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**Background: Severe combined immunodeficiency (SCID) comprises rare inherited disorders of immunity that require definitive treatment through hematopoietic cell transplantation (HCT) or gene therapy for survival. Despite successes of allogeneic HCT, many SCID patients experience incomplete immune reconstitution, persistent T-cell lymphopenia, and poor long-term outcomes.**

**Objective: We hypothesized that $CD4^+$ T-cell lymphopenia could be associated with a state of T-cell exhaustion in previously transplanted SCID patients.**

**Methods: We analyzed markers of exhaustion in blood samples from 61 SCID patients at a median of 10.4 years after HCT.**

**Results: Compared to post-HCT SCID patients with normal $CD4^+$ T-cell counts, those with poor T-cell reconstitution showed lower frequency of naive $CD45RA^+/CCR7^+$ T cells, recent thymic emigrants, and TCR excision circles. They also had a restricted TCR repertoire, increased expression of inhibitory receptors (PD-1, 2B4, CD160, BTLA, CTLA-4), and increased activation markers (HLA-DR, perforin) on their total and naive $CD8^+$ T cells, suggesting T-cell exhaustion and**

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**aberrant activation, respectively. The exhaustion score of CD8$^+$ T cells was inversely correlated with CD4$^+$ T-cell count, recent thymic emigrants, TCR excision circles, and TCR diversity. Exhaustion scores were higher among recipients of unconditioned HCT, especially when further in time from HCT. Patients with fewer CD4$^+$ T cells showed a transcriptional signature of exhaustion.**

**Conclusions: Recipients of unconditioned HCT for SCID may develop late post-HCT T-cell exhaustion as a result of diminished production of T-lineage cells. Elevated expression of inhibitory receptors on their T cells may be a biomarker of poor long-term T-cell reconstitution. (J Allergy Clin Immunol 2023;151:260-71.)**

***Key words:*** *Conditioning chemotherapy, hematopoietic cell transplantation (HCT), immune reconstitution, severe combined immunodeficiency (SCID), T-cell exhaustion*

*Abbreviations used*

DE: Differently expressed
FDR: False discovery rate
GvHD: Graft-versus-host disease
HCT: Hematopoietic cell transplantation
IR: Inhibitory receptor
IS: Immunosuppression
MAC: Myeloablative conditioning
MMRD: Mismatched related donor
PD-1: Programmed cell death 1
PIDTC: Primary Immune Deficiency Treatment Consortium
RIC: Reduced-intensity conditioning
RTE: Recent thymic emigrant
SCID: Severe combined immunodeficiency
TREC: TCR excision circle

SCID is a rare but life-threatening inborn error of immunity encompassing at least 14 monogenic diseases that result in defective T-cell development.[1,2] Both cellular and humoral immunity are compromised, leading to fatal infections in infancy unless effective immunity can be established. Currently, allogeneic hematopoietic cell transplantation (HCT) remains the standard treatment for SCID. While HCT has dramatically increased survival,[3,4] up to 30% of SCID patients have incomplete immune reconstitution after HCT, remaining at increased risk for recurrent infections and autoimmunity.[5] Although low T-cell numbers after HCT correlate with reduced overall survival,[5-8] little is known of qualitative donor T-cell defects in transplanted SCID patients. We hypothesized that poor engraftment of hematopoietic stem cells could lead to insufficient T-cell reconstitution in a subset of transplanted SCID patients; in this setting, poor T-cell output could be associated with T-cell exhaustion.

Exhaustion is a T-cell differentiation state in which T cells become progressively unable to provide robust sterilizing immunity as a result of diminished renewal capacity and defective effector functions, including reduced cytotoxicity, impaired cytokine production, and poor antigen-specific proliferation.[9,10] Exhausted T cells are identified by expression of inhibitory receptors (IRs) and a specific transcriptional signature.[11,12] Although T-cell exhaustion has been described largely in the context of chronic viral infections[13-16] and cancer,[17-20] this phenomenon is increasingly recognized in patients who have undergone HCT for malignant diseases.[21-23] Several factors may render T cells susceptible to exhaustion after HCT, such as chronic T-cell lymphopenia[24,25] or the presence of minor and/or major histocompatibility alloantigens.[26-28]

This study addressed whether inadequate T-cell reconstitution in SCID patients at least 2 years after HCT could be associated with T-cell exhaustion and explored factors promoting T-cell exhaustion.

## METHODS

### Study participants

Our cohort consisted of individuals who had been followed for >2 years after allogeneic HCT for SCID at a participating Primary Immune Deficiency Treatment Consortium (PIDTC) institution before January 2020. Patients were excluded if they had acute or chronic graft-versus-host disease (GvHD) or if they had PCR-confirmed cytomegalovirus, Epstein-Barr virus, or adenovirus infection in the 6 months preceding sample collection, as GvHD and DNA viral infections are known drivers of T-cell exhaustion.[29] Patient and HCT details are listed in Table I; post-HCT details are provided in Table E1 in this article's Online Repository (available at www.jacionline.org). Poor immune reconstitution at least 2 years after HCT was defined as CD4$^+$ T cells <500 cells/mm$^3$, while low CD3$^+$ T-cell number was defined as <1000 cells/mm$^3$. Conditioning regimens were categorized as none, immunosuppression (IS, serotherapy alone or combined with fludarabine or cyclophosphamide), reduced-intensity conditioning (RIC; melphalan, anti-CD45, total-body irradiation of 200-400 cGy, or total busulfan dose <12 mg/kg), and myeloablative conditioning (MAC; total busulfan dose ≥12 mg/kg), as previously published.[3] For analysis, the conditioning regimens were separated into 2 categories: None/IS and RIC/MAC. Blood was also obtained from healthy volunteers matched in age to the oldest patients of our cohort (age range, 19-28 years; n = 13).

### Study approval

Subjects were recruited via written informed consent through institutional review board–approved protocols of the PIDTC under studies NCT01186913 and NCT01346150 (ClinicalTrials.gov).

### Flow cytometry immunophenotyping

Immunophenotyping was performed on whole blood shipped overnight at room temperature. Cells were stained within 24 hours of blood procurement with monoclonal antibodies (see Table A in the Online Repository at www.jacionline.org) and then treated with FACS Lysing Solution 1× (BD Biosciences, San Jose, Calif) for 10 minutes at room temperature before flow cytometry. Intracellular staining was performed on cells fixed with Cytofix/Cytoperm (BD Biosciences) according to the manufacturer's instructions. Flow cytometry was performed (LSRFortessa II, BD Biosciences), with data analyzed by FlowJo 9.7.6 software (Treestar, Ashland, Ore). Gates were set using fluorescence minus one controls.

### T-cell repertoire diversity

T-cell receptor (TCR) excision circles (TRECs) and polyclonal Vβ TCR analyses were performed at the PIDTC core lab (UCSF Department of Pediatrics) at the same time points as exhaustion samples using methods described previously.[30,31] Briefly, DNA extracted from dried blood spots was used for quantitative PCR to yield TREC copy number, and total RNA extracted from peripheral blood mononuclear cell was used to amplify 24 TCR Vβ families to classify TCR repertoire by spectratyping.

### T-cell exhaustion score

The $z$ scores relative to the mean of the control group for each IR (programmed cell death 1 [PD-1], 2B4, CD160) were calculated by the following formula:

$$\frac{\%\ IR\ patient - Mean\ \%\ IR\ Ctrl}{SD\ \%\ IR\ Ctrl}.$$

Each patient's total exhaustion score was the sum of the individual IR $z$ scores.

### Cell sorting, library preparation, and RNA sequencing

Total and naive $CD8^+$ T-cell populations were obtained for RNA sequencing from 9 patients with *IL2RG*- and 1 with *JAK3*-deficient SCID, who had received HCT from mismatched related donors (MMRDs; 3 patient samples from the initial cohort and 6 additional samples; see the Methods in the Online Repository at www.jacionline.org, along with Table B and Table E2 in the Online Repository at www.jacionline.org).

