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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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

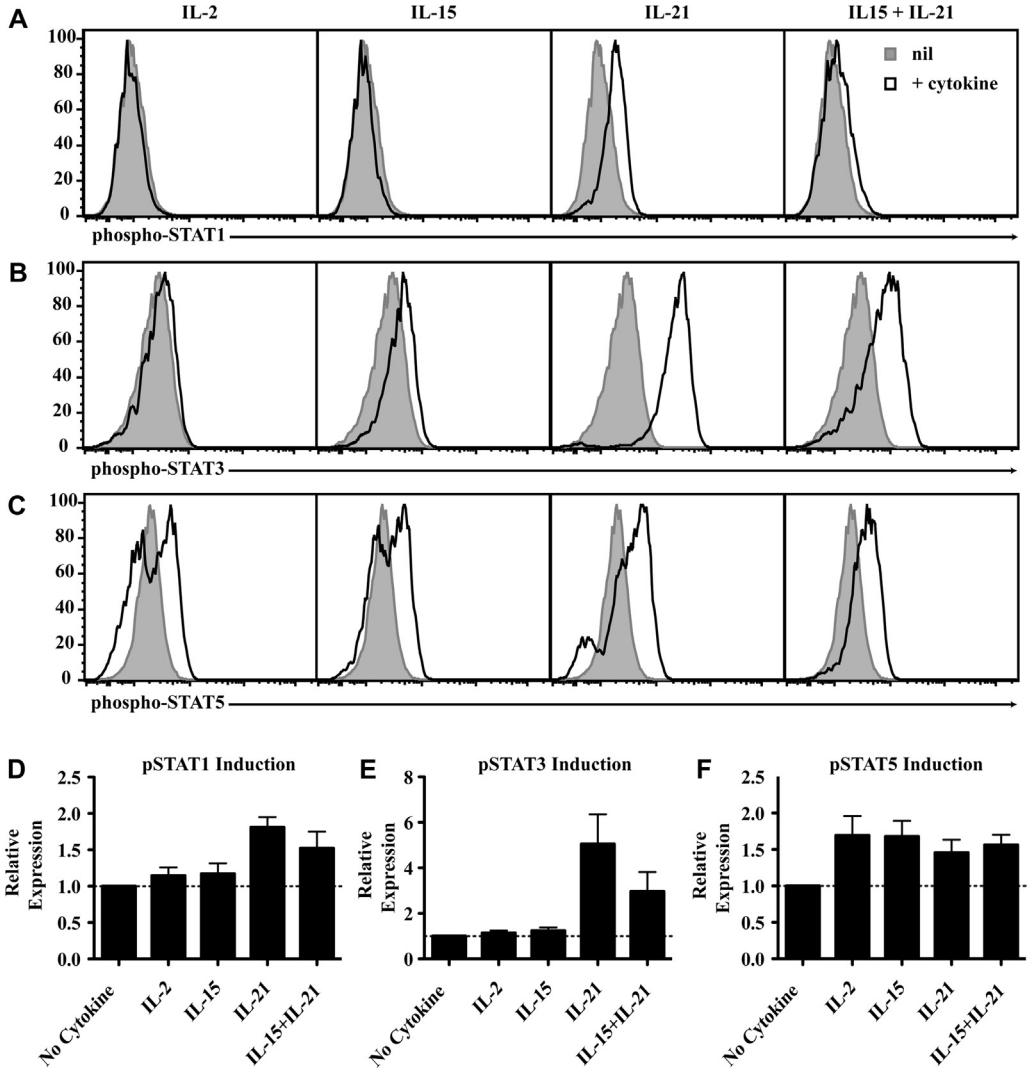

FIG 1. IL-21 predominately activates STAT1, STAT3, and STAT5 in human CD8$^+$ T cells. **A-C,** Naive CD8$^+$ T cells were activated for 4 days with TAE beads and recultured with cytokines for 30 minutes to determine phosphorylation of STAT1, STAT3, and STAT5. Histograms show nil or cytokine cultures 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-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.

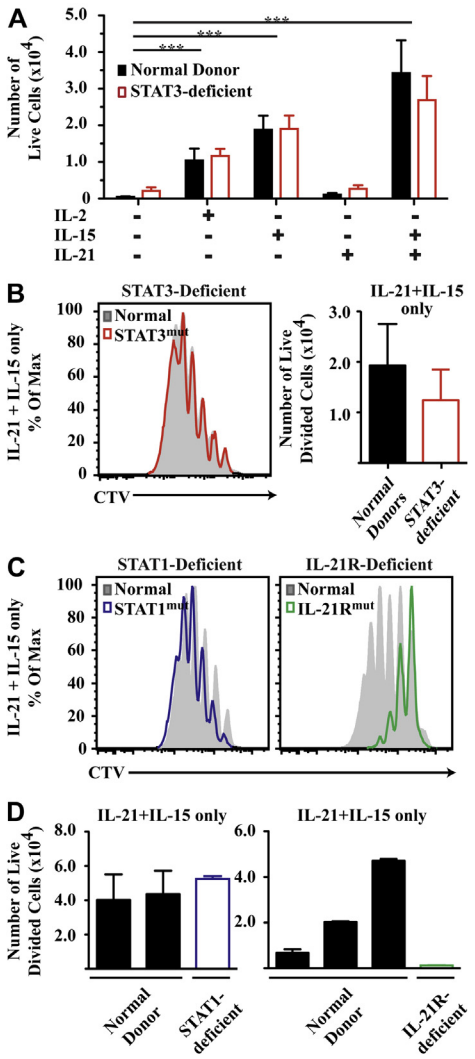

FIG 2. Cytokine-induced proliferation is impaired in IL-21R–deficient, but not STAT1- or STAT3-deficient, naive $CD8^+$ T cells. **A,** Naive $CD8^+$ T cells were cultured with cytokines only, and the numbers of live cells were determined (mean ± SEM, n = 5). **B** and **C,** Histograms show representative 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. ***$P < .001$.

