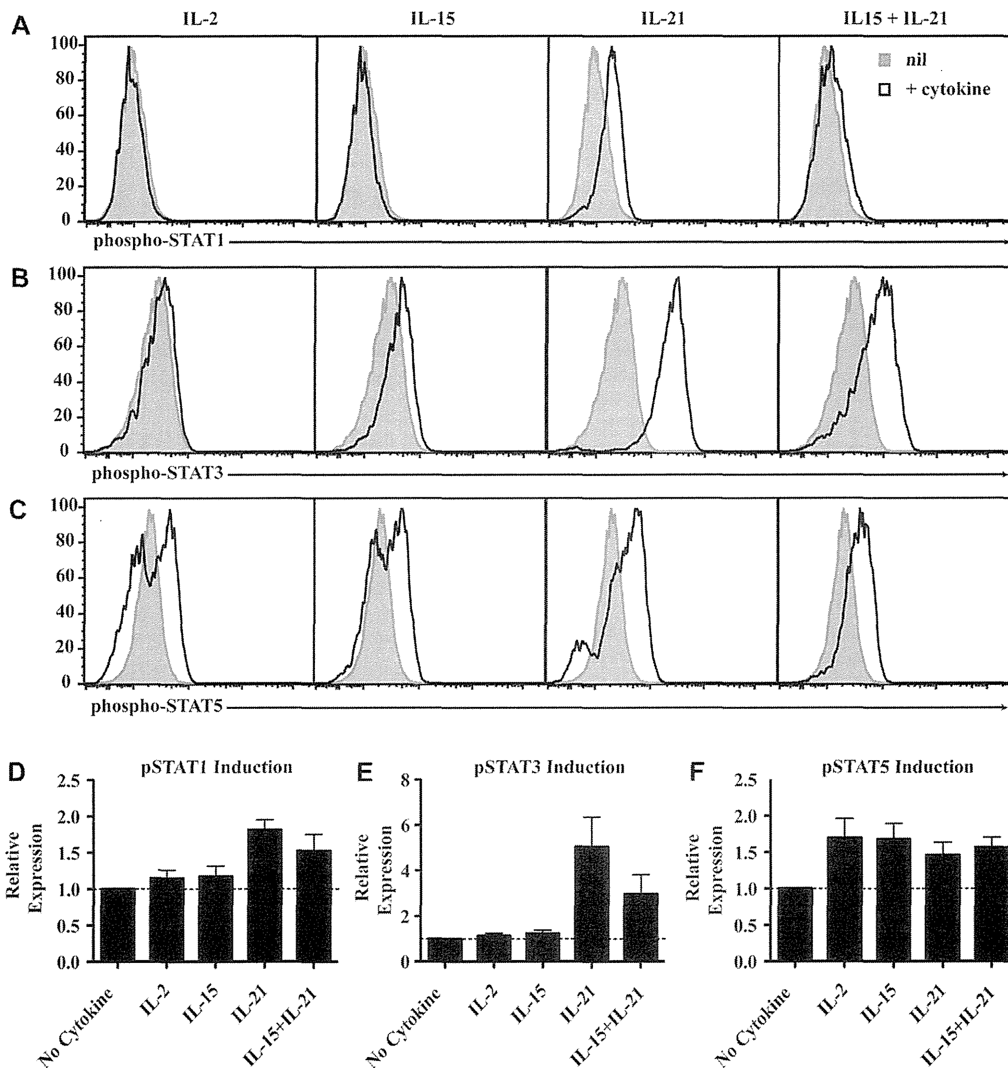

FIG 1. IL-21 predominately activates STAT1, STAT3, and STAT5 in human $CD8^+$ T cells. **A-C,** Naive $CD8^+$ T cells were activated for 4 days with TAE beads and recultured with cytokines for 30 minutes to determine phosphorylation of STAT1, STAT3, and STAT5. Histograms show nil or cytokine cultures and are representative of 4 experiments. **D-F,** Graphs represent the fold increase in mean fluorescence intensity (mean ± SEM, n = 4) of cells stimulated with cytokine over nil cultures. The *dashed lines* indicate a fold change of 1 (ie, no change).

$CD8^+$ T cells with TAE beads plus IL-21 increased the total percentage of divided cells and the average number of divisions the cells had undergone compared with those cultured with TAE beads alone (Fig 4, *B* and *C*). Interestingly, we detected no significant defect in the ability of TAE-activated naive $STAT3^{MUT}$ (Fig 4, *A-C*) or $STAT1^{MUT}$ (Fig 4, *D*) $CD8^+$ T cells to respond to IL-21 or IL-21/IL-15 costimulation. However, mutations in *IL21R* decreased recovery of viable cells and progression through division in cultures containing IL-21 (Fig 4, *E*). IL-7, another γ-chain cytokine, also enhanced proliferation of normal naive $CD8^+$ T cells that had been stimulated with TAE beads (data not shown). Although previous studies have found that IL-7 can activate STAT3,[44,45] the ability of IL-7 to promote naive $CD8^+$ T-cell proliferation was unaffected by mutations in *STAT3* (data not shown).

In cultures receiving cytokines alone, *STAT3* mutations impaired the ability of IL-21 to upregulate granzyme B (Fig 3). Therefore we determined whether signals provided by TCR/costimulation modulated this impairment. Addition of IL-2, IL-15, IL-21, or IL-21/IL-15 to TAE-stimulated cultures strongly (ie, >20-fold) upregulated granzyme B expression in normal naive $CD8^+$ T cells (Fig 5, *A* and *D*). In contrast to these cytokines, the effect of IL-7 on granzyme B induction was modest (ie, <10% granzyme B^+ cells; see Parmigiani et al[16] and data not shown). Mutations in *STAT3* (Fig 5, *A* and *D*) or *STAT1* (Fig 5, *B* and *D*) did not impair the ability of TAE-stimulated T cells to upregulate granzyme B in response to any of the cytokines tested. However, $IL\text{-}21R^{MUT}$ naive $CD8^+$ T cells were unable to upregulate granzyme B after IL-21 stimulation, and this was partially recovered by IL-15 (Fig 5, *C* and *D*). These results demonstrate that stimulation through TCR/costimulation alters the activation of the naive $CD8^+$ T cells such that *STAT3* mutations no longer prevent IL-21–induced expression of the cytotoxic mediator granzyme B.

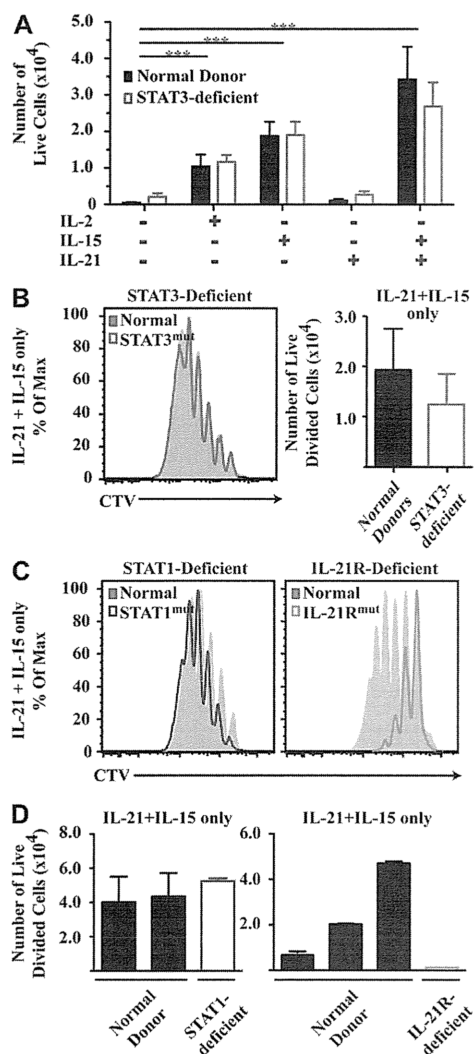

FIG 2. Cytokine-induced proliferation is impaired in IL-21R–deficient, but not STAT1- or STAT3-deficient, naive CD8$^+$ T cells. **A,** Naive CD8$^+$ T cells were cultured with cytokines only, and the numbers of live cells were determined (mean ± SEM, n = 5). **B** and **C,** Histograms show representative CTV profiles of cells stimulated with IL-21/IL-15. The graph in Fig 2, *B*, shows the number of divided cells in IL-21/IL-15 cultures (mean ± SEM, n = 5). **D,** Each *bar* represents an individual patient or healthy donor for experiments by using cells from STAT1- and IL-21R–deficient patients. $***P < .001$.