### Statistical analysis

Statistical significance was determined by Wilcoxon-Mann-Whitney or Kruskal-Wallis test to compare groups for variables that did not follow a normal distribution. Analyses were performed by ABI Prism 6 software (GraphPad Software, La Jolla, Calif), with data displayed as means ± SEMs. Correlation coefficients for normal variables were calculated by Pearson correlation. Univariate and multivariable analyses were conducted by linear regression to examine whether genotype, HCT product, donor type, or conditioning regimen was associated with exhaustion. Significance was set as $*P < .05$, $**P < .01$, $***P < .001$, and $****P < .0001$.

## RESULTS

### Patient and transplant characteristics

Of 69 patients from whom blood samples were obtained, data from 61 were analyzed. Two samples from patients with recent chronic GvHD were excluded, as were 6 samples with shipping or processing problems (see Table E3 in the Online Repository at www.jacionline.org). The median patient age at HCT was 5.8 months (Table I), with 41% having undergone HCT before 3.5 months of age. Blood samples were collected and analyzed at a median time of 10.4 years (range, 2-28.8 years) after HCT. Patients were grouped by genotype and phenotypic profile (Table I), with the most common grouping being $T^-B^+NK^-$ *IL2RG/JAK3* (51%), followed by $T^-B^-NK^+$ *RAG1/RAG2* (13%) defects. There were 5 patients with $T^-B^+NK^+$ *IL7R*/CD3/CD45 defects, 5 with radiation-sensitive *DCLRE1C* defects, 3 with *ADA* deficiency, 3 with cartilage hair hypoplasia, 1 with *ZAP70* deficiency, and 5 $T^-B^+NK^+$ defects with unknown genotypes. In this study, 88% of patients had received a single HCT, and 69% had received bone marrow. Fifty-one percent (51%) of patients received MMRD grafts, and only 33% received either RIC or MAC. Posttransplantation autoimmunity, acute GvHD, and resolved chronic GvHD occurred in 16%, 26%, and 13% of patients, respectively (Table E1).

**TABLE I.** Patient and HCT characteristics

| Characteristic | Value |
|---|---|
| No. of subjects | 61 |
| Age at HCT | |
| ≤3.5 months | 25 (41) |
| >3.5 months | 36 (59) |
| Median (range) (months) | 5.8 (0.3-109.6) |
| Time of blood sample after HCT (months), median (range) | 124.8 (24-346) |
| Genotype grouping, phenotype profile | |
| *IL2RG/JAK3*, $T^-B^+NK^-$ | 31 (51) |
| *RAG1/RAG2*, $T^-B^-NK^+$ | 8 (13) |
| *DCLRE1C*, $T^-B^-NK^+$, radiation sensitive | 5 (8) |
| *IL7R*, CD3, CD45, $T^-B^+NK^+$ | 5 (8) |
| *ADA*, $T^-B^-NK^-$ | 3 (5) |
| *RMRP*, cartilage hair hypoplasia | 3 (5) |
| *ZAP70* | 1 (2) |
| Unknown or not tested, $T^-B^+NK^+$ | 5 (8) |
| SCID therapy received before definitive HCT | |
| None | 54 (88) |
| Enzyme replacement therapy | 2 (3) |
| 1 HCT | 4 (7) |
| 2 HCTs | 1 (2) |
| Product type | |
| Bone marrow | 42 (69) |
| Peripheral blood $CD34^+$ cells | 11 (18) |
| UCB | 7 (11) |
| Bone marrow plus UCB | 1 (2) |
| Graft type | |
| MMRD | 31 (51)*† |
| MRD | 11 (18) |
| MUD | 8 (13) |
| Unrelated UCB | 7 (11) |
| MORD | 3 (5) |
| MMUD | 1 (2) |
| Conditioning regimen | |
| None | 32 (52) |
| IS | 9 (15) |
| RIC | 9 (15) |
| MAC | 11 (18) |
| Degree of compatibility | |
| Mismatch | 37 (61)‡ |
| Match | 24 (39) |

Data are presented as numbers (%) unless otherwise indicated.
*ADA*, Adenosine deaminase; *DCLRE1C*, DNA cross-link repair 1C; *IL2RG*, IL-2 receptor gamma; *IL7R*, IL-7 receptor; *JAK3*, Janus kinase 3; *MMUD*, mismatched unrelated donor; *MORD*, matched other related donor; *MRD*, matched related donor; *MUD*, matched unrelated donor; *RAG*, recombinase activating gene; *RMRP*, RNA component of mitochondrial RNA processing endoribonuclease; *UCB*, umbilical-cord blood; *ZAP70*, zeta chain of TCR-associated protein kinase 70kDa.
*Only 2 patients received a conditioning regimen: 1 RIC and 1 MAC.
†For ≤4/8 graft, n = 13, >4/8 graft, n = 18.
‡For ≤4/8 graft, n = 15.

### Poor T-cell reconstitution correlated with low thymic output and restricted T-cell diversity

Low $CD4^+$ T-cell counts in the first 2 years after HCT are known to predict reduced event-free and overall survival and compromised cellular immune reconstitution.[5,8,32] To identify immunological markers associated with poor T-cell reconstitution, we separated our cohort according to T-cell number, defining poor T-cell reconstitution as <500 $CD4^+$ T cells/$mm^3$ two years or more after HCT.[3] There were 24 patients (39%) with low $CD3^+$ counts (median 654 $CD3^+$ cells/$mm^3$, range 144-994 cells/$mm^3$ vs normal median 1628 cells/$mm^3$, range 1100-5306 cells/$mm^3$), and 29 (48%) with low $CD4^+$ counts (median 283 $CD4^+$ cells/$mm^3$, range 44-474 cells/$mm^3$ vs normal median 885 cells/$mm^3$, range 528-2700 cells/$mm^3$) (Fig 1, *A*). We evaluated the relative composition of various T-cell subsets, including