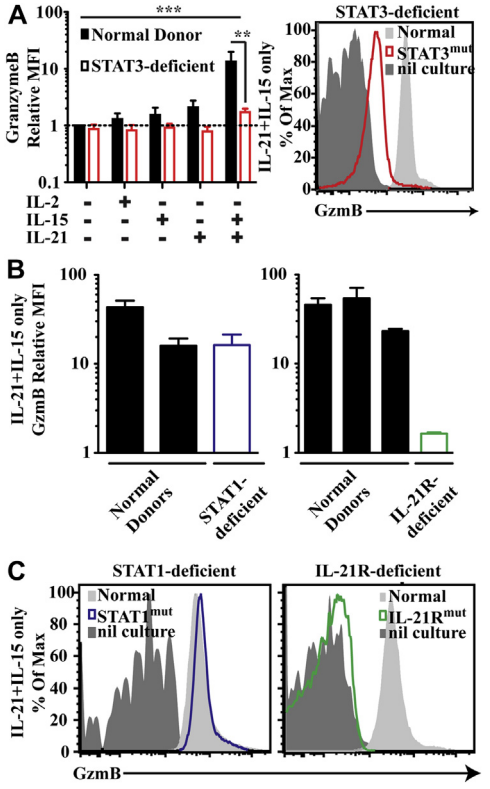

FIG 3. Cytokine-induced expression of granzyme B is impaired in IL-21R–deficient or STAT3-deficient naive $CD8^+$ T cells. **A** and **B,** Naive $CD8^+$ T cells were cultured with cytokines only, and graphs depict the fold increase (mean ± SEM, n = 5) in mean fluorescence intensity *(MFI)* of granzyme 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 $IL\text{-}21R^{MUT}$ (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

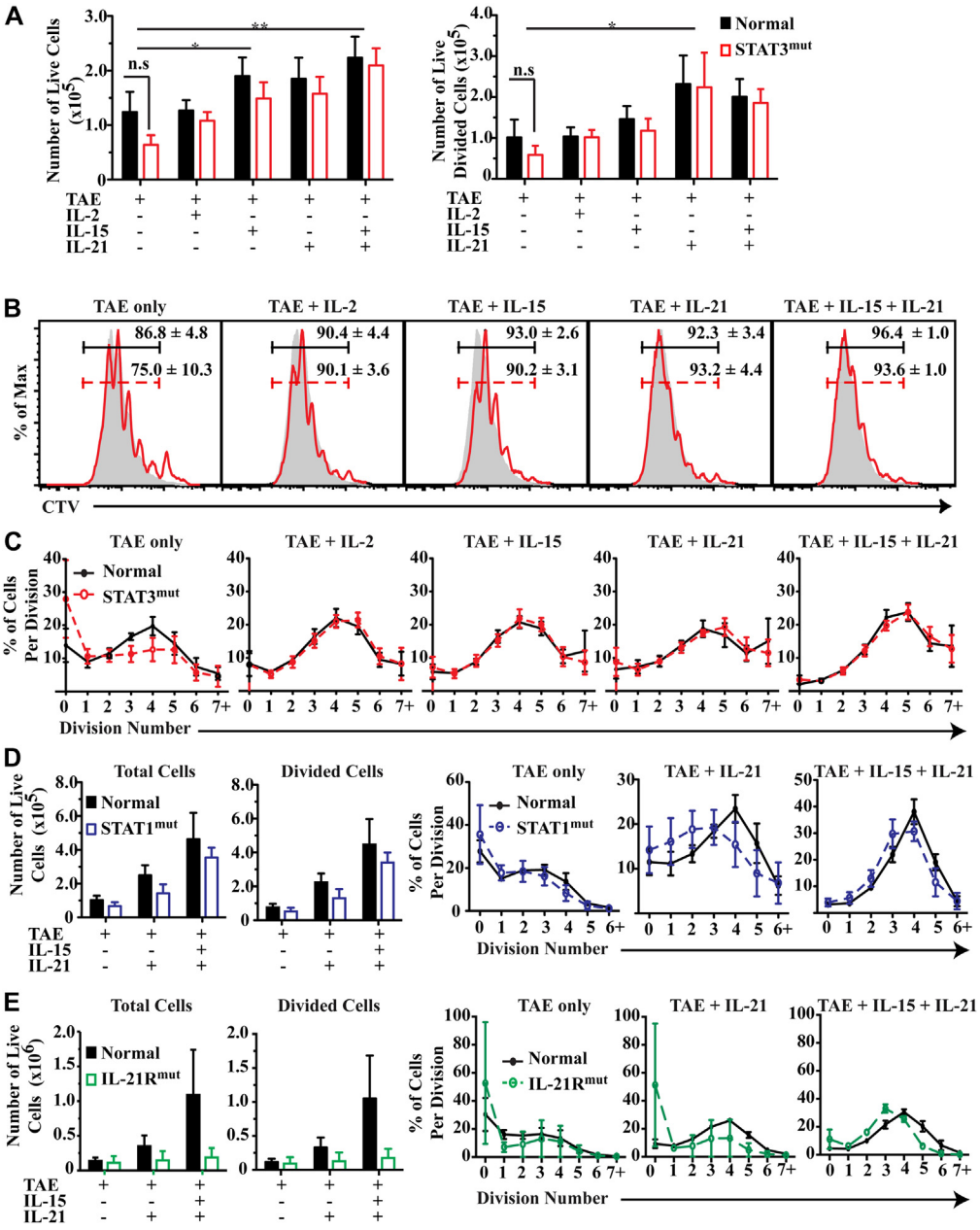

FIG 4. STAT3-deficient naive CD8$^+$ T cells proliferate normally in response to TCR engagement and activating cytokines. **A-E,** Naive CD8$^+$ T cells from healthy donors (n = 6-8 [Fig 4, *A-C*]; n = 3 [Fig 4, *D*]; and n = 5 [Fig 4, *E*]) and STAT3-deficient (Fig 4, *A-C*; n = 6-8), STAT1-deficient (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).