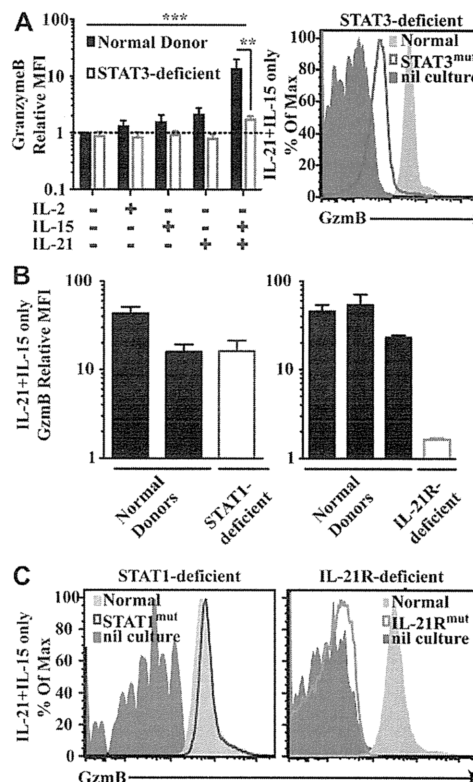

FIG 3. Cytokine-induced expression of granzyme B is impaired in IL-21R–deficient or STAT3-deficient naive CD8$^+$ T cells. **A** and **B,** Naive CD8$^+$ T cells were cultured with cytokines only, and graphs depict the fold increase (mean ± SEM, n = 5) in mean fluorescence intensity *(MFI)* of granzyme B over normal cells cultured with media alone (Fig 3, *A*). Each bar graph in Fig 3, *B*, represents means and ranges of duplicate cultures from an individual patient or normal donor for experiments using cells from STAT1- and IL-21R–deficient patients. **A** and **C,** Representative plots of cells from normal donors or the indicated patients stimulated with IL-21/IL-15 and normal donor cells from unstimulated cultures. $**P < .01$ and $***P < .001$.

Mutations in *STAT3* and *IL21R* alter the frequencies of memory CD8$^+$ T cells

Because IL-21 signals through IL-21R to activate STAT1 and STAT3 and regulates effector function, we speculated that impaired IL-21R, STAT1, or STAT3 function might also affect CD8$^+$ T-cell differentiation *in vivo*. Therefore we examined PB CD8$^+$ T-cell populations and phenotypes in patients with mutations in these molecules. We found that CD8$^+$ T cells represented 21.0% ± 1.1% of PB lymphocytes in healthy donors. This did not differ for STAT3^{MUT} (23.5% ± 1.5%), or IL-21R^{MUT} (17.4% ± 2.6%) patients (Fig 6, *A*).

In human subjects the CD8$^+$ T-cell population can be divided into subsets based on differential expression of CD45RA and CCR7.[46] The CD8$^+$ T-cell compartment of healthy donors thus comprises naive (35.5% ± 2.3%), T_{CM} (9.0% ± 7.0%), T_{EM} (33.5% ± 1.8%), and T_{EMRA} (22.1% ± 2.1%) cells (Fig 6, *B*). The distribution of these subsets in STAT1-deficient patients did not differ from those in healthy control subjects (Fig 6, *B*). However, the frequency of naive CD8$^+$ T cells in STAT3-deficient patients was significantly increased (62.6% ± 5.7%, $P < .001$) compared with that seen in healthy control subjects. This was associated with substantial decreases in T_{EM}, T_{EMRA}, and T_{CM} cell numbers in STAT3-deficient patients (19.9% ± 3.8%, 14.0% ± 2.7%, and 3.6% ± 0.8%, respectively; Fig 6, *B*). Furthermore, analysis of CD8$^+$ T-cell subsets from the 3 IL-21R–deficient patients suggested that memory in these patients might also be dysregulated, with increased naive (57.5% ± 6.3%) and reduced T_{EMRA} (8.2% ± 5.0%) cell numbers (Fig 6, *B*). It was recently reported that populations of memory and effector CD8$^+$ T cells reach adult levels by approximately 5 to 10 years of age.[47,48] This is consistent with our finding that the proportions of memory and effector CD8$^+$ T cells in the cohort of STAT1-deficient patients, the average age of which was 13 years (see Table E1), were normal (Fig 6, *B*). Thus it is unlikely that the decreased frequencies of nonnaive CD8$^+$ T cells in STAT3- and IL-21R–deficient subjects reflects the inclusion of some younger patients in these cohorts. These results suggest that STAT3, but not STAT1, plays an important role in generating, maintaining, or both memory CD8$^+$ T cells. Analysis of further IL-21R–deficient patients