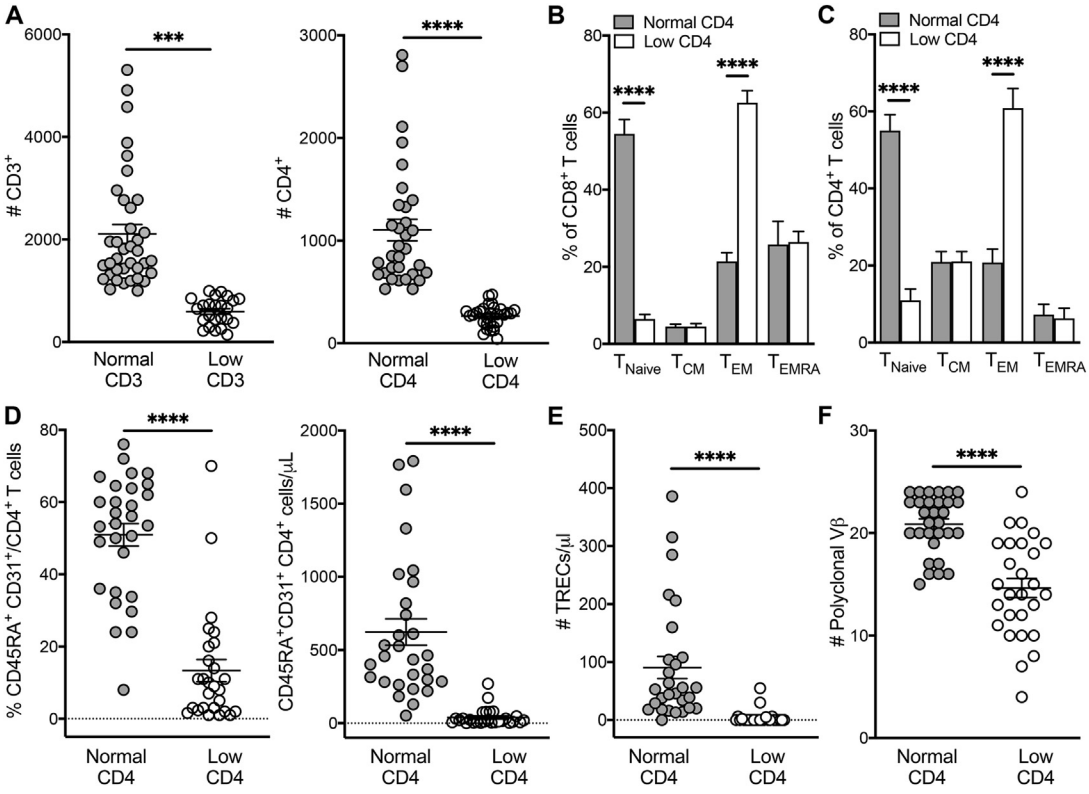


**FIG 1.** Low $CD4^+$ counts after HCT were correlated with few naive T cells, reduced RTEs, and restricted T-cell diversity. **(A)** Patients were separated into those with normal (n = 37) and low (n = 24) absolute $CD3^+$ T-cell counts *(left)* and those with normal (n = 32) and low (n = 29) absolute $CD4^+$ T-cell counts *(right)*. **(B** and **C)** Frequency of $CD8^+$ *(B)* and $CD4^+$ *(C)* T-cell subsets in normal $CD4^+$ (n = 32) and low $CD4^+$ (n = 29) groups. T-cell populations are indicated as follows: $T_{Naive}$, naive T cells ($CD45RA^+/CCR7^+$); $T_{CM}$, central memory T cells ($CD45RA^-/CCR7^+$); $T_{EM}$, effector memory T cells ($CD45RA^-/CCR7^-$); and $T_{EMRA}$, effector memory T cells reexpressing CD45RA ($CD45RA^+/CCR7^-$). **(D)** RTE $CD4^+$ T-cell ($CD45RA^+$ $CD31^+/CD4^+$) frequency *(left)* and counts *(right)* among the normal $CD4^+$ (n = 29) and low $CD4^+$ (n = 27) groups. **(E)** TRECs in normal $CD4^+$ (n = 28) and low $CD4^+$ (n = 27) groups. **(F)** Number of polyclonal Vβ TCR peaks in spectratype analysis of normal $CD4^+$ (n = 30) and low $CD4^+$ (n = 27) patients. Error bars indicate means ± SEMs. Statistical significance was assessed by Wilcoxon-Mann-Whitney test. $*P < .05$, $**P < .01$, $***P < .001$, $****P < .0001$.

naive ($T_{Naive}$, $CD45RA^+/CCR7^+$), central memory ($T_{CM}$, $CD45RA^-/CCR7^+$), effector memory ($T_{EM}$, $CD45RA^-/CCR7^-$), and effector memory cells reexpressing CD45RA, aka EMRA ($T_{EMRA}$, $CD45RA^+/CCR7^-$) T cells. The subjects with low $CD4^+$ T-cell counts had far fewer naive $CD8^+$ and $CD4^+$ T cells than subjects with normal $CD4^+$ T-cell numbers after HCT, with a shift favoring $T_{EM}$ cells (Fig 1, *B* and *C*). Similarly, proportions and absolute counts of recent thymic emigrants (RTEs; $CD45RA^+$ $CD31^+/CD4^+$) as well as TRECs were lower in the low $CD4^+$ T-cell group (Fig 1, *D* and *E*), indicating reduced thymic output in these patients. In contrast to patients with low $CD4^+$ T-cell counts, patients with normal $CD4^+$ T-cell counts had increased numbers of polyclonal Vβ TCR peaks in spectratype analysis (Fig 1, *F*), indicating that better T-cell reconstitution was correlated with higher TCR diversity.

## Poor T-cell reconstitution was associated with increased expression of IRs and an activated T-cell state

Presence of T-cell IRs (PD-1, 2B4, CD160, TIGIT, BTLA, CTLA-4) and surface markers associated with T-cell activation (CD27, CD38, CD39, HLA-DR) were assessed.[10,33-35] Compared to healthy controls and patients with normal $CD4^+$ T-cell counts, patients with low $CD4^+$ T cells exhibited markedly increased frequency of $CD8^+$ T cells expressing 2B4 and CD160, as well as more cells expressing BTLA and CTLA-4 (Fig 2, *A*); 2B4 and CD160 expression were inversely correlated with the number of both $CD4^+$ T cells and naive $CD4^+$ T cells (Fig E1 in the Online Repository available at www.jacionline.org). Moreover, while fewer $CD8^+$ T cells expressed CD27 and CD38, the frequency of $HLA\text{-}DR^+/CD8^+$ T cells was increased in patients with poor T-cell reconstitution compared to patients with low $CD4^+$ T cells or controls (Fig 2, *B*). Again, changes in the expression of activation markers correlated with $CD4^+$ and RTE counts (Fig E1). $CD8^+$ T-cell perforin expression was significantly more frequent in patients with low $CD4^+$ T cells, a phenomenon observed with terminal exhaustion (Fig 2, *C*).[36] The pattern of expression of IRs and activation markers on $CD4^+$ T cells largely resembled that seen on $CD8^+$ T cells (Fig 2, *D* and *E*).

Because IRs are preferentially expressed on differentiated $CD8^+$ T cells[37] and patients with poor T-cell reconstitution had an effector memory phenotype, we analyzed the presence of these