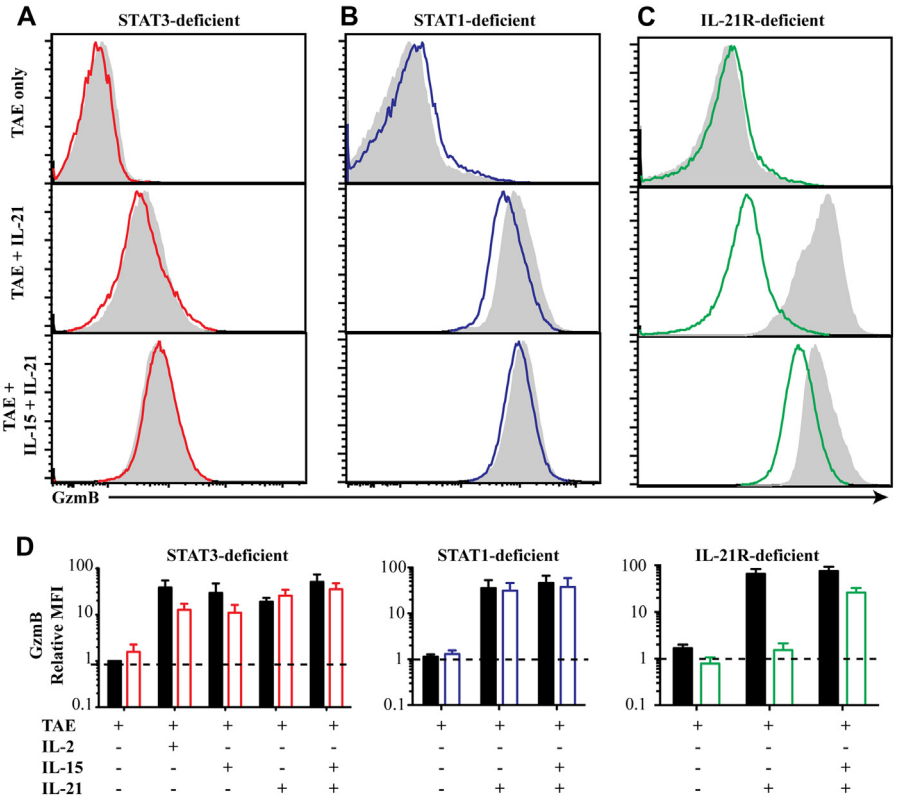

FIG 5. IL-21–induced granzyme B production is intact in TCR-stimulated STAT3-deficient CD8$^+$ T cells. **A-C,** Naive CD8$^+$ T cells from STAT3-deficient (n = 6-8; Fig 5, *A*), STAT1-deficient (n = 3; Fig 5, *B*), or IL-21R–deficient (n = 2; Fig 5, *C*) patients or healthy donors (n = 6-8) were cultured 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