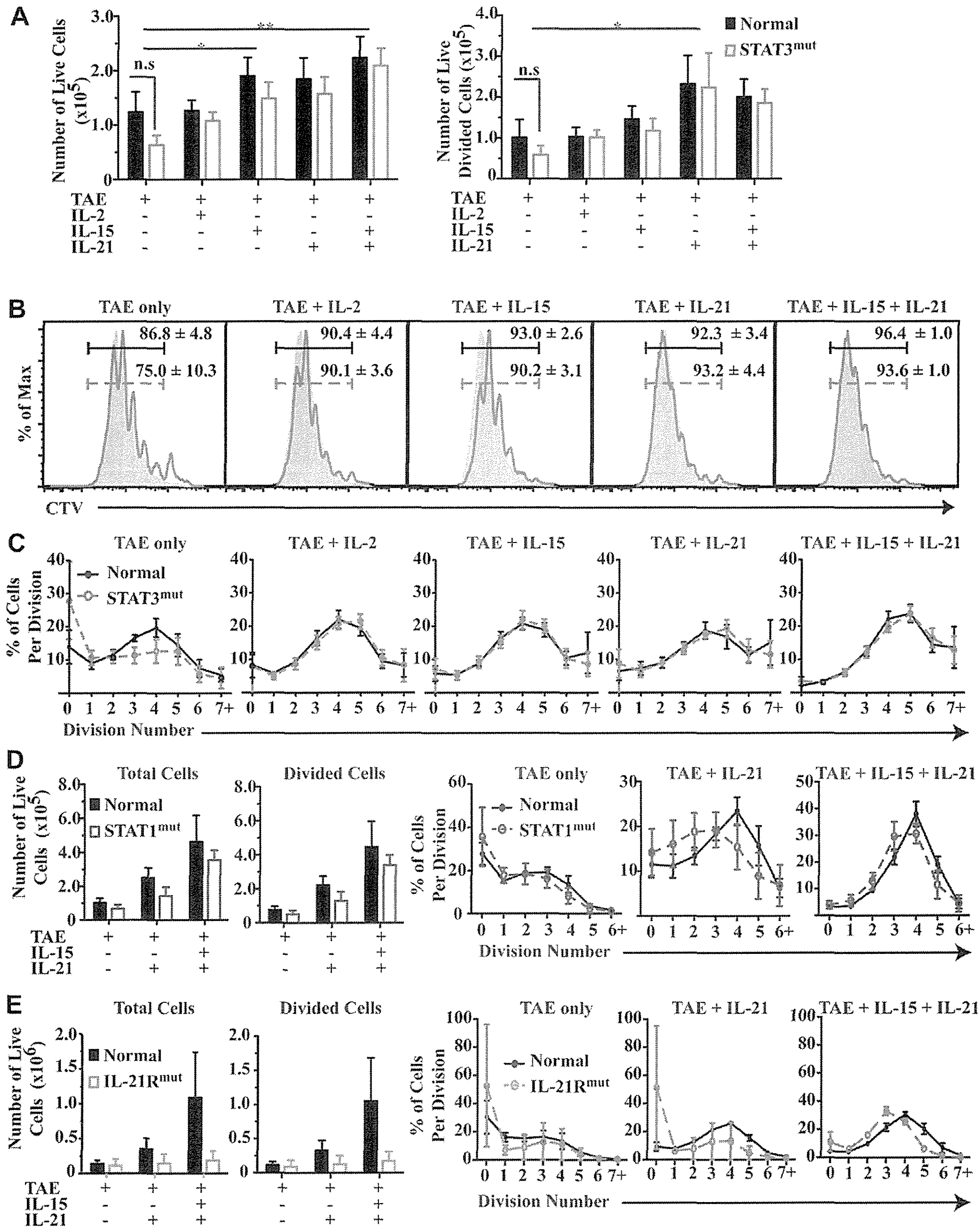

FIG 4. STAT3-deficient naive CD8$^+$ T cells proliferate normally in response to TCR engagement and activating cytokines. **A-E,** Naive CD8$^+$ T cells from healthy donors (n = 6-8 [Fig 4, *A-C*]; n = 3 [Fig 4, *D*]; and n = 5 [Fig 4, *E*]) and STAT3-deficient (Fig 4, *A-C*; n = 6-8), STAT1-deficient (Fig 4, *D*; n = 3), or IL-21R–deficient (Fig 4, *E*; n = 2) patients were cultured with TAE beads alone or together with cytokines. Total numbers of live cells that had entered division (Fig 4, *A*, *D*, and *E*) from each culture were determined (mean ± SEM). Fig 4, *B*, Histograms show representative CTV. Numbers indicate percentages of divided cells (mean ± SEM). Fig 4, *C-E*, Percentage of CD8$^+$ T cells in each division was determined. *$P < .05$, **$P < .01$, and *n.s*, not significant.

will be required to determine whether the required STAT3 activation is occurring downstream of IL-21 signaling or whether other cytokines, such as IL-10,[49] are also involved.

To further understand the decrease in memory CD8$^+$ T-cell numbers in STAT3-deficient patients, we analyzed expression of genes that control their differentiation and survival (see Fig E1 in this article's Online Repository at www.jacionline.org). *BCL2* was significantly higher in STAT3^{MUT} naive compared to normal naive CD8$^+$ T cells (see Fig E1). This suggests BCL2 might play a role in the survival and thus increased frequency of naive CD8$^+$ T cells in STAT3-deficient patients. However, we observed no other differences in expression of proapoptotic or antiapoptotic molecules between patients and control subjects (see Fig E1). Similarly, although transcription factors responsible for CD8$^+$ T-cell function and differentiation were differentially expressed across CD8$^+$ T-cell populations, we observed no significant differences between normal and STAT3^{MUT} CD8$^+$ T-cell numbers (see Fig E1).

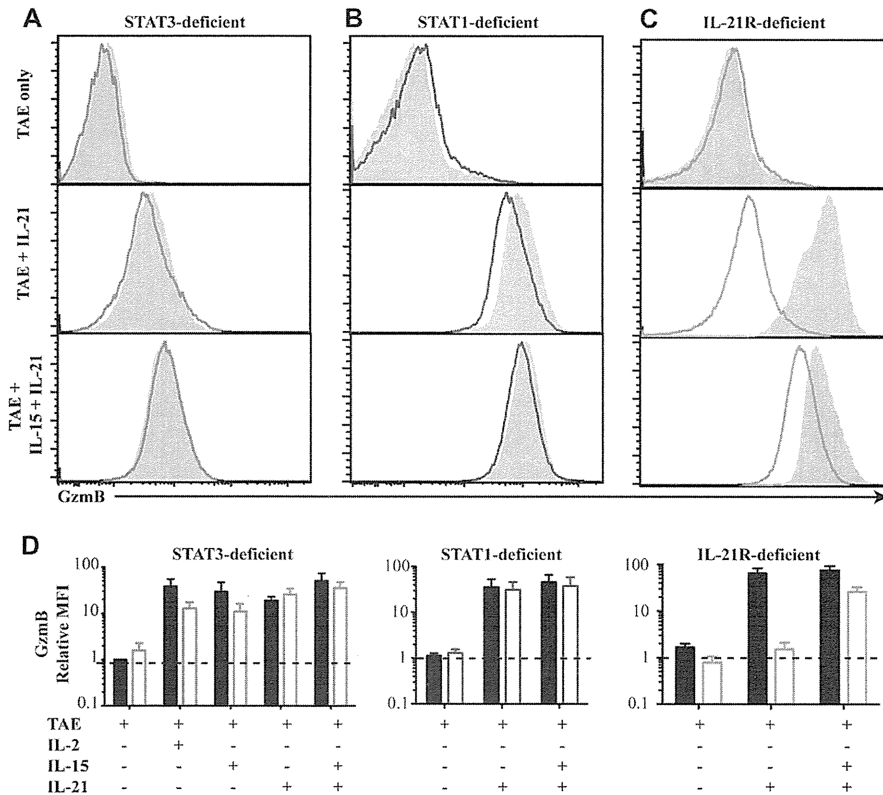

FIG 5. IL-21–induced granzyme B production is intact in TCR-stimulated STAT3-deficient $CD8^+$ T cells. **A-C,** Naive $CD8^+$ T cells from STAT3-deficient (n = 6-8; Fig 5, *A*), STAT1-deficient (n = 3; Fig 5, *B*), or IL-21R–deficient (n = 2; Fig 5, *C*) patients or healthy donors (n = 6-8) were cultured with TAE beads alone or together with cytokines. Representative histograms of granzyme B expression (*solid*, normal subjects; *colored lines*, patients) are depicted in Fig 5, *A* to *C*. **D,** Graphs show the fold increase (mean ± SEM) in mean fluorescence intensity *(MFI)* of granzyme B expression by cytokine-stimulated normal and patient cells over those cultured with TAE beads alone.

