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Authors

Labrosse, Roxane

Boufaied, Ines

Bourdin, Benoîte

et al.

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Aberrant T-cell exhaustion in SCID survivors with poor T-cell reconstitution post transplant

Roxane Labrosse, MD, MMSc[1], **Ines Boufaied, MSc**[2], **Benoîte Bourdin, PhD**[2], **Saideep Gona, MSc**[3], **Haley E. Randolph, Ph.D. candidate**[3], **Brent R. Logan, PhD**[4], **Sara Bourbonnais, MSc**[2], **Chloé Berthe, MSc**[2], **Wendy Chan, MD**[5], **Rebecca H. Buckley, MD**[6], **Roberta E. Parrott, BS**[6], **Geoffrey D.E. Cuvelier, MD**[7], **Neena Kapoor, MD**[8], **Sharat Chandra, MD**[9], **Blachy J. Dávila Saldaña, MD**[10], **Hesham Eissa, MD**[11], **Fred D. Goldman, MD**[12], **Jennifer Heimall, MD**[13], **Richard O'Reilly, MD**[14], **Sonali Chaudhury, MBBS, MD**[15], **Edward A. Kolb, MD**[16], **Shalini Shenoy S, MD**[17], **Linda M. Griffith, MD, MHS, PhD**[18], **Michael Pulsipher, MD**[8], **Donald B. Kohn, MD**[19], **Luigi D. Notarangelo, MD**[20], **Sung-Yun Pai, MD**[21], **Morton J. Cowan, MD**[5], **Christopher C. Dvorak, MD**[5], **Élie Haddad, MD, PhD**[1], **Jennifer M. Puck, MD**[5], **Luis B. Barreiro, PhD**[3], **Hélène Decaluwe, MD, PhD**[1,2]

[1]Pediatric Immunology and Rheumatology Division, Department of Pediatrics, University of Montreal, QC, Canada

[2]Cytokines and Adaptive Immunity Laboratory, Sainte-Justine University Hospital and Research Center, Montreal, QC, Canada

[3]Genetics, Genomics, and Systems Biology, Department of Medicine, Section of Genetic Medicine, University of Chicago, Chicago, IL

[4]Division of Biostatistics, Medical College of Wisconsin, Milwaukee, WI

Correspondence: Hélène Decaluwe, CHU Sainte-Justine Research Center. 3175, ch. de la Côte-Sainte-Catherine, Montréal, QC, Canada (H3T 1C5), Phone: 1-514-345-4931 (ext. 5075), helene.decaluwe@umontreal.ca.

AUTHOR CONTRIBUTIONS

HD conceptualized the project and designed the study and experiments.

BB, HR, SB, CB, WC performed the experiments.

IB, BB, SG, BL, LBB, HD analyzed the data, interpreted the results, and prepared the figures. LMG, RB, RP, GDC, NK, SC, BDS, HE, FDG, JH, ROR, SC, NK, SS identified patients for the study.

RL and HD wrote the manuscript, with the help of JMP.

LMG, MP, DBK, LDN, SYP, MJC, CCD, EH and JMP edited the manuscript and provided critical input as members of the PIDTC Steering Committee.

RL and IB share first authorship. RL wrote the manuscript while IB analyzed all of the flow cytometry data and prepared the related figures.

Study approval

Subjects were recruited with written, informed consent through IRB-approved protocols of the Primary Immune Deficiency Treatment Consortium (PIDTC) under studies NCT01186913 and NCT01346150 (www.clinicaltrials.gov).

CONFLICT OF INTEREST STATEMENT

CCD consults for Jazz Pharmaceuticals, Omeros Corporation, and Alexion Inc. HD sat on an Eli Lilly scientific advisory board. JMP reports royalties from UpToDate and spousal employment at Invitae, a DNA sequencing company. MJC is a member of the Scientific Advisory Board and holds stock in Homology Medicine, Inc; is an UpToDate author; and is a member of Data and Safety Monitoring Boards for bluebird bio, Rocket Pharma, and Leadiant, Inc. All other authors declare no conflicts.