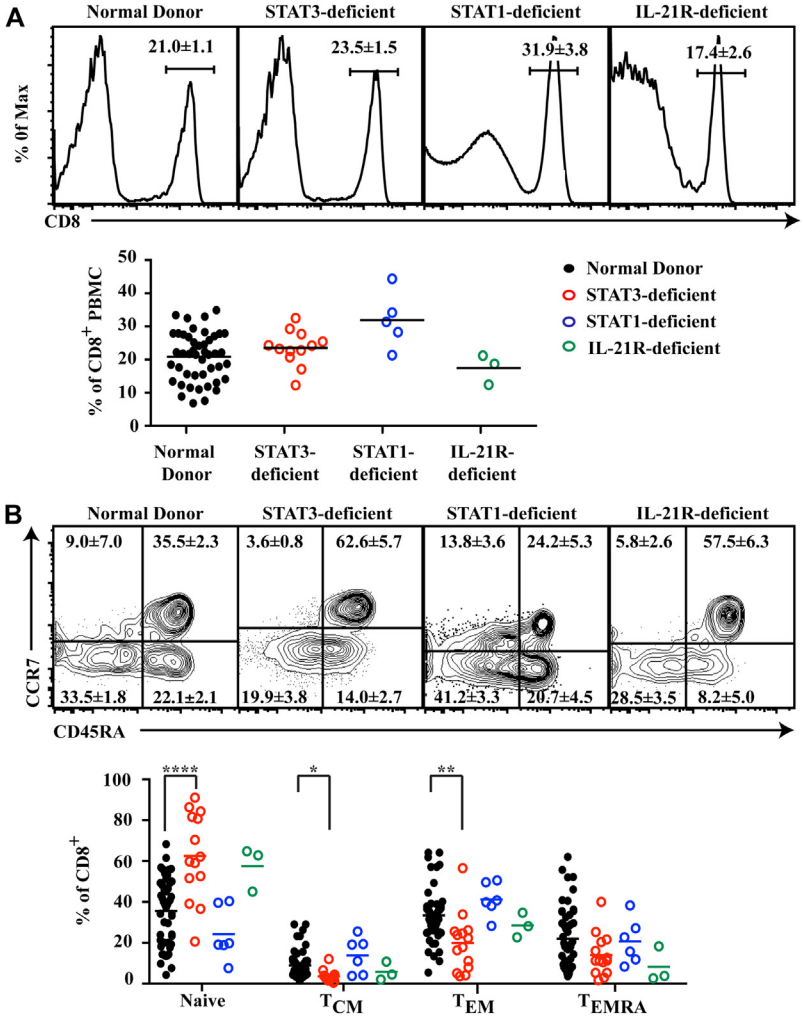

FIG 6. Mutations in *STAT3*, but not *STAT1*, impair generation of effector/memory $CD8^+$ T cells *in vivo*. PB from healthy donors (n = 46 or 51) or STAT3-deficient (n = 12-14), STAT1-deficient (n = 5 or 6), or IL-21R–deficient (n = 3) patients were assessed for the percentage of total $CD8^+$ T cells **(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

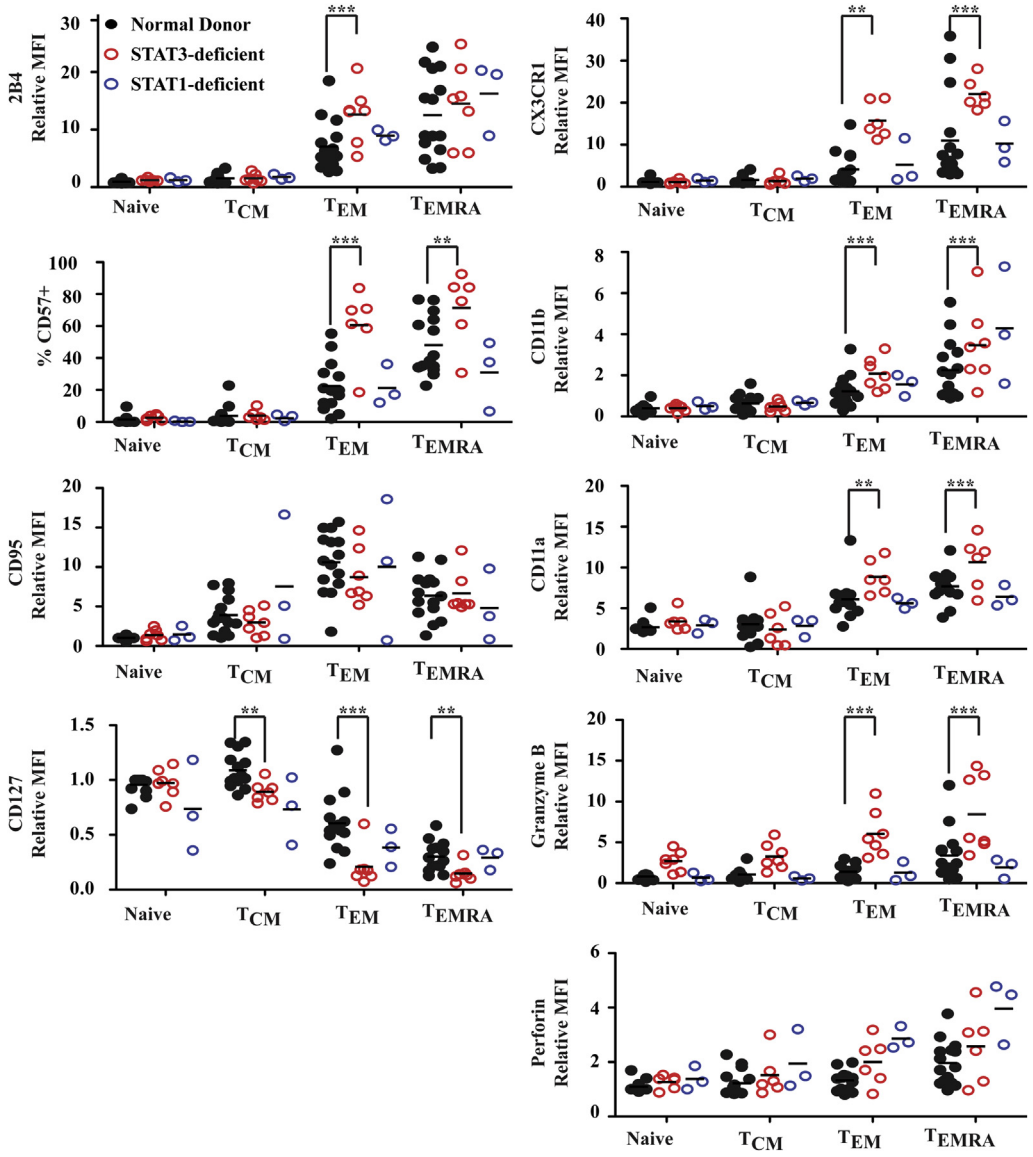

FIG 7. $CD8^+$ T cells from STAT3-deficient patients display a more activated phenotype. Subsets of naive, T_{CM}, T_{EM}, and T_{EMRA} $CD8^+$ T cells in PB of healthy donors (n = 15) or STAT3-deficient (n = 7) and STAT1-deficient (n = 3) patients were assessed for expression of 2B4, CD57, CD95, CD127, 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.**