STAT3-deficient T_{EM} and T_{EMRA} $CD8^+$ T cells have a phenotype suggestive of sustained activation

$CD8^+$ T-cell subsets were further assessed for expression of a range of molecules that change during differentiation from naive to effector cells.[50,51] $STAT3^{MUT}$ cells showed dysregulated expression of many of these molecules (Fig 7). For example, 2B4 was highest on normal $CD8^+$ T_{EM} and T_{EMRA} cells but was increased 2- to 4-fold on $STAT3^{MUT}$ T_{EM} cells ($P < .001$). In contrast, 2B4 was expressed at normal levels on $STAT1^{MUT}$ $CD8^+$ T cells (Fig 7 and see Figs E2, *A*, and E3, *A*, in this article's Online Repository at www.jacionline.org). CD57 expression is associated with poorly proliferative, terminally differentiated T cells.[51,52] Consistent with this, the greatest frequency of $CD57^+$ cells was found in the T_{EMRA} population. However, proportions of $CD57^+$ cells in the T_{EM} and T_{EMRA} populations of $STAT3^{MUT}$ $CD8^+$ T cells were significantly increased relative to those seen in normal and $STAT1^{MUT}$ cells (Fig 7). CD127 (IL-7 receptor α) is highly expressed on naive and T_{CM} cells and downregulated on T_{EM} and T_{EMRA} cells. However, in $STAT3^{MUT}$ patients all memory populations displayed significantly decreased levels of CD127 compared with healthy donors (Fig 7). Not all activation molecules displayed altered expression, however, because CD95 was not altered on $CD8^+$ T cells from either the $STAT1^{MUT}$ or $STAT3^{MUT}$ patients (Fig 7 and see Figs E2, *A*, and E3, *A*).

Chemokine receptors and adhesion molecules are important for regulating migration to secondary lymphoid organs and inflamed tissues. CX3CR1, CD11a, and CD11b levels are highest on T_{EMRA} and T_{EM} cells from healthy donors. However, their expression on $STAT3^{MUT}$, but not $STAT1^{MUT}$, T_{EM} and T_{EMRA} cells was significantly (2- to 3-fold) higher than on normal cells (Fig 7 and see Figs E2, *B*, and E3, *B*). Interestingly, $STAT3^{MUT}$ T_{EM} and T_{EMRA} $CD8^+$ T cells had significantly higher expression of granzyme B but not perforin. The altered expression of these molecules by $STAT3^{MUT}$ $CD8^+$ T cells suggests they have undergone aberrant differentiation *in vivo*, and the residual effector memory populations exhibit a more senescent/exhausted phenotype.

DISCUSSION

Naive $CD8^+$ T cells must proliferate and acquire effector function, processes believed to be regulated by IL-21, to establish protective antiviral and antitumor immunity. Indeed, IL-21 levels have been found to increase *in vitro* proliferation and survival of mouse and human $CD8^+$ T cells,[6-12,38] as well as induce the effector molecules IFN-γ, granzyme B, and perforin and enhance their overall cytotoxicity.[7-10,16,42,43] Furthermore, *in vivo* delivery of IL-21 to patients with cancer upregulated granzyme B and perforin in $CD8^+$ T cells.[17] Consistent with previous studies in multiple cell types,[6,15,17,26] IL-21 induced phosphorylation of STAT1, STAT3, and STAT5 in human $CD8^+$ T cells. Yet the contribution of individual STATs and

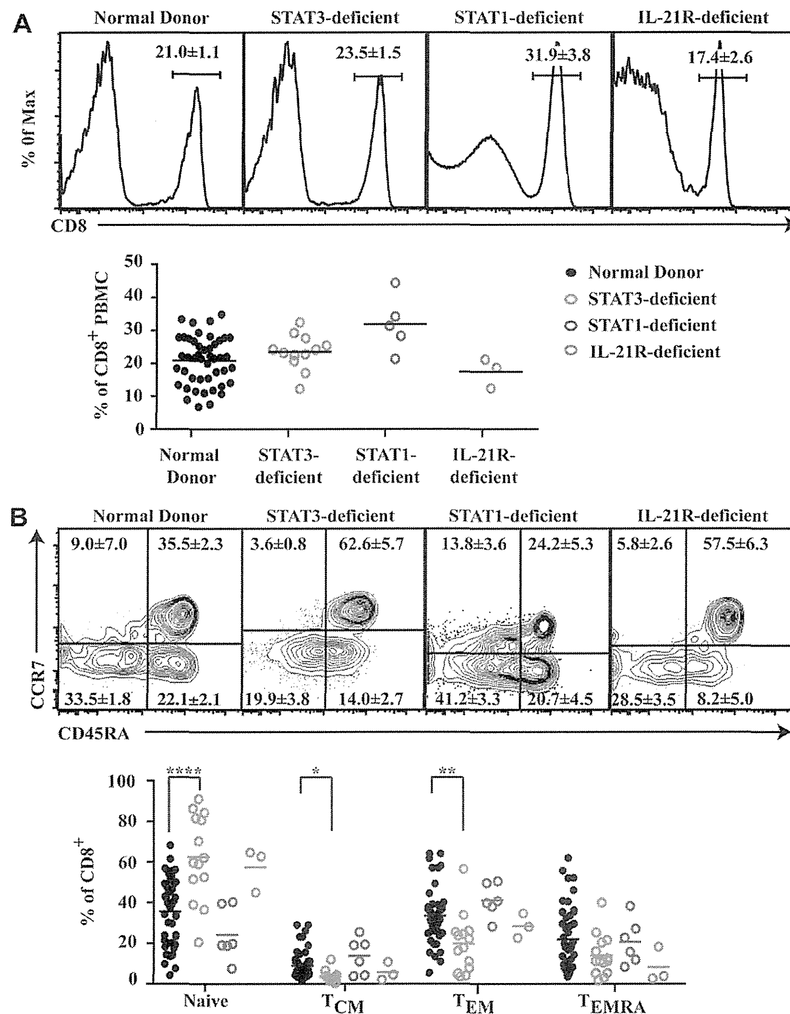

FIG 6. Mutations in *STAT3,* but not *STAT1,* impair generation of effector/memory $CD8^+$ T cells *in vivo.* PB from healthy donors (n = 46 or 51) or STAT3-deficient (n = 12-14), STAT1-deficient (n = 5 or 6), or IL-21R–deficient (n = 3) patients were assessed for the percentage of total $CD8^+$ T cells **(A)** or naive, T_{CM}, T_{EM}, and T_{EMRA} cells **(B)**. *Each symbol* corresponds to an individual donor or patient, and *lines* represent means. Histograms and dot plots are from 1 representative donor or patient. *$P < .05$, **$P < .01$, and ****$P < .0001$.

cytokines to the development and effector function of human $CD8^+$ T cells has not previously been defined. To clarify the requirements for these molecules in human $CD8^+$ T-cell function, we analyzed $CD8^+$ T-cell differentiation *in vivo* and IL-21 signaling *in vitro* in subjects with loss-of function mutations in *IL21R*, *STAT1*, or *STAT3*.