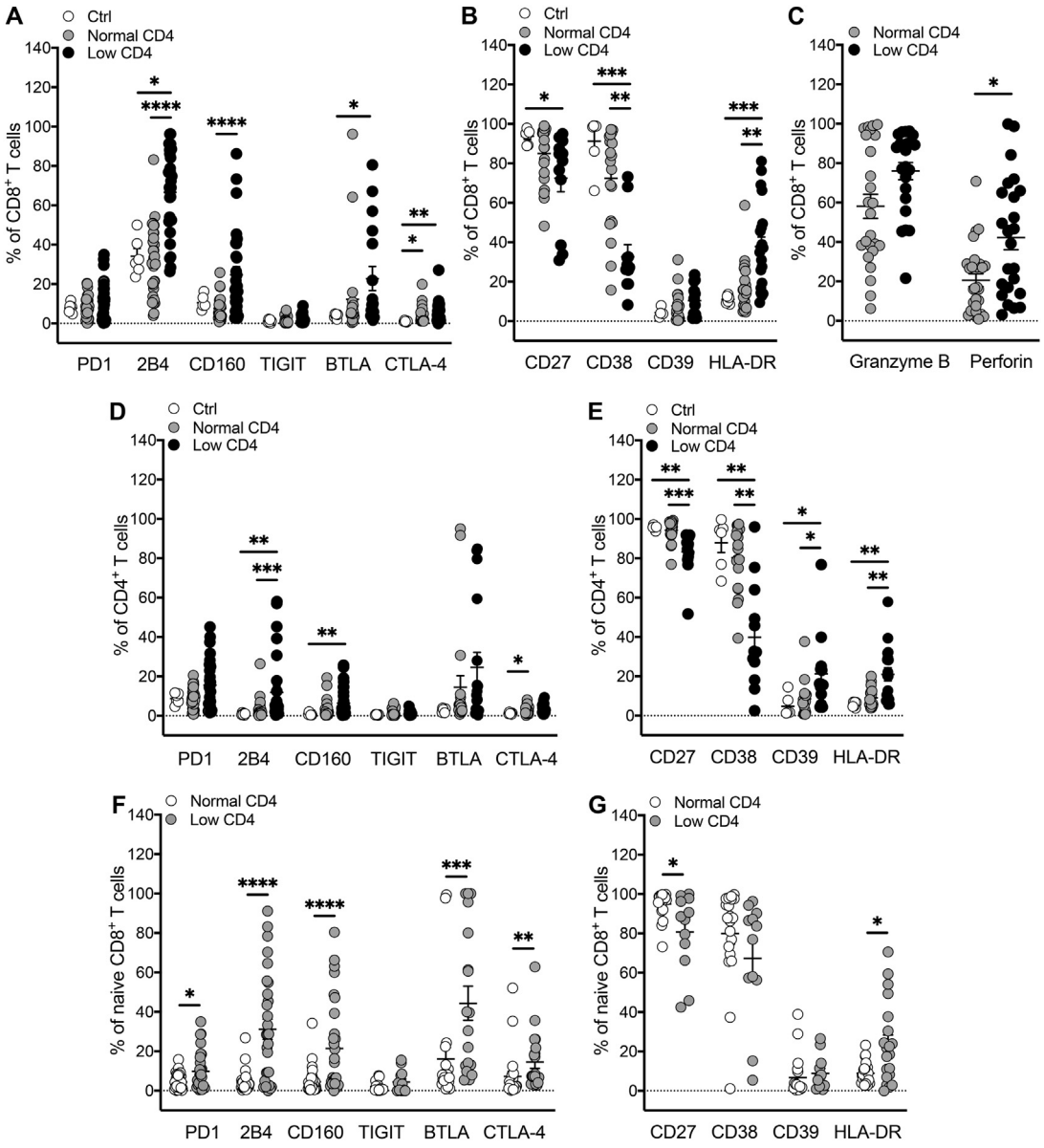


**FIG 2.** Poor T-cell reconstitution was associated with increased expression of IRs and an activated T-cell state, even within naive cells. **(A)** Frequency of the indicated IRs and **(B)** activation markers on $CD8^+$ T cells in healthy controls (n = 6) and patients with normal (n = 20-32) or low (n = 12-29) $CD4^+$ T cells. **(C)** Expression of intracellular granzyme B and perforin in $CD8^+$ T cells from patients with normal (n = 27) or low (n = 24) $CD4^+$ T cells. **(D)** Frequency of the indicated IRs and **(E)** activation markers on $CD4^+$ T cells in healthy controls (n = 6) and patients with normal (n = 20-32) or low $CD4^+$ T cells (n = 13-29). **(F)** Expression of the indicated IRs and (**G**) activation markers on $CD8^+$ $T_{Naive}$ cells in patients with normal (n = 17-32) or low (n = 12-29) $CD4^+$ T cells. Error bars indicate means ± SEMs. Statistical significance was assessed by Kruskal-Wallis test *(A, B, D, E)* or Wilcoxon-Mann-Whitney *(C, F, G)*. *$P < .05$, **$P < .01$, ***$P < .001$, ****$P < .0001$.

same receptors on naive $CD45RA^+$ $CCR7^+$ $CD8^+$ T cells to circumvent potential bias. Strikingly, with the exception of TIGIT, all IRs were highly expressed on naive $CD8^+$ T cells from the low $CD4^+$ T-cell group, in contrast to the normal $CD4^+$ T-cell group, confirming true diverging patterns of IR expression in these 2 subsets of patients after HCT (Fig 2, *F*). Similarly, activation markers remained distinct on naive $CD8^+$ T cells, with more cells expressing HLA-DR and fewer expressing CD27 in the low $CD4^+$ T-cell group (Fig 2, *G*). This contrasted with the expression observed on other cell subsets, where most IR and activation expression were comparable between groups, except on $T_{EMRA}$ cells, where 2B4, CD160, and HLA-DR was increased in the low $CD4^+$ T-cell group (Fig E2 in the Online Repository available at www.jacionline.org). Taken together, in our cohort, poor immune reconstitution after HCT coincided with a dysregulated pattern of expression of inhibitory and activation molecules, consistent with a state of aberrant T-cell activation and exhaustion.

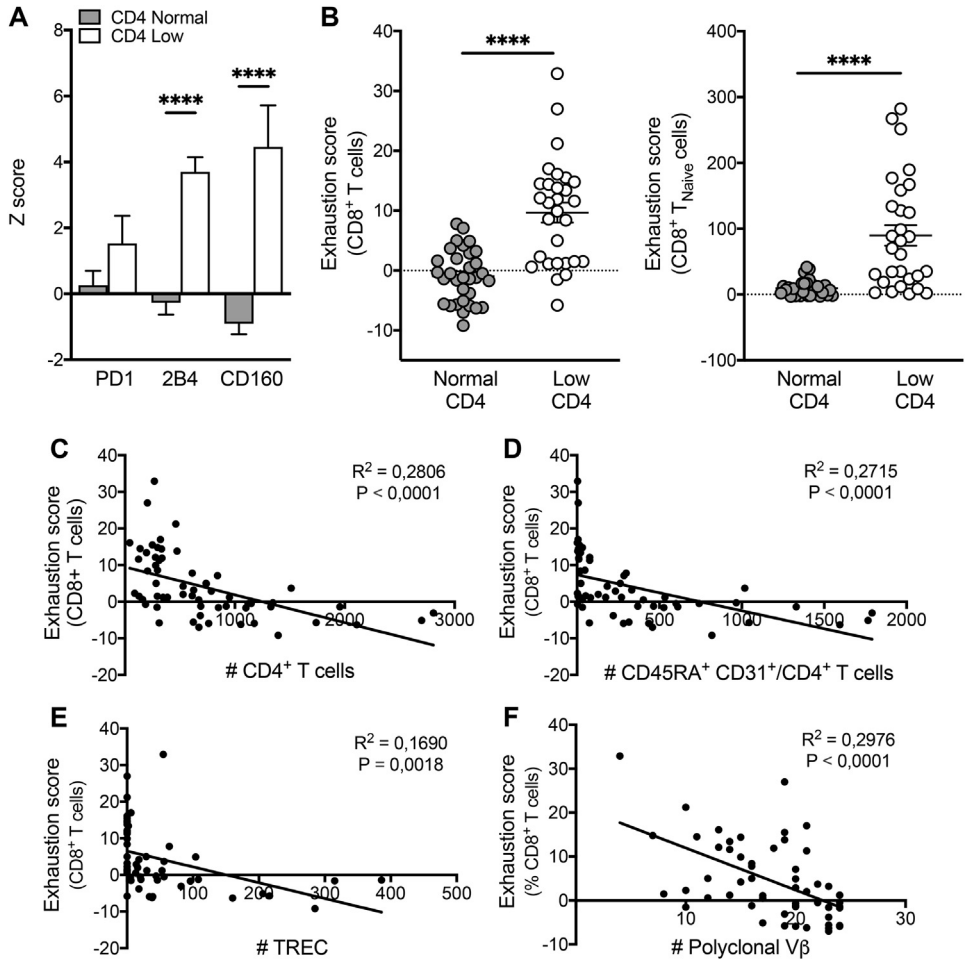


**FIG 3.** Exhaustion score was inversely correlated to quality of T-cell reconstitution. **(A)** The *z* score of the indicated IR on $CD8^+$ T cells in patients with normal (n = 32) and low (n = 29) $CD4^+$ T cells. **(B)** Exhaustion scores of patients with normal (n = 32) and low (n = 29) $CD4^+$ T cells. The exhaustion score is the sum of the *z* scores of PD-1, 2B4, and CD160 IRs on total $CD8^+$ T cells *(left)* or naive $CD8^+$ T cells *(right)*. **(C-F)** Correlations between the exhaustion score on $CD8^+$ T cells and the number of *(C)* $CD4^+$ T cells (n = 61), *(D)* $CD45RA^+$ $CD31^+/CD4^+$ T cells (n = 56), *(E)* TRECs (n = 55), and *(F)* polyclonal Vβ TCR peaks by spectratyping (n = 57). Error bars indicate means ± SEMs. Statistical significance was assessed by Wilcoxon-Mann-Whitney *(A, B)*. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001. Correlation was assessed by Pearson correlation coefficient *(C-F)*. Coefficient $R^2$ and *P* values are shown.