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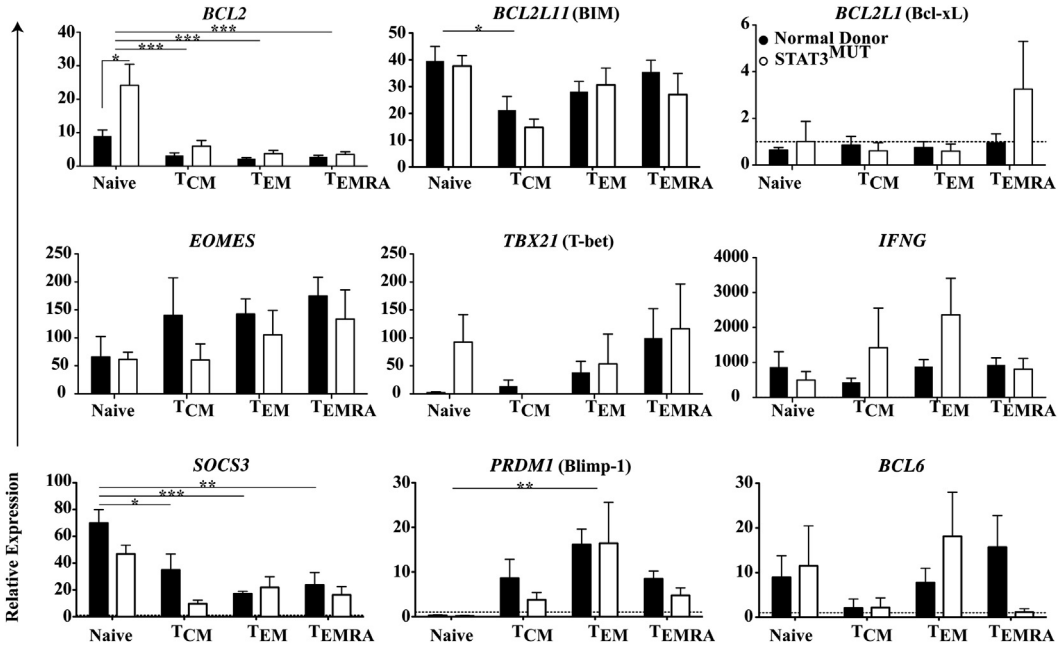

FIG E1. Differential expression of transcription factors and apoptotic family members in subsets of $CD8^+$ T cells. Expression of the proapoptotic regulator *BCL2* and the antiapoptotic regulators *BCL2L11* and *BCL2L1*; the transcription factors *EOMES*, *TBX21*, *SOCS3*, *PRDM1*, and *BCL6*; and the cytokine *IFNG* was determined in sorted purified subsets of $CD8^+$ T cells from normal donors and STAT3-deficient patients by means of quantitative PCR. The results are means ± SEMs (n = 5) and are expressed relative to glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*. **P* < .05, ***P* < .01, and ****P* < .001.

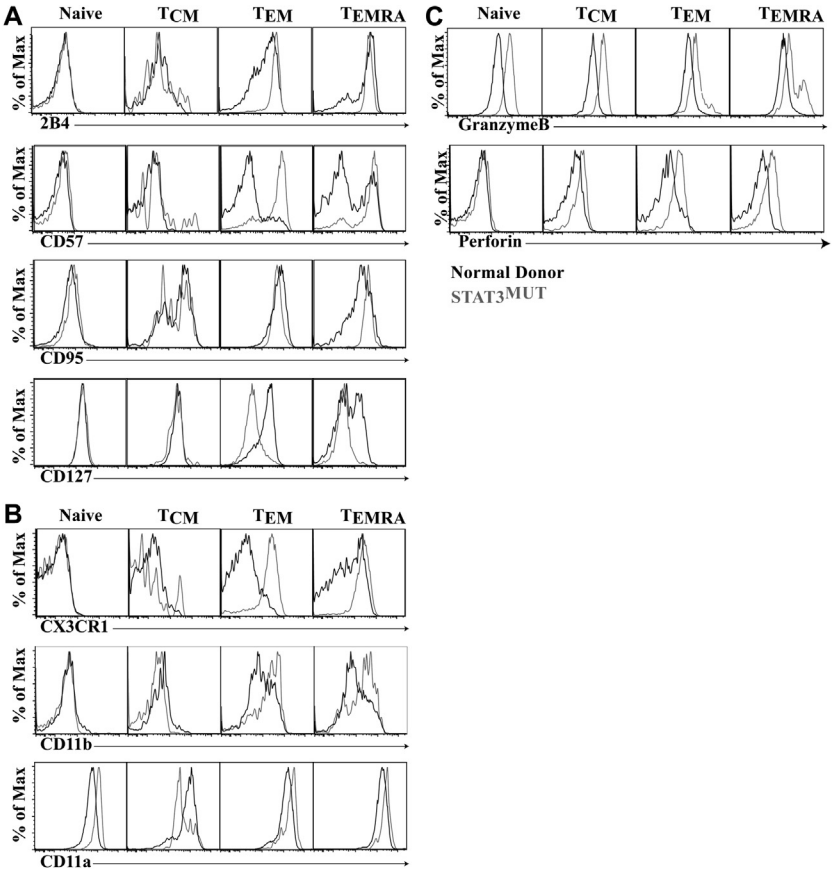

FIG E2. The *ex vivo* phenotype of STAT3-deficient $CD8^+$ T cells. **A-C,** Naive, T_{CM}, T_{EM}, and T_{EMRA} $CD8^+$ T cells in PB of normal donors *(black histogram)* and STAT3-deficient patients *(gray histogram)* were assessed for expression of 2B4, CD57, CD95, CD127, CX3CR1, CD11a, CD11b, granzyme B, or perforin. Histogram plots are from 1 representative healthy donor and 1 STAT3-deficient patient.