The ability of IL-21 to promote proliferation of naive $CD8^+$ T cells was unaffected by mutations in *STAT1* or *STAT3*. In contrast, impaired STAT3 signaling abolished upregulation of granzyme B in response to IL-15/IL-21 when no exogenous TCR stimulus was provided. This paralleled our observations from cells unable to signal through the IL-21R, thereby suggesting that intact signaling through the IL-21R/STAT3 axis is required for *GZMB* transcription and subsequent cytotoxicity in human $CD8^+$ T cells stimulated with IL-15/IL-21. These findings are physiologically relevant because they infer that the increase in expression of granzyme B observed in $CD8^+$ T cells of patients with cancer who were administered IL-21 in the absence of specific T-cell activation[17] was STAT3 dependent. This is supported by work showing that IL-21–induced phospho-STAT3 binds upstream of *Gzmb* in murine $CD4^+$ T cells.[53] The inability of STAT3-deficient $CD8^+$ T cells to upregulate granzyme B in response to IL-15/IL-21 would be further compounded by impaired production of IL-21 by STAT3-deficient $CD4^+$ T cells.[54] Surprisingly, we observed that when naive $STAT3^{MUT}$ $CD8^+$ T cells were provided with extrinsic TCR stimulus and IL-21, they were capable of upregulating granzyme B to similar levels as controls. IL-21–induced granzyme B upregulation was also intact in STAT1-deficient naive $CD8^+$ T cells, suggesting that TCR/costimulation did not result in a switch from a STAT3 to a STAT1 pathway downstream of IL-21R for granzyme B regulation. Rather, TCR/costimulatory signaling is likely to alter the sensitivity of cells to STAT3, such that residual STAT3 activity in $STAT3^{MUT}$ $CD8^+$ T cells is sufficient to induce a cytotoxic response.

Studies in mice have suggested that IL-21 is important for controlling some viral infections, such as vaccina[18] and chronic LCMV.[19-21] Patients with AD-HIES generally do not exhibit heightened susceptibility to primary viral infection, but some (<20%) do have an impaired ability to control reactivation of herpes viruses (HSV, EBV, and varicella zoster virus).[29,30] On the

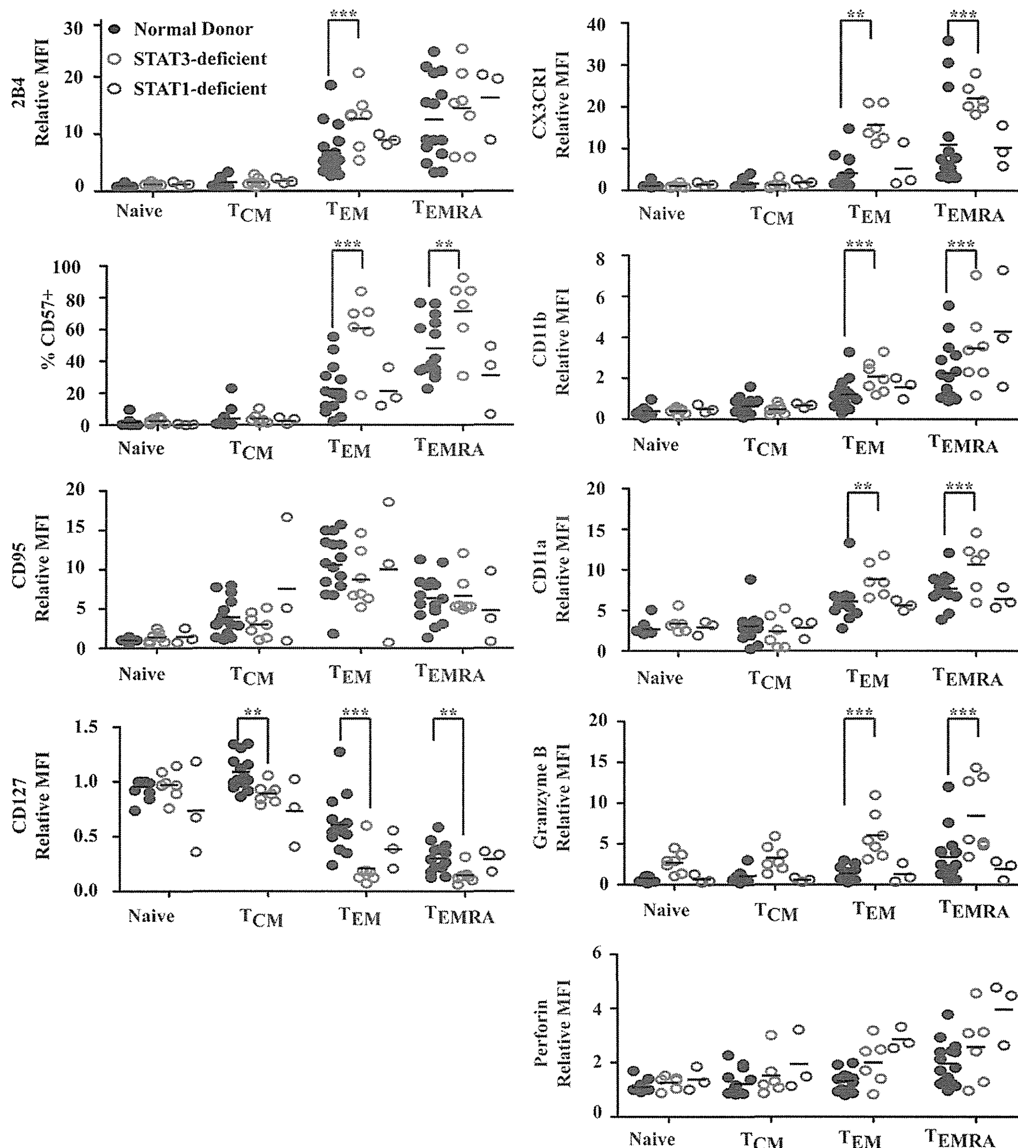

FIG 7. $CD8^+$ T cells from STAT3-deficient patients display a more activated phenotype. Subsets of naive, T_{CM}, T_{EM}, and T_{EMRA} $CD8^+$ T cells in PB of healthy donors (n = 15) or STAT3-deficient (n = 7) and STAT1-deficient (n = 3) patients were assessed for expression of 2B4, CD57, CD95, CD127, CX3CR1, CD11a, CD11b, granzyme B, or perforin. Graphs represent the fold change in mean fluorescent intensity *(MFI)* of the molecules relative to naive cells or the percentage of positive cells. $^{**}P < .01$ and $^{***}P < .001$.

other hand, the few IL-21R–deficient subjects documented to date appear capable of mounting protective responses to these viruses yet experience ongoing infection with norovirus and rhinovirus.[37] However, both of these PIDs are associated with increased susceptibility to infection with bacterial and fungal pathogens (*Staphylococcus aureus*, *Candida albicans*, *Pneumocystis* species, and cryptosporidia).[28,29,37,55,56] Our findings actually provide an explanation for the relatively mild susceptibility of $STAT3^{MUT}$ and $IL\text{-}21R^{MUT}$ patients to primary viral infection, despite predictions from mouse models.[18-21,49] First, delivery of strong TCR and costimulatory signals during viral infection would facilitate normal induction of granzyme B in IL-21–stimulated $STAT3^{MUT}$ cells. Second, granzyme B induction by IL-2 and IL-15 is intact in $STAT3^{MUT}$ and $IL\text{-}21R^{MUT}$ $CD8^+$ T cells. Thus during most viral infections, combined signals by TCR/costimulation, IL-2, IL-15, and/or IL-21 in naive $CD8^+$ T cells would be sufficient to generate a protective cytotoxic response, thereby circumventing the dependency on signaling through IL-21R or STAT3.