## Quality of T-cell reconstitution was inversely correlated to level of T-cell exhaustion

The sustained and simultaneous expression of multiple IRs is a hallmark of T-cell exhaustion,[9] and coexpression of several IRs may have a synergistic effect on T-cell dysfunction.[11] We therefore calculated an overall exhaustion score from the sum of *z* scores of individual IRs to quantify the level of deviation from healthy controls. Patients with low $CD4^+$ T-cell counts had higher single IR *z* scores than those with normal counts (Fig 3, *A*). Likewise, overall exhaustion score of $CD8^+$ T cells was significantly higher in patients with low $CD4^+$ T-cell counts, both in total $CD8^+$ T cells (4.3970 vs −0.9219, $P < .0001$) and in naive $CD8^+$ T cells (89.790 vs 9.517, $P < .0001$) (Fig 3, *B*). In addition, exhaustion scores were negatively correlated with absolute $CD4^+$ T-cell counts ($R^2 = 0.2806$, $P < .0001$) (Fig 3, *C*). Similarly, thymic output was markedly decreased with increasing exhaustion scores, as evidenced by lower RTEs and TRECs (Fig 3, *D* and *E*). Finally, TCR diversity by spectratyping also showed an inverse correlation with exhaustion scores (Fig 3, *F*). We thus observed a strong inverse relationship between the level of exhaustion and the presence of newly formed T cells after HCT.

## Absence of conditioning and of donor myeloid engraftment were associated with T-cell exhaustion

We investigated the association of patient and/or transplant characteristics with T-cell exhaustion. Patients who received no conditioning or IS alone had higher exhaustion scores than those who received either RIC or MAC (6.695 vs −1.160, $P = .0003$) (Fig 4, *A*). This association remained significant in a multivariable analysis, while we found no correlation between the exhaustion score and SCID genotype, graft source, donor type or HLA-compatibility (Table II, Fig 4, *B-D*). Similarly, neither autoimmunity nor chronic, but resolved GvHD after HCT were correlated with exhaustion scores (see Fig E3, *A* and *B*, in the Online Repository at www.jacionline.org). However, patients with donor

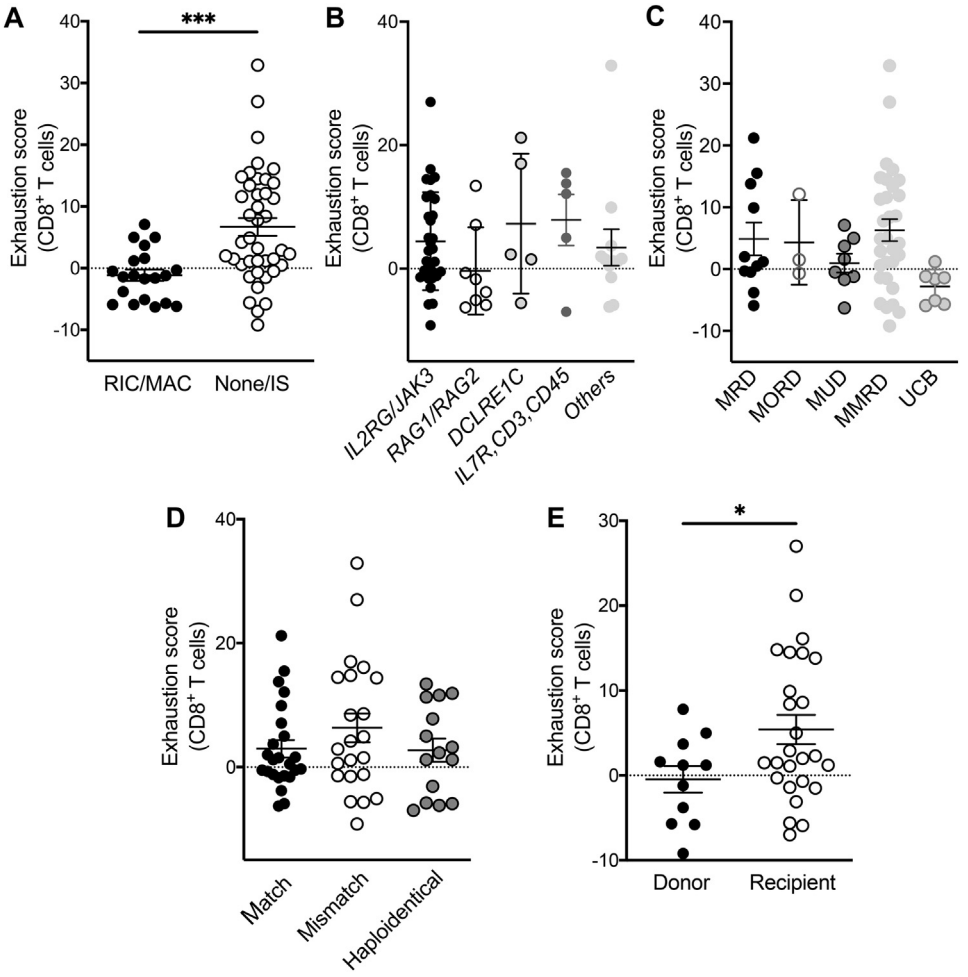


**FIG 4.** Elevated exhaustion score was associated with lack of HCT conditioning regimen and low donor myeloid engraftment. **(A)** Exhaustion score of $CD8^+$ T cells in RIC or MAC (n = 20) versus unconditioned (None/IS; n = 41) patients. **(B)** Exhaustion score on total $CD8^+$ T cells according to SCID genotypes: *IL2RG/JAK3* (n = 31), *RAG1/RAG2* (n = 8), *DCLRE1C* (n = 5), *IL7R*/CD3/CD45 (n = 5), and others (n = 12). **(C)** $CD8^+$ T-cell exhaustion score according to graft type: MRD (n = 11), MORD (n = 3), MUD (n = 8), MMRD (n = 31), and UCB (n = 7). **(D)** Exhaustion score according to HLA compatibility: match (n = 24), mismatch (n = 22), and haploidentical (n = 15). **(E)** Exhaustion score in patients with donor (n = 11, more than 80% of myeloid cells of donor origin) or recipient (n = 26, less than 5% of myeloid cells of donor origin) myeloid chimerism. Error bars indicate means ± SEMs. Statistical significance was assessed by Wilcoxon-Mann-Whitney *(A)*, Kruskal-Wallis *(B-D)*, or Student *t* test *(E)*. **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001. *cGvHD,* Chronic GvHD; *DCLRE1C,* DNA cross-link repair 1C; *IL2RG,* IL-2 receptor gamma; *IL7R,* IL-7 receptor ; *JAK3,* Janus kinase 3; *MORD,* matched other related donor; *MRD,* matched related donor; *MUD,* matched unrelated donor; *RAG*, recombinase activating gene; *UCB,* umbilical-cord blood.

myeloid chimerism <5% had higher exhaustion scores than patients with full donor myeloid chimerism >80% (5.412 vs −0.4727, $P = .0463$) (Fig 4, *E*). Interestingly, patients who did not receive conditioning also showed skewed T-cell differentiation away from naive T cells and a restricted TCR repertoire, similar to patients with low $CD4^+$ T cells (see Fig E4, *A-D,* in the Online Repository at www.jacionline.org). IRs and activation marker expression were also more frequent in these patients (Fig E4, *E* and *F*).