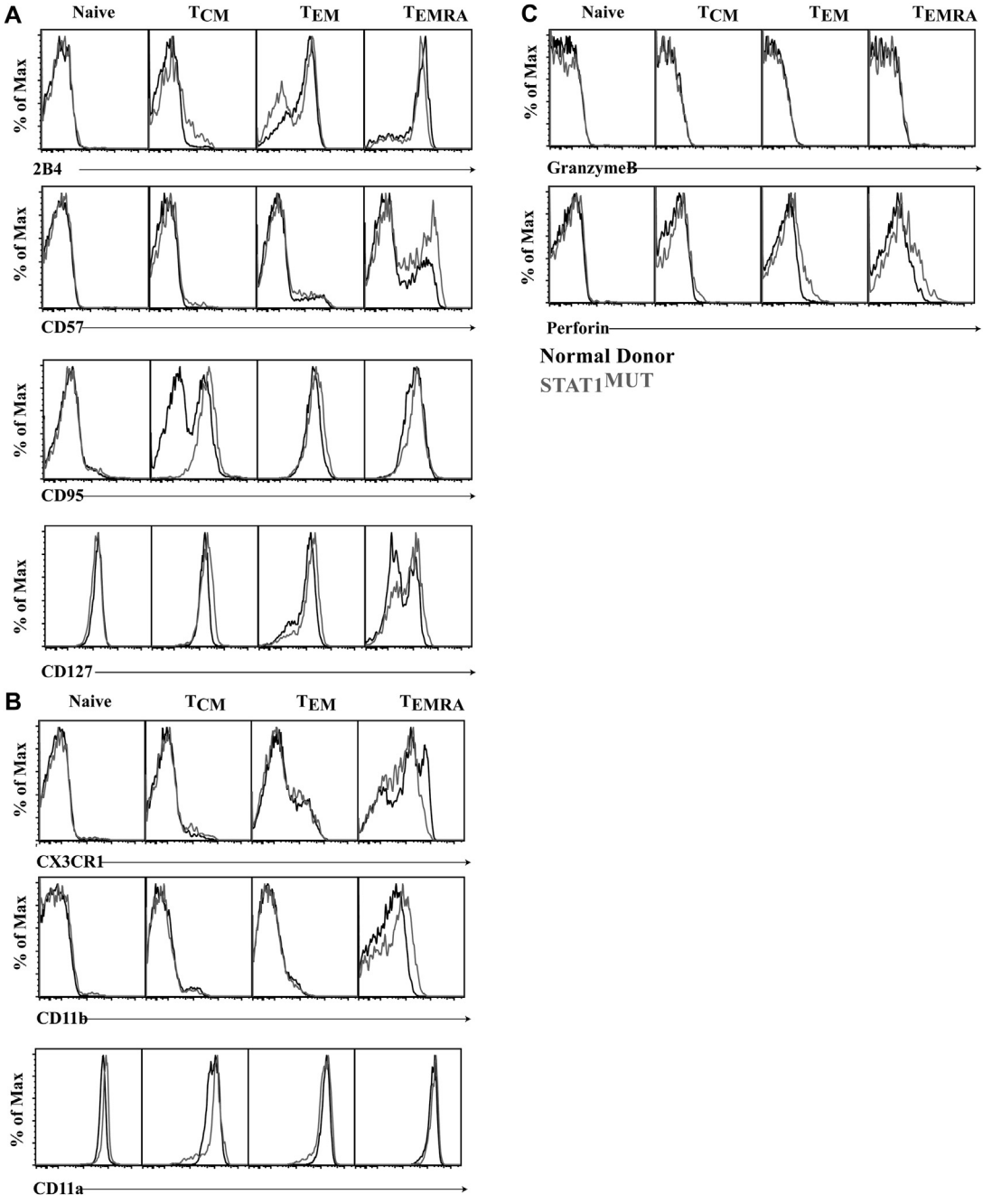

FIG E3. The *ex vivo* phenotype of STAT1-deficient $CD8^+$ T cells. **A-C,** Naive, T_{CM}, T_{EM}, and T_{EMRA} $CD8^+$ T cells in PB of normal donors *(black histogram)* and STAT1-deficient patients *(gray histogram)* were assessed for expression of 2B4, CD57, CD95, CD127, CX3CR1, CD11a, CD11b, granzyme B, or perforin. Histogram plots are from 1 representative healthy donor and 1 STAT1-deficient patient.