An interesting feature of AD-HIES is predisposition to B-cell lymphoma.[29,31,32,57] It is possible that during an antitumor response, immune activation is not as strong as during viral infection; thus the relative contribution of IL-21R/STAT3 signaling in regulating granzyme B might be greater. Consequently, *STAT3* mutations could contribute to impaired $CD8^+$ T-cell immune surveillance against B-cell malignancies. This is reminiscent of the susceptibility of perforin-deficient subjects to hematologic neoplasms, including B-cell lymphoma.[58]

The reported role of IL-21 in memory cell development[12,18,49] prompted us to investigate the phenotype of $CD8^+$ T cells from

STAT1- and STAT3-deficient patients. Recent studies reported reduced frequencies of T_{CM} cells in patients with AD-HIES,[30] thereby implicating STAT3 in the development of "central memory" $CD8^+$ T cells.[30,49] We found significant decreases not only in T_{CM} but also in T_{EM} cell numbers in STAT3-deficient, but not STAT1-deficient, subjects. These changes were not due to alterations in total percentages of $CD8^+$ T cells or age-related variations in the number of naive versus memory cells (see Fig E4 in this article's Online Repository at www.jacionline.org). T_{EM} and T_{EMRA} cell populations from STAT3-deficient patients also displayed a phenotype of exaggerated differentiation often associated with increased/sustained exposure to antigen.[50,51,59] Assessment of IL-21R–deficient patients suggested IL-21 might contribute to establishing some of the memory populations in STAT3-deficient subjects. However, analysis of additional IL-21R–deficient patients will be required to determine whether IL-21 is the STAT3-activating cytokine required for maintaining $CD8^+$ T-cell memory.

Several explanations can be proposed for the memory $CD8^+$ T-cell deficiency seen in patients with AD-HIES. First, *STAT3* mutations might affect $CD8^+$ T-cell homeostasis or differentiation. However, proliferation of $STAT3^{MUT}$ naive $CD8^+$ T cells in response to IL-7 or IL-21/IL-15, which regulate the homeostasis of $CD8^+$ T cells,[2,9,38,42,60-64] was normal. Thus there is no evidence that the homeostatic proliferation and survival of naive T cells induced by these cytokines requires STAT3, implying that the memory cell deficit is unlikely to be caused by impaired proliferation.

Second, impaired STAT3 function might alter $CD8^+$ memory T-cell numbers through effects on differentiation. $CD8^+$ T-cell differentiation is regulated by multiple transcription factors that control opposing fates: Eomes and Bcl-6 favor "central memory" $CD8^+$ T-cell development, whereas T-bet and Blimp-1 promote differentiation to "effector" $CD8^+$ T cells.[13,65-69] The observations that *Socs3*, *Tbx21*, *Bcl6*, and *Prdm1* are direct targets of STAT3,[53] together with reduced expression of these genes in $Stat3^{-/-}$ murine $CD8^+$ T cells after infection with LCMV[49] and of *SOCS3* and *BCL6* in *ex vivo* isolated STAT3-deficient human naive $CD8^+$ T cells,[30] suggest that STAT3 regulates $CD8^+$ T-cell differentiation by controlling expression of transcription factors. Consistent with this, we observed decreased *SOCS3* expression in IL-21–stimulated STAT3-deficient naive $CD8^+$ T cells *in vitro*. Thus, as proposed previously,[49] decreased SOCS3 expression might contribute to aberrant differentiation, resulting in the enhanced activated phenotype we observed in T_{EM} and T_{EMRA} cells in $STAT3^{MUT}$ patients. However, we saw no significant differences in the levels of other transcription factors between normal and STAT3-deficient $CD8^+$ T cells either directly *ex vivo* (see Fig E1) or after IL-21 stimulation (see Fig E5 in this article's Online Repository at www.jacionline.org). This suggests that the IL-21/STAT3 axis proposed to drive Bcl-6 expression and $CD8^+$ T_{CM} cell generation might be an oversimplification. Instead, STAT3 appears to have broader effects, being required for the generation or maintenance of not only T_{CM} cells but also T_{EM} and T_{EMRA} cell populations.

Lastly, reduced memory $CD8^+$ T-cell frequencies in $STAT3^{MUT}$ patients might reflect reduced signaling through the IL-7 receptor (CD127). IL-7 is another homeostatic cytokine important for memory T-cell maintenance.[39,61,63,64] Interestingly, CD127 expression was significantly decreased on all memory populations from STAT3-deficient patients compared with healthy donors, which is consistent with a more activated phenotype. Thus reduced expression of CD127 on STAT3-deficient $CD8^+$ T cells might limit their responsiveness to IL-7 signals, thereby compromising the prosurvival effects of IL-7 and compounding any decrease in memory cell numbers caused by reduced memory cell differentiation.

These findings reveal STAT3, but not STAT1, as an important downstream component of IL-21 signaling that mediates induction of effector function in $CD8^+$ T cells. However, a high level of redundancy for the induction of cytotoxic function seems to exist, suggesting that in most circumstances a functional level of killing would still be generated. This is consistent with mild susceptibility to viral infections in patients with either AD-HIES or IL-21R deficiency. In contrast, STAT3 signals, possibly initiated by IL-21, are critical for regulating the pool of all memory $CD8^+$ T-cell subsets in human subjects. Collectively, these insights significantly add to our understanding of the function of IL-21 and STAT3 in human $CD8^+$ T-cell development and behavior and disease pathogenesis in subjects with mutations in *STAT3* and *IL21R*.

We thank the patients and their families for their involvement in this work.

Key messages

- **Loss-of function mutations in *STAT3*, causing AD-HIES, and *IL21R*, compromise differentiation of human $CD8^+$ T cells to memory and effector cells.**
- **Mutations in *STAT3*, but not *STAT1*, abrogate the ability of human $CD8^+$ T cells to differentiate into granzyme B–expressing effector cells in response to IL-21.**

REFERENCES

1. Becker TC. Interleukin 15 is required for proliferative renewal of virus-specific memory CD8 T cells. J Exp Med 2002;195:1541-8.
2. Lodolce JP, Burkett PR, Koka RM, Boone DL, Ma A. Regulation of lymphoid homeostasis by interleukin-15. Cytokine Growth Factor Rev 2002;13:429-39.
3. Surh CD, Sprent J. Homeostasis of naive and memory T cells. Immunity 2008;29:848-62.
4. Pipkin ME, Sacks JA, Cruz-Guilloty F, Lichtenheld MG, Bevan MJ, Rao A. Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells. Immunity 2010;32:79-90.
5. Kalia V, Sarkar S, Subramaniam S, Haining WN, Smith KA, Ahmed R. Prolonged interleukin-2Rα expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo. Immunity 2010;32:91-103.
6. Zeng R, Spolski R, Casas E, Zhu W, Levy DE, Leonard WJ. The molecular basis of IL-21-mediated proliferation. Blood 2007;109:4135-42.
7. Casey KA, Mescher MF. IL-21 promotes differentiation of naive CD8 T cells to a unique effector phenotype. J Immunol 2007;178:7640-8.
8. Kasaian MT, Whitters MJ, Carter LL, Lowe LD, Jussif JM, Deng B, et al. IL-21 limits NK cell responses and promotes antigen-specific T cell activation: a mediator of the transition from innate to adaptive immunity. Immunity 2002;16:559-69.
9. Zeng R, Spolski R, Finkelstein SE, Oh S, Kovanen PE, Hinrichs CS, et al. Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function. J Exp Med 2005;201:139-48.
10. Barker BR, Parvani JG, Meyer D, Hey AS, Skak K, Letvin NL. IL-21 induces apoptosis of antigen-specific CD8+ T lymphocytes. J Immunol 2007;179:3596-603.
11. Li Y, Bleakley M, Yee C. IL-21 influences the frequency, phenotype, and affinity of the antigen-specific CD8 T cell response. J Immunol 2005;175:2261-9.
12. Nguyen H, Weng N-p. IL-21 preferentially enhances IL-15-mediated homeostatic proliferation of human CD28+ CD8 memory T cells throughout the adult age span. J Leukoc Biol 2010;87:43-9.
13. Ichii H, Sakamoto A, Hatano M, Okada S, Toyama H, Taki S, et al. Role for Bcl-6 in the generation and maintenance of memory CD8+ T cells. Nat Immunol 2002;3:558-63.