## Poor $CD4^+$ T-cell recovery was sufficient to explain the association between unconditioned HCT and T-cell exhaustion

Because 68% of patients who had received None/IS conditioning before HCT also had low $CD4^+$ T cells, we questioned if exhaustion was driven by the lack of conditioning and/or low $CD4^+$ T-cell counts. We divided the None/IS patients according to their $CD4^+$ T-cell counts (Fig 5, *A*). Within this subgroup analysis, unconditioned patients with poor $CD4^+$ T-cell recovery had highly reduced $CD4^+$ and $CD8^+$ naive T-cell counts (Fig 5, *B* and *C*), with poor thymic output and limited TCR repertoire (see Fig E5, *A-C,* in the Online Repository at www.jacionline.org). Strikingly, the exhaustion scores of these poorly reconstituted, unconditioned patients were much higher both on total $CD8^+$ T cells and on naive $CD8^+$ T cells (Fig 5, *D* and *E*), with only 10 (36%) of 28 None/IS patients with low $CD4^+$ T cells having a normal $CD8^+$ T-cell exhaustion score compared to 100% of patients with normal $CD4^+$ counts (Fig 5, *D*). Individual IRs and activation markers also differed in patients with low $CD4^+$ T cells versus those with normal $CD4^+$ T cells (Fig E5, *D* and *E*). Similar

**TABLE II.** Univariate analysis of independent variables for exhaustion score

| Variable and categories | No. of patients | Mean *z* score | 95% confidence interval | *P* value |
|---|---|---|---|---|
| Genotype grouping, phenotype profile | | | | .261 |
| *IL2RG/JAK3* | 31 | 4.43 | 1.52-7.33 | |
| *RAG1/RAG2* | 8 | −0.36 | −6.26-5.53 | |
| *DCLRE1C* | 5 | 7.27 | −6.79-21.32 | |
| *IL7R*, CD3, CD45 | 5 | 7.86 | −3.59-19.32 | |
| *ADA* | 3 | −1.12 | −11.49-9.25 | |
| *RMRP*, cartilage hair hypoplasia | 3 | 11.72 | −34.18-57.61 | |
| Other/not tested | 6 | 1.52 | −3.83-6.86 | |
| *ZAP70* | 1 | | | |
| Unknown or not tested | 5 | | | |
| Product type | | | | .053 |
| Bone marrow | 42 | 5.61 | 2.85-8.37 | |
| Peripheral blood stem cells | 11 | 3.32 | −2.59-9.22 | |
| UCB | 7 | −2.83 | −5.38-0.28 | |
| Bone marrow + UCB | 1 | | | |
| Graft type | | | | .063 |
| MMRD | 31 | 6.28 | 2.68-9.89 | |
| MRD | 11 | 4.88 | −1.02-10.77 | |
| MORD/MUD | 11 | 1.89 | −1.44-5.21 | |
| UCB | 7 | −2.82 | −5.38-0.28 | |
| MMUD | 1 | | | |
| Conditioning regimen | | | | |
| None/IS | 41 | 6.69 | 3.79-9.58 | |
| RIC/MAC | 20 | −1.16 | −3.09-0.77 | |
| None/IS vs RIC/MAC | | 7.85 | 3.53-12.16 | .001* |

*ADA,* Adenosine deaminase*; DCLRE1C,* DNA cross-link repair 1C*; IL2RG,* IL2 receptor gamma*; IL7R,* IL-7 receptor*; JAK3,* Janus kinase 3*; MMUD,* mismatched unrelated donor*; MORD,* matched other related donor*; MRD,* matched related donor*; MUD,* matched unrelated donor*; RAG,* recombinase activating gene*; RMRP,* RNA component of mitochondrial RNA processing endoribonuclease*; UCB,* umbilical-cord blood*; ZAP70,* zeta chain of TCR-associated protein kinase 70kDa.
*In multivariable analysis, only conditioning regimen was significantly associated with exhaustion score ($P < .001$).

to the global cohort, the magnitude of exhaustion inversely correlated with the levels of total $CD4^+$ T cells, RTEs, TRECs, and TCR polyclonality (Fig E5, *F-I*). Thus, while patients who had no conditioning were at risk of poor immune reconstitution and T-cell dysfunction, those with normal $CD4^+$ T-cell counts did not display abnormal T-cell differentiation or an exhausted T-cell state.

To further interrogate parameters that could be driving T-cell exhaustion after unconditioned transplantations, we compared the characteristics of recipients of None/IS HCT with low $CD4^+$ counts who had either normal (n = 10) or high (n = 18) exhaustion scores (see Table E4 in the Online Repository at www.jacionline.org). Patients with high exhaustion scores submitted samples further beyond the time of their HCT compared to those with normal scores; indeed, None/IS patients showed a direct correlation between time after HCT and exhaustion scores ($R^2$ = 0.3538, $P < .0001$, Fig 5, *F, top*). In contrast, time since HCT did not affect the exhaustion score of conditioned patients (Fig 5, *F, bottom*). We next assessed the effect of $CD4^+$ T-cell counts relative to time in unconditioned patients. When comparing patients with normal versus low $CD4^+$ T cells, the latter group demonstrated heightened $CD8^+$ T-cell exhaustion 15 years after HCT (Fig 5, *G*). In unconditioned patients with low $CD4^+$ T-cell recovery, time after HCT favored exhaustion ($R^2$ = 0.2976, $P = .0027$, Fig 5, *H, top*), while it did not in those with normal $CD4^+$ T-cell counts (Fig 5, *H, bottom*), suggesting that unconditioned HCT drives the exhaustion phenotype and is exacerbated with time.

## T cells of patients with poor $CD4^+$ T-cell recovery after unconditioned HCT displayed an exhausted transcriptional signature

We investigated functional T-cell exhaustion[38] in patients with low $CD4^+$ T-cell counts by RNA sequencing of total and naive $CD8^+$ T cells in 9 patients (Table E2 in the Online Repository at www.jacionline.org) with *IL2RG* or *JAK3* genotype who received MMRD HCTs. Three patients underwent conditioning and 6 did not. At sample collection (median 9.25 years after HCT; no differences between groups), 3 of 6 unconditioned patients had low $CD4^+$ T counts, while all 3 conditioned patients had normal $CD4^+$ T-cell counts.

Among the 6 unconditioned patients, at a false discovery rate (FDR) of 10%, 105 genes were differently expressed (DE) between total $CD8^+$ T cells from those with low versus normal $CD4^+$ T-cell counts (Fig 6, *A*). At a less stringent FDR of 20%, the number of DE genes rose to 486 (see Table E5 in the Online Repository at www.jacionline.org). Gene set enrichment analyses showed a striking enrichment for exhaustion signature genes[38] among DE genes between individuals with low $CD4^+$ T cells (Fig 6, *B*). Specifically, individuals with low $CD4^+$ T-cell counts showed increased expression of many genes known to be upregulated in exhausted cells, including *PDCD1* (the gene encoding PD-1), *LAG3,* and genes encoding transcription factors driving and associated with terminal T-cell exhaustion (*TOX, PRDM1* encoding Blimp-1, *EOMES*) compared to the normal $CD4^+$ count individuals (Fig 6, *C*).[39-42] Conversely, expression of genes associated with naive T cells (eg, *TCF7, SELL, LEF1, CCR7*) were downregulated in individuals