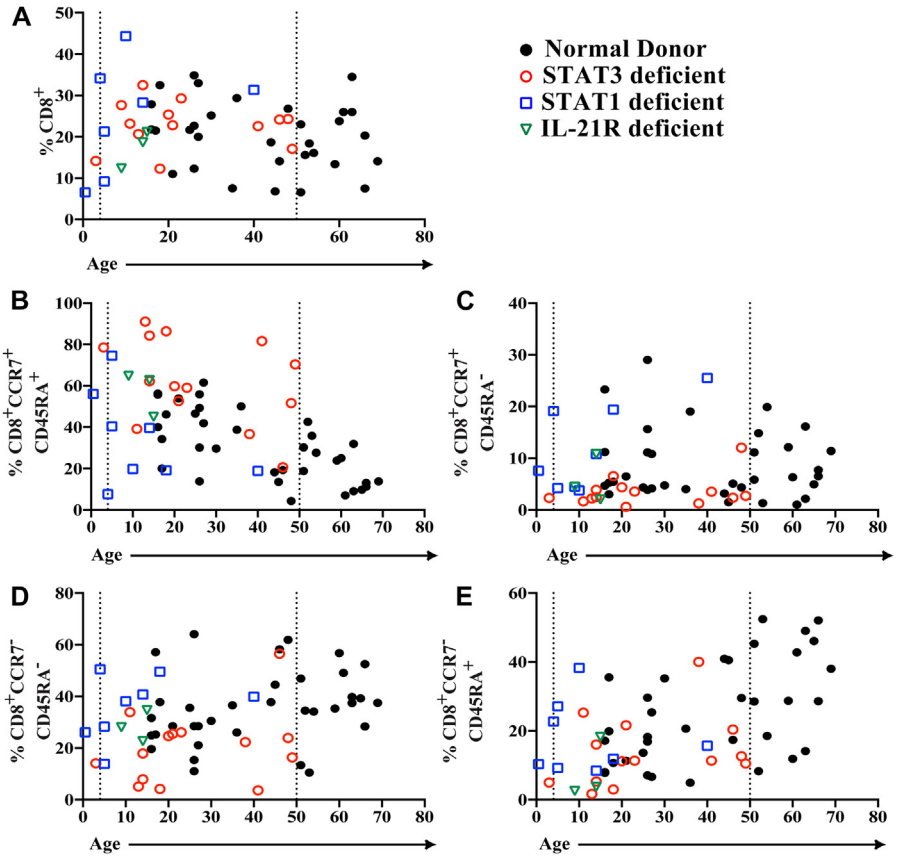

FIG E4. $CD8^+$ T-cell subsets in STAT3-deficient and STAT1-deficient patients. **A-E,** Percentages of $CD8^+$ (Fig E4, *A*), naive (Fig E4, *B*), T_{CM} (Fig E4, *C*), T_{EM} (Fig E4, *D*), and T_{EMRA} (Fig E4, *E*) cells are plotted against the age of each subject at the time the PB samples were analyzed from healthy donors (n = 31), STAT3-deficient patients (n = 14), STAT1-deficient patients (n = 8), or IL-21R–deficient patients (n = 3). Each value represents an individual donor or patient.