14. Ostiguy V, Allard EL, Marquis M, Leignadier J, Labrecque N. IL-21 promotes T lymphocyte survival by activating the phosphatidylinositol-3 kinase signaling cascade. J Leukoc Biol 2007;82:645-56.
15. Strengell M, Sareneva T, Foster D, Julkunen I, Matikainen S. IL-21 up-regulates the expression of genes associated with innate immunity and Th1 response. J Immunol 2002;169:3600-5.
16. Parmigiani A, Pallin MF, Schmidtmayerova H, Lichtenheld MG, Pahwa S. Interleukin-21 and cellular activation concurrently induce potent cytotoxic function and promote antiviral activity in human CD8 T cells. Hum Immunol 2011;72: 115-23.
17. Frederiksen KS, Lundsgaard D, Freeman JA, Hughes SD, Holm TL, Skrumsager BK, et al. IL-21 induces in vivo immune activation of NK cells and CD8(+) T cells in patients with metastatic melanoma and renal cell carcinoma. Cancer Immunol Immunother 2008;57:1439-49.
18. Novy P, Huang X, Leonard WJ, Yang Y. Intrinsic IL-21 signaling is critical for CD8 T cell survival and memory formation in response to vaccinia viral infection. J Immunol 2011;186:2729-38.
19. Elsaesser H, Sauer K, Brooks DG. IL-21 is required to control chronic viral infection. Science 2009;324:1569-72.
20. Frohlich A, Kisielow J, Schmitz I, Freigang S, Shamshiev AT, Weber J, et al. IL-21R on T cells is critical for sustained functionality and control of chronic viral infection. Science 2009;324:1576-80.
21. Yi JS, Du M, Zajac AJ. A vital role for interleukin-21 in the control of a chronic viral infection. Science 2009;324:1572-6.
22. Ma HL, Whitters MJ, Konz RF, Senices M, Young DA, Grusby MJ, et al. IL-21 activates both innate and adaptive immunity to generate potent antitumor responses that require perforin but are independent of IFN-gamma. J Immunol 2003;171:608-15.
23. Di Carlo E, Comes A, Orengo AM, Rosso O, Meazza R, Musiani P, et al. IL-21 induces tumor rejection by specific CTL and IFN-gamma-dependent CXC chemokines in syngeneic mice. J Immunol 2004;172:1540-7.
24. Moroz A, Eppolito C, Li Q, Tao J, Clegg CH, Shrikant PA. IL-21 enhances and sustains CD8+ T cell responses to achieve durable tumor immunity: comparative evaluation of IL-2, IL-15, and IL-21. J Immunol 2004;173:900-9.
25. Markley JC, Sadelain M. IL-7 and IL-21 are superior to IL-2 and IL-15 in promoting human T cell-mediated rejection of systemic lymphoma in immunodeficient mice. Blood 2010;115:3508-19.
26. Asao H, Okuyama C, Kumaki S, Ishii N, Tsuchiya S, Foster D, et al. Cutting edge: the common gamma-chain is an indispensable subunit of the IL-21 receptor complex. J Immunol 2001;167:1-5.
27. Habib T, Senadheera S, Weinberg K, Kaushansky K. The common gamma chain (gamma c) is a required signaling component of the IL-21 receptor and supports IL-21-induced cell proliferation via JAK3. Biochemistry 2002; 41:8725-31.
28. Grimbacher B, Holland SM, Puck JM. Hyper-IgE syndromes. Immunol Rev 2005;203:244-50.
29. Chandesris MO, Melki I, Natividad A, Puel A, Fieschi C, Yun L, et al. Autosomal dominant STAT3 deficiency and hyper-IgE syndrome: molecular, cellular, and clinical features from a French national survey. Medicine (Baltimore) 2012;91: e1-19.
30. Siegel A, Heimall J, Freeman A, Hsu A, Brittain E, Brenchley J, et al. A critical role for STAT3 transcription factor signaling in the development and maintenance of human T cell memory. Immunity 2011;35:806-18.
31. Kumánovics A, Perkins S, Gilbert H, Cessna M, Augustine N, Hill H. Diffuse large B cell lymphoma in hyper-IgE syndrome due to STAT3 mutation. J Clin Immunol 2010;30:886-93.
32. Jiao H, Toth B, Erdos M, Fransson I, Rakoczi E, Balogh I, et al. Novel and recurrent STAT3 mutations in hyper-IgE syndrome patients from different ethnic groups. Mol Immunol 2008;46:202-6.
33. Holland SM, DeLeo FR, Elloumi HZ, Hsu AP, Uzel G, Brodsky N, et al. STAT3 mutations in the hyper-IgE syndrome. N Engl J Med 2007;357:1608-19.
34. Minegishi Y, Saito M, Tsuchiya S, Tsuge I, Takada H, Hara T, et al. Dominant-negative mutations in the DNA-binding domain of STAT3 cause hyper-IgE syndrome. Nature 2007;448:1058-62.
35. Boisson-Dupuis S, Kong XF, Okada S, Cypowyj S, Puel A, Abel L, et al. Inborn errors of human STAT1: allelic heterogeneity governs the diversity of immunological and infectious phenotypes. Curr Opin Immunol 2012;24:364-78.
36. Casanova JL, Holland SM, Notarangelo LD. Inborn errors of human JAKs and STATs. Immunity 2012;36:515-28.
37. Kotlarz D, Zietara N, Uzel G, Weidemann T, Braun CJ, Diestelhorst J, et al. Loss-of-function mutations in the IL-21 receptor gene cause a primary immunodeficiency syndrome. J Exp Med 2013;210:433-43.
38. Alves NL, Arosa FA, van Lier RAW. IL-21 Sustains CD28 expression on IL-15-activated human naive CD8+ T cells. J Immunol 2005;175:755-62.
39. Melchionda F, Fry TJ, Milliron MJ, McKirdy MA, Tagaya Y, Mackall CL. Adjuvant IL-7 or IL-15 overcomes immunodominance and improves survival of the CD8+ memory cell pool. J Clin Invest 2005;115:1177-87.
40. Trapani JA, Smyth MJ. Functional significance of the perforin/granzyme cell death pathway. Nat Rev Immunol 2002;2:735-47.
41. Tamand DL, Redelman D, Alves BN, Vollger L, Bethley C, Hudig D. Induction of granzyme B and T cell cytotoxic capacity by IL-2 or IL-15 without antigens: multiclonal responses that are extremely lytic if triggered and short-lived after cytokine withdrawal. Cytokine 2006;36:148-59.
42. White L, Krishnan S, Strbo N, Liu H, Kolber MA, Lichtenheld MG, et al. Differential effects of IL-21 and IL-15 on perforin expression, lysosomal degranulation, and proliferation in CD8 T cells of patients with human immunodeficiency virus-1 (HIV). Blood 2007;109:3873-80.
43. Strengell M, Matikainen S, Sirén J, Lehtonen A, Foster D, Julkunen I, et al. IL-21 in synergy with IL-15 or IL-18 enhances IFN-γ production in human NK and T cells. J Immunol 2003;170:5464-9.
44. Lin JX, Migone TS, Tsang M, Friedmann M, Weatherbee JA, Zhou L, et al. The role of shared receptor motifs and common Stat proteins in the generation of cytokine pleiotropy and redundancy by IL-2, IL-4, IL-7, IL-13, and IL-15. Immunity 1995;2:331-9.
45. Yu CR, Young HA, Ortaldo JR. Characterization of cytokine differential induction of STAT complexes in primary human T and NK cells. J Leukoc Biol 1998;64:245-58.
46. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 1999;401:708-12.
47. van Gent R, van Tilburg CM, Nibbelke EE, Otto SA, Gaiser JF, Janssens-Korpela PL, et al. Refined characterization and reference values of the pediatric T- and B-cell compartments. Clin Immunol 2009;133:95-107.
48. Schatorje EJ, Gemen EF, Driessen GJ, Leuvenink J, van Hout RW, de Vries E. Paediatric reference values for the peripheral T cell compartment. Scand J Immunol 2012;75:436-44.
49. Cui W, Liu Y, Weinstein JS, Craft J, Kaech SM. An interleukin-21-interleukin-10-STAT3 pathway is critical for functional maturation of memory CD8+ T cells. Immunity 2011;35:792-805.
50. Appay V, van Lier RAW, Sallusto F, Roederer M. Phenotype and function of human T lymphocyte subsets: consensus and issues. Cytometry A 2008;73A: 975-83.
51. Focosi D, Bestagno M, Burrone O, Petrini M. CD57+ T lymphocytes and functional immune deficiency. J Leukoc Biol 2010;87:107-16.
52. Brenchley JM, Karandikar NJ, Betts MR, Ambrozak DR, Hill BJ, Crotty LE, et al. Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. Blood 2003;101:2711-20.
53. Kwon H, Thierry-Mieg D, Thierry-Mieg J, Kim HP, Oh J, Tunyaplin C, et al. Analysis of interleukin-21-induced Prdm1 gene regulation reveals functional cooperation of STAT3 and IRF4 transcription factors. Immunity 2009;31:941-52.
54. Ma CS, Avery DT, Chan A, Batten M, Bustamante J, Boisson-Dupuis S, et al. Functional STAT3 deficiency compromises the generation of human T follicular helper cells. Blood 2012;119:3997-4008.
55. Minegishi Y. Hyper-IgE syndrome. Curr Opin Immunol 2009;21:487-92.
56. Freeman AF, Holland SM. Clinical manifestations, etiology, and pathogenesis of the hyper-IgE syndromes. Pediatr Res 2009;65:32R-7R.
57. Heimall J, Freeman A, Holland S. Pathogenesis of hyper IgE syndrome. Clin Rev Allerg Immunol 2010;38:32-8.
58. Brennan AJ, Chia J, Trapani JA, Voskoboinik I. Perforin deficiency and susceptibility to cancer. Cell Death Differ 2010;17:607-15.
59. Randall KL, Chan SS, Ma CS, Fung I, Mei Y, Yabas M, et al. DOCK8 deficiency impairs CD8 T cell survival and function in humans and mice. J Exp Med 2011; 208:2305-20.
60. Berard M, Brandt K, Paus SB, Tough DF. IL-15 promotes the survival of naive and memory phenotype CD8+ T cells. J Immunol 2003;170:5018-26.
61. Schluns KS, Kieper WC, Jameson SC, Lefrancois L. Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo. Nat Immunol 2000;1: 426-32.
62. Zhang X, Sun S, Hwang I, Tough DF, Sprent J. Potent and selective stimulation of memory-phenotype CD8+ T cells in vivo by IL-15. Immunity 1998;8:591-9.
63. Kaech SM, Tan JT, Wherry EJ, Konieczny BT, Surh CD, Ahmed R. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nat Immunol 2003;4:1191-8.
64. Maraskovsky E, Teepe M, Morrissey P, Braddy S, Miller R, Lynch D, et al. Impaired survival and proliferation in IL-7 receptor-deficient peripheral T cells. J Immunol 1996;157:5315-23.
65. Banerjee A, Gordon SM, Intlekofer AM, Paley MA, Mooney EC, Lindsten T, et al. Cutting edge: the transcription factor eomesodermin enables CD8+