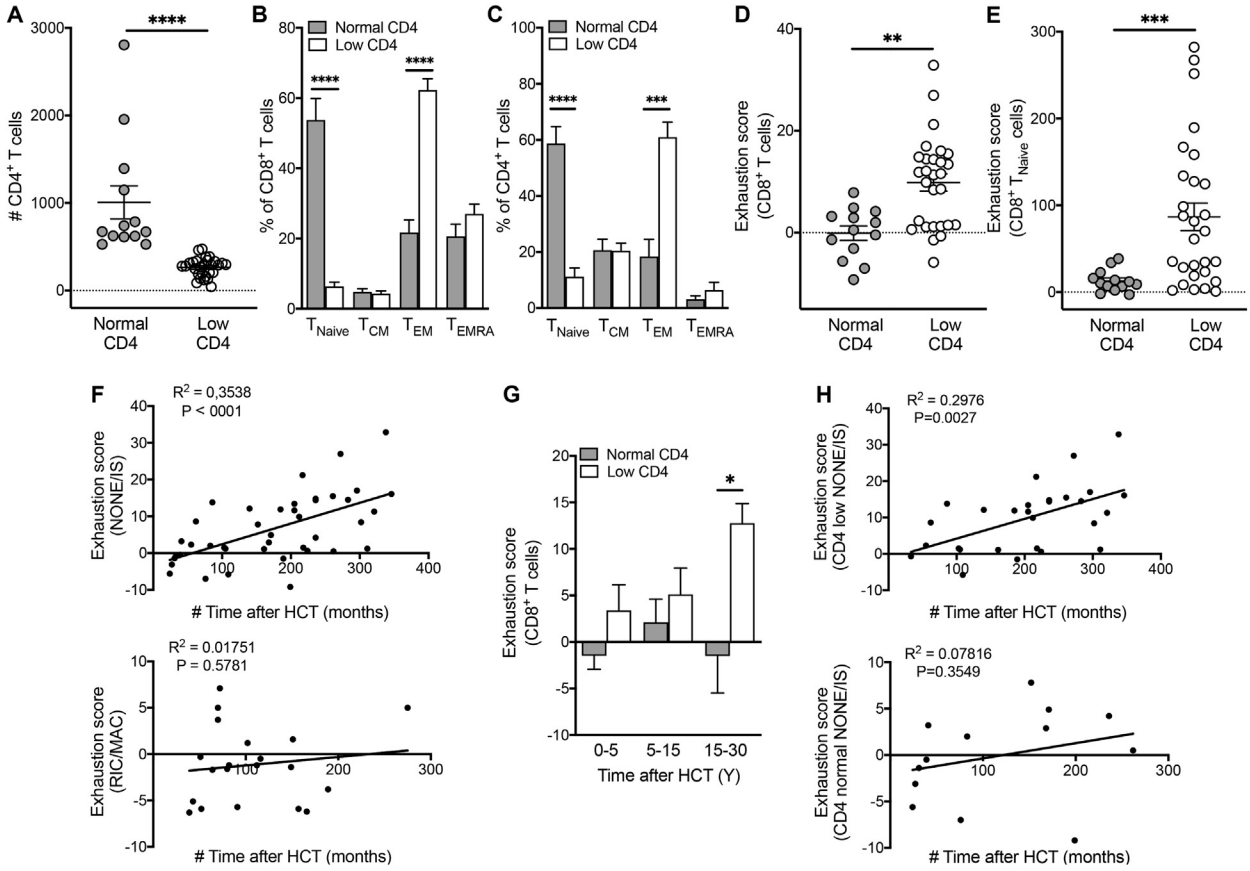


**FIG 5.** Unconditioned SCID patients with low $CD4^+$ counts had skewed T-cell differentiation and high exhaustion scores that correlated with their poor T-cell reconstitution. **(A)** $CD4^+$ cell number in nonconditioned (None/IS) patients with normal (n = 13) versus low (n = 28) $CD4^+$ T cells. **(B** and **C)** Percentage of $CD8^+$ *(B)* and $CD4^+$ *(C)* T-cell subsets in None/IS patients with normal (n = 13) versus low (n = 28) $CD4^+$ T cells. T-cell populations are indicated as follows: $T_{Naive}$, naive T cells ($CD45RA^+/CCR7^+$); $T_{CM}$, central memory T cells ($CD45RA^-/CCR7^+$); $T_{EM}$, effector memory T cells ($CD45RA^-/CCR7^-$); and $T_{EMRA}$, effector memory T cells reexpressing CD45RA ($CD45RA^+/CCR7^-$). **(D** and **E)** Exhaustion score in None/IS patients with normal (n = 13) versus low (n = 28) $CD4^+$ T cells. The exhaustion score is the sum of the *z* scores of PD-1, 2B4, and CD160 IRs on $CD8^+$ *(D)* or naive $CD8^+$ *(E)* T cells. **(F)** Correlation between the exhaustion score on $CD8^+$ T cells and the time after HCT (months) in None/IS patients (n = 41; *top*) and RIC/MAC patients (n = 20; *bottom*). **(G)** Exhaustion score in None/IS patients with normal (n = 13) versus low (n = 28) $CD4^+$ T cells on $CD8^+$ T cells at 0-5, 5-15 and more than 15 years after HCT (Y, years). **(H)** Correlation between the exhaustion score on $CD8^+$ T cells and the time after HCT (months) in None/IS patients with low $CD4^+$ counts (n = 28; *top*) and normal $CD4^+$ counts (n = 13; *bottom*). Error bars indicate means ± SEMs. Statistical significance was assessed by Wilcoxon-Mann-Whitney test *(A-F)*. $*P < .05$, $**P < .01$, $***P < .001$, $****P < .0001$. Correlation was assessed by Pearson correlation coefficient *(F, H)*. Coefficient $R^2$ and *P* values are shown.

with low $CD4^+$ cells.[39-42] In accordance with observed protein marker expression, we found similar exhaustion signatures in the naive $CD8^+$ T cells of unconditioned individuals with low $CD4^+$ T cells (in the Online Repository at www.jacionline.org, see Fig E6 [FDR = $2.8 \times 10^{-4}$] and Table E5), suggesting a true state of exhaustion independent of the stage of T-cell differentiation. In contrast, we found no differences when comparing genes expressed in $CD8^+$ T cells between conditioned and unconditioned individuals with $CD4^+$ T-cell counts of >500 cells/$mm^3$ (data not shown), suggesting that $CD4^+$ T-cell lymphopenia may be the primary driver of $CD8^+$ T-cell exhaustion after HCT.

## DISCUSSION

To our knowledge, this evaluation is the first to study T-cell exhaustion after HCT for SCID. We have explored factors contributing to development of T-cell exhaustion in the large North American SCID cohort under study by the PIDTC. Surface expression of IRs during exhaustion restrain T-cell effector functions.[10,11] Dysfunctional T cells are a risk factor for infections and allow for immune evasion of tumors, as demonstrated in both mouse models and humans.[13,17,19,43-50] Further, in post-HCT leukemic patients, the presence of exhausted T cells is associated with relapse.[21-23] Thus, T-cell exhaustion is likely to have deleterious effects on the long-term fitness of transplanted SCID patients.