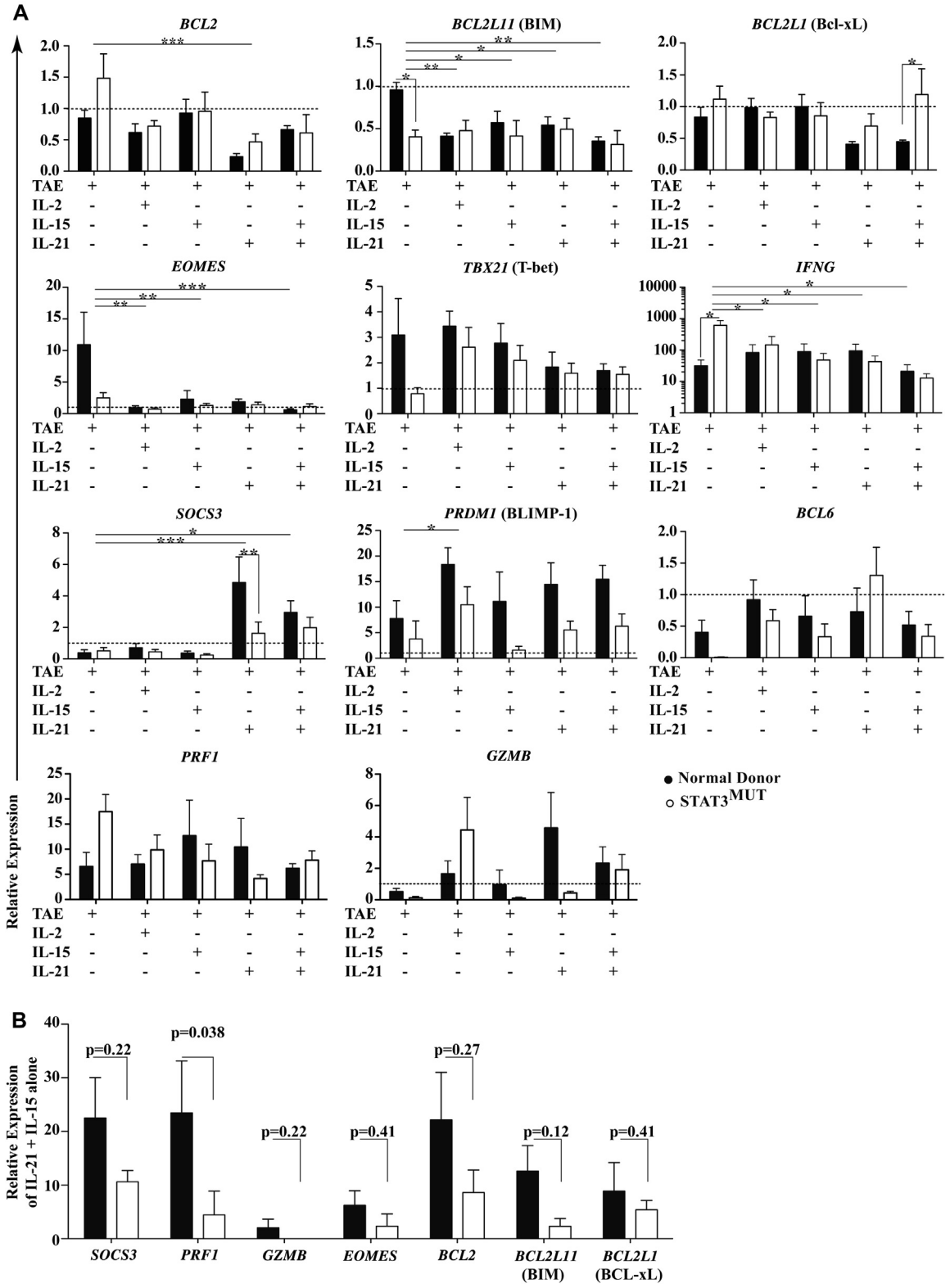

FIG E5. Differential expression of transcription factors and apoptotic family members in cultured naive $CD8^+$ T cells. Expression of the proapoptotic regulator *BCL2* and the antiapoptotic regulators *BCL2L11* and *BCL2L1*; the transcription factors *EOMES*, *TBX21*, *SOCS3*, *PRDM1*, and *BCL6*; and the effector molecules *IFNG*, *GZMB*, and *PFR1* was determined by means of quantitative PCR in naive $CD8^+$ T cells from normal donors and STAT3-deficient patients that were cultured for either 4 days with TAE beads in the absence or presence of the indicated cytokines **(A)** or for 10 days with IL-21 and IL-15 alone **(B)**. The results are means ± SEMs (n = 4-7) and expressed relative to glyceraldehyde-3-phosphate dehydrogenase *(GAPDH)*. $*P < .05$, $**P < .01$, and $***P < .001$.

TABLE E1. Patients with PIDs

Disease	Patient ID	Mutation/genotype	Age at analysis (y)	References
AD-HIES	HIES#1	R382Q	20	E1-E4
(STAT3 deficiency)	HIES#2	V637M	21	
(Heterozygous)	HIES#4	H437P	11	
	HIES#5	Q644P	46	
	HIES#6	S465F	13	
	HIES#7	Y657N	48	
	HIES#8	R382W	9	
	HIES#9+	L706M	23	
	HIES#10+	L706M	49	
	HIES#11	R382W	14	
	HIES#13*	V463Δ	18	
	HIES#14*	V463Δ	41	
	HIES#15	R539P	14	
	HIES#16	V463Δ	38	
	HIES#17	F621L	3	
MSMD + viral infection	$STAT1_{MUT}1$	1928insA (homozygous)	0.5	E5,E6
(STAT1 deficiency)	$STAT1_{MUT}2$†	P696S (homozygous)	18	
	$STAT1_{MUT}3$†	P696S (homozygous)	14	
MSMD only	$STAT1_{MUT}4$	Q463H/WT	10	E7,E8
	$STAT1_{MUT}5$	L706S/WT	5	
(STAT1 deficiency)	$STAT1_{MUT}9$	E320Q/WT	4	
	$STAT1_{MUT}10$‡	Y701C/WT	40	E9
	$STAT1_{MUT}11$‡	Y701C/WT	5	
IL-21R deficiency	$IL21R_{MUT}1$§	C81_H82del (homozygous)	9	E10
	$IL21R_{MUT}2$§	C81_H82del (homozygous)	14	E10
	$IL21R_{MUT}3$	W138S (homozygous)	15.5	Unpublished

MSMD, Mendelian susceptibility to mycobacterial disease.
+,*,†,‡,§Related subjects.

TABLE E2. Primary mAbs

mAb	Clone	Company
CCR7-FITC	150503	R&D Systems, Minneapolis, Minn
Phospho-STAT5–A488		BD, Franklin Lakes, NJ
CD11b-PE	D12	BD
CD45RO-PE	UCHL1	eBioscience, San Diego, Calif
CD57-PE	HCD57	BioLegend, San Diego, Calif
CD95-PE	DX2	BD
244-PE	2B4; C1.7	Beckman Coulter, Fullerton, Calif
Perforin-PE	dG9	eBioscience
Phospho-STAT3–PE		BD
CD45RA-PerCpCy5.5	HI100	eBioscience
CD8-PeCy7	RPA-T8	BD
CD28-PeCy7	CD28.2	BioLegend
CD11a-APC	HI111	BD
CD27-APC	O323	eBioscience
CX3CR1-APC	2A9-1	BioLegend
Granzyme B–APC	GB11	Invitrogen, Carlsbad, Calif
Phospho-STAT1–A647		BD
CD27-APC.Cy7	O323	BioLegend
CD8-Pacific Blue	RPA-T8	BD
CD4-e450	OKT-4	eBioscience
CD127-e450	eBioRDR5	eBioscience

APC, Allophycocyanin; *FITC*, fluorescein isothiocyanate; *PE*, phycoerythrin; *PerCP*, peridinin-chlorophyll-protein complex.

TABLE E3. Real-time PCR primers

Gene	Forward	Reverse
BCL2	5′-ttgacagaggatcatgctgtactt-3′	5′-atctttatttcatgaggcacgtt-3′
BCL2L11/BIM	5′-catcgcggtattcggttc-3′	5′-gctttgccatttggtcttttt-3′
BCL2L1/Bcl-xL	5′-tttctccttcggcggggcact-3′	5′-aaaagtatcccagccgccgttc-3′
BCL6	5′-gagctctgttgattcttagaactgg-3′	5′-gccttgcttcacagtccaa-3′
EOMES	5′-gtggggaggtcgaggttc-3′	5′-tgttctggaggtccatggtag-3′
GAPDH	5′-ctctgctcctcctgttcgac-3′	5′-acgaccaaatccgttgactc-3′
GZMB	5′-agatgcaaccaatcctgctt-3′	5′-catgtcccccgatgatct-3′
IFNG	5′-ggcattttgaagaattggaaag-3′	5′-tttggatgctctggtcatctt-3′
PRDM1/BLIMP-1	5′-aacgtgtgggtacgaccttg-3′	5′-attttcatggtccccttggt-3′
PRF1	5′-ccgcttctctatacgggattc-3′	5′-gcagcagcaggagaaggat-3′
SOCS3	5′-agacttcgattcgggacca-3′	5′-aacttgctgtgggtgacca-3′
TBX21/T-BET	5′-tgtggtccaagtttaatcagca-3′	5′-tgacaggaatgggaacatcc-3′