T cells to compete for the memory cell niche. J Immunol 2010;185: 4988-92.
66. Ichii H, Sakamoto A, Arima M, Hatano M, Kuroda Y, Tokuhisa T. Bcl6 is essential for the generation of long-term memory CD4+ T cells. Int Immunol 2007;19:427-33.
67. Ichii H, Sakamoto A, Kuroda Y, Tokuhisa T. Bcl6 acts as an amplifier for the generation and proliferative capacity of central memory CD8+ T cells. J Immunol 2004;173:883-91.
68. Intlekofer AM, Takemoto N, Wherry EJ, Longworth SA, Northrup JT, Palanivel VR, et al. Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. Nat Immunol 2005;6:1236-44.
69. Rutishauser RL, Martins GA, Kalachikov S, Chandele A, Parish IA, Meffre E, et al. Transcriptional repressor Blimp-1 promotes CD8+ T cell terminal differentiation and represses the acquisition of central memory T cell properties. Immunity 2009;31:296-308.

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REFERENCES

E1. Avery DT, Deenick EK, Ma CS, Suryani S, Simpson N, Chew GY, et al. B cell-intrinsic signaling through IL-21 receptor and STAT3 is required for establishing long-lived antibody responses in humans. J Exp Med 2010;207:155-71.

E2. Ma CS, Avery DT, Chan A, Batten M, Bustamante J, Boisson-Dupuis S, et al. Functional STAT3 deficiency compromises the generation of human T follicular helper cells. Blood 2012;119:3997-4008.

E3. Ma CS, Chew GY, Simpson N, Priyadarshi A, Wong M, Grimbacher B, et al. Deficiency of Th17 cells in hyper IgE syndrome due to mutations in STAT3. J Exp Med 2008;205:1551-7.

E4. Woellner C, Gertz EM, Schäffer AA, Lagos M, Perro M, Glocker EO, et al. Mutations in STAT3 and diagnostic guidelines for hyper-IgE syndrome. J Allergy Clin Immunol 2010;125:424-32.e8.

E5. Chapgier A, Boisson-Dupuis S, Jouanguy E, Vogt G, Feinberg J, Prochnicka-Chalufour A, et al. Novel STAT1 alleles in otherwise healthy patients with mycobacterial disease. PLoS Genet 2006;2:e131.

E6. Chapgier A, Kong XF, Boisson-Dupuis S, Jouanguy E, Averbuch D, Feinberg J, et al. A partial form of recessive STAT1 deficiency in humans. J Clin Invest 2009;119:1502-14.

E7. Chapgier A, Wynn RF, Jouanguy E, Filipe-Santos O, Zhang S, Feinberg J, et al. Human complete Stat-1 deficiency is associated with defective type I and II IFN responses in vitro but immunity to some low virulence viruses in vivo. J Immunol 2006;176:5078-83.

E8. Dupuis S, Dargemont C, Fieschi C, Thomassin N, Rosenzweig S, Harris J, et al. Impairment of mycobacterial but not viral immunity by a germline human STAT1 mutation. Science 2001;293:300-3.

E9. Hirata O, Okada S, Tsumura M, Kagawa R, Miki M, Kawaguchi H, et al. Heterozygosity for the Y701C STAT1 mutation in a multiplex kindred with multifocal osteomyelitis. Haematologica 2013 [Epub ahead of print].

E10. Kotlarz D, Zietara N, Uzel G, Weidemann T, Braun CJ, Diestelhorst J, et al. Loss-of-function mutations in the IL-21 receptor gene cause a primary immunodeficiency syndrome. J Exp Med 2013;210:433-43.