In this study, we developed an exhaustion score with the goal of being able to easily discriminate between patients with enhanced exhaustion while using a minimal amount of IR markers to facilitate potential future clinical applications. We focused on 2B4, CD160, and PD-1, three members from distinct families of IRs, because we noticed high expression of 2B4 and CD160 in

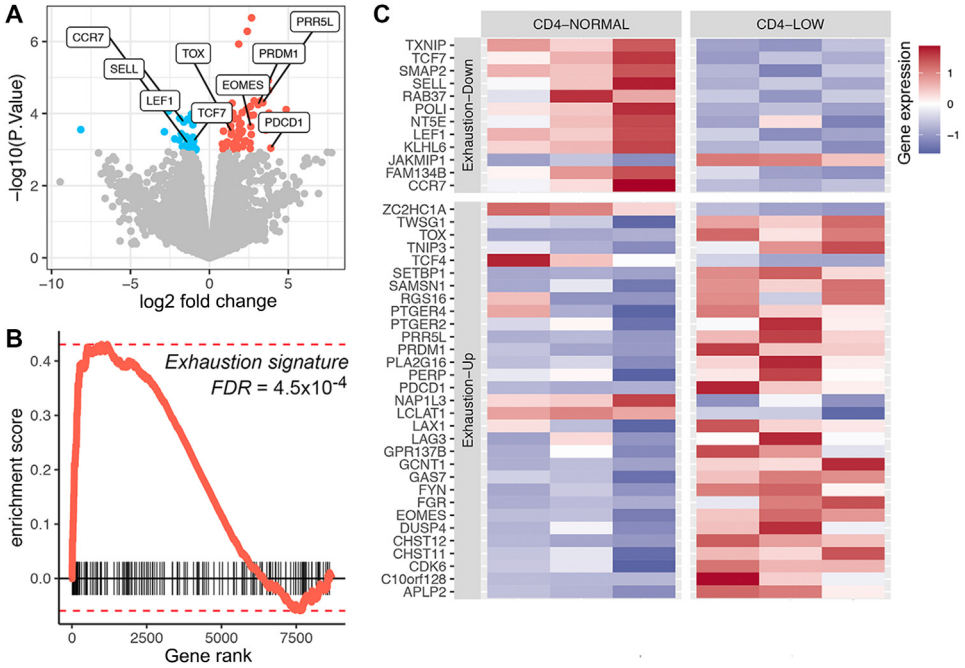


**FIG 6.** Unconditioned SCID patients with low CD4$^+$ counts showed a transcriptional signature of exhaustion. **(A)** Volcano plot of genes DE between CD8$^+$ T cells from individuals with normal versus low CD4$^+$ T cells after unconditioned HCT. Labels indicate DE (FDR < 10%) known to be associated with T-cell exhaustion. **(B)** Gene set enrichment analyses showing that genes known to be upregulated in exhausted T cells were enriched among upregulated genes in individuals with low CD4$^+$ T-cell counts relative to individuals with normal counts. Genes on the x-axis were ranked from the most upregulated to the most downregulated in patients with low versus normal CD4$^+$ T cells. **(C)** Heat map showing expression levels for exhaustion-associated genes that were differentially expressed between patients with normal versus low CD4$^+$ T cells.

patients with low CD4$^+$ T cells, and because PD-1, the best-studied IR, acts as a hallmark for T-cell exhaustion.[12] We found that T-cell exhaustion in post-HCT SCID patients was inversely correlated with the number of CD4$^+$ T cells and the quality of immune reconstitution. These findings are clinically pertinent because low total and naive CD4$^+$ T cells predict poor outcomes after HCT for SCID, including waning long-term T-cell reconstitution, increased susceptibility to infections and autoimmunity, need for long-term immunoglobulin supplementation, and higher mortality.[5,8,32,51] The direct relationship between poor immune reconstitution and emergence of an exhausted T-cell state, however, has been unclear. Our results suggest that CD4$^+$ T-cell lymphopenia may be a major driver of CD8$^+$ T-cell exhaustion. Indeed, a paucity of CD4$^+$ T cells may promote exhaustion through reduced CD4$^+$ T cell help provided to CD8$^+$ T cells.[13,52,53] In human immunodeficiency virus (HIV)–infected patients, high PD-1 expression on HIV-specific CD8$^+$ T cells was inversely related to CD4$^+$ T-cell counts, supporting this hypothesis.[15] Further, the lymphopenic environment might increase the availability of cytokines that drive T-cell exhaustion, such as IL-15.[54] Finally, loss of protective IL-21 signals in the absence of CD4$^+$ T-cell help combined with increased IL-15 signaling may favor exhaustion.[55] Nevertheless, because patients with low CD4$^+$ T cells also tended to be generally lymphopenic, we cannot completely exclude that CD8$^+$ lymphopenia could also contribute to CD8$^+$ T-cell exhaustion in patients with low CD4$^+$ T cells. However, in our study, CD4$^+$ T-cell counts in patients with low CD4$^+$ T cells did not correlate with CD8$^+$ T-cell counts (data not shown), making this less likely.

Another key finding in this study was the association of absent pre-HCT conditioning with post-HCT T-cell exhaustion. While the benefit of conditioning for achieving numerical T-cell recovery and B-cell functional reconstitution in SCID has been established,[8,56,57] the impact on the quality of T-cell reconstitution has been less well documented, especially long after HCT. In this study, patients who received RIC or MAC conditioning were less likely to have an exhausted T-cell phenotype compared to their unconditioned counterparts, and myeloid donor chimerism was also inversely related to T-cell exhaustion. Because so few patients in our cohort had a mixed myeloid donor chimerism, a myeloid donor chimerism threshold below which exhaustion would be likely could not be determined. Together, these findings suggest that better stem cell engraftment may favor T-cell reconstitution of higher quality and durability via donor stem cell engraftment, more sustained thymopoiesis, or both.[5,58] Nonetheless, the immediate and long-term toxicity associated with alkylating agents used for conditioning must be recognized.[59] Notably, within the group of patients receiving unconditioned HCT, those with normal CD4$^+$ T-cell counts did not demonstrate T-cell exhaustion either at the protein expression level or in their transcriptional signature. In contrast, patients with CD4$^+$ T-cell lymphopenia more than 15 years after an unconditioned

transplantation harbored exhausted total and naive CD8$^+$ T cells. Nonetheless, specific factors among the unconditioned HCT recipients that could predict robust, durable immune reconstitution remain unknown. A prospective study is now ongoing to determine whether lower doses of busulfan can open marrow niches for sufficient hematopoietic stem cell engraftment to generate donor B cells and prevent T-cell exhaustion while minimizing toxicity (NCT03619551). Additional trials are also ongoing in an attempt to maximize engraftment while minimizing toxicity in the conditioning of SCID patients, such as with the use of an anti-CD177 (c-kit) monoclonal antibody (NCT02963064).

The cross-sectional nature of our study prevented us from determining whether patients who ultimately developed T-cell exhaustion had manifested aberrant activation and differentiation of total and naive CD8$^+$ T cells early after HCT. Furthermore, few samples were available from RIC/MAC recipients >15 years after HCT; thus, we cannot exclude the possibility that such individuals might develop exhaustion later on, and that conditioning may merely delay exhaustion rather than permanently preventing it. Another limitation is that the study was not designed to correlate exhaustion scores with clinical outcomes. Further long-term prospective studies must therefore be undertaken to establish whether post-HCT T-cell exhaustion directly increases infection frequency, chronic GvHD, autoimmunity, or malignancy. In addition, T-cell functions typically impaired in an exhausted state such as cytotoxicity, cytokine secretion, antigen-dependent proliferation, and homeostatic proliferation were not assessed because of sample limitations; such studies could better define functional T-cell impairments induced by IR expression in this unique context. Nonetheless, our observations suggest that conditioning SCID patients may improve the overall quality of immune reconstitution after HCT and reduce T-cell exhaustion. Further, monitoring post-HCT SCID patients for T-cell exhaustion, perhaps with the exhaustion score described here, could help identify those at risk for protracted infections or cancer, thus possibly leading to consideration of further interventions, such as repeat HCT or gene therapy.

## Conclusions

In a cohort of 61 SCID patients studied at least 2 years after allogeneic transplantation, T-cell exhaustion occurred preferentially in those with low CD4$^+$ T-cell numbers; the degree of exhaustion was inversely correlated with markers of thymic output and T-cell diversity. Furthermore, the absence of HCT conditioning and subsequent lack of donor myeloid chimerism were risk factors for higher exhaustion scores, particularly late after transplantation, although individual patients treated with unconditioned HCT who obtained normal CD4$^+$ T-cell numbers did not exhibit T-cell exhaustion.

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**Clinical implications: HCT for SCID may require conditioning to guarantee durable production of new T cells, thereby preventing development of CD4$^+$ T-cell lymphopenia and CD8$^+$ T-cell exhaustion.**

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