[5]Division of Allergy, Immunology and Blood and Marrow Transplantation, Department of Pediatrics, University of California San Francisco and UCSF Benioff Children's Hospital, San Francisco, CA

[6]Duke University Medical Center, Durham, NC

[7]Manitoba Blood and Marrow Transplant Program, CancerCare Manitoba, University of Manitoba, Winnipeg, MB, Canada

[8]Blood and Marrow Transplant Program, Division of Hematology, Oncology and Blood and Marrow Transplantation, Children's Hospital Los Angeles, Keck School of Medicine, University of Southern California, Los Angeles, CA

[9]Division of Bone Marrow Transplantation and Immune Deficiency, Cincinnati Children's Hospital Medical Center, Cincinnati, OH, Department of Pediatrics, University of Cincinnati College of Medicine, Cincinnati, OH

[10]Division of Blood and Marrow Transplantation, Children's National Hospital, George Washington University School of Medicine and Health Sciences, Washington, DC

[11]Children's Hospital of Colorado, University of Colorado School of Medicine, Aurora, CO

[12]Department of Pediatrics, The University of Alabama at Birmingham, Birmingham, AL

[13]Allergy and Immunology, The Children's Hospital of Philadelphia, Philadelphia, PA

[14]Department of Pediatrics, Bone Marrow Transplant Service, Memorial Sloan Kettering Cancer Center, New York, NY

[15]Division of Hematology, Oncology, and Stem Cell Transplantation, Ann & Robert H. Lurie Children's Hospital of Chicago, Northwestern University Feinberg School of Medicine, Chicago, IL

[16]Nemours Children's Health, Center for Cancer and Blood Disorders, Wilmington, DE

[17]Division of Pediatric Hematology/Oncology, Department of Pediatrics, Washington University School of Medicine, St. Louis, MO

[18]Division of Allergy, Immunology and Transplantation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD

[19]Pediatrics, David Geffen School of Medicine at University of California, Los Angeles, Los Angeles, CA

[20]Laboratory of Clinical Immunology and Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD

[21]Immune Deficiency Cellular Therapy Program, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD

Abstract

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 post-HCT.

Results: Compared to post-HCT SCID patients with normal $CD4^+$ T-cell counts, those with poor T-cell reconstitution showed lower frequency of naïve $CD45RA^+/CCR7^+$ T cells, recent thymic emigrants (RTEs), and T-cell receptor excision circles (TRECs). They also had a restricted TCR repertoire, increased expression of inhibitory receptors (PD1, 2B4, CD160, BTLA, CTLA-4), and increased activation markers (HLA-DR, perforin) on their total and naïve $CD8^+$ T cells, suggesting T-cell exhaustion and aberrant activation, respectively. The exhaustion score of $CD8^+$ T cells was inversely correlated with $CD4^+$ T-cell count, RTEs, TRECs, 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.

Conclusion: Recipients of unconditioned HCT for SCID may develop late post-HCT T-cell exhaustion due to 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.

Keywords

conditioning chemotherapy; hematopoietic cell transplantation (HCT); immune reconstitution; severe combined immunodeficiency (SCID); T-cell exhaustion

INTRODUCTION

Severe combined immunodeficiency (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 following HCT, remaining at increased risk for recurrent infections and autoimmunity(5). Although low T-cell numbers following HCT correlate with reduced overall survival, (5, 6, 7, 8) little is known of qualitative donor T-cell defects in transplanted SCID patients. We hypothesized that poor engraftment of hematopoietic stem cells (HSC) 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 due to 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 following HCT, such as chronic T-cell lymphopenia (24, 25) or the presence of minor and/or major histocompatibility allo-antigens (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 post allogeneic HCT for SCID at a participating Primary Immune Deficiency Treatment Consortium (PIDTC) institution before January 2020. Patients were excluded if they suffered from acute or chronic graft vs. host disease (GvHD) or from PCR-confirmed cytomegalovirus (CMV), Epstein-Barr virus (EBV) or adenovirus infection in the six months preceding sample collection, as GvHD and DNA viral infections are known drivers of T-cell exhaustion(29). Patient and HCT details in Table 1 include age at HCT, SCID genotype, any therapies prior to definitive HCT, cell product, donor matching, and conditioning regimen. For post-HCT details see Supplemental Table E1 in the Online Repository, including whether myeloid chimerism was achieved; GvHD, and autoimmunity. Poor immune reconstitution at least two 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 two categories: none/IS and RIC/MAC. Blood was also obtained from healthy volunteers aged-matched to the oldest patients of our cohort (age range: 19–28 years, n=13).

Flow cytometry immunophenotyping

Immunophenotyping was performed on whole blood shipped overnight at room temperature (RT). Cells were stained within 24 hours of blood procurement with monoclonal antibodies (mAbs) (Supplement for methods Table A in the Online Repository) and then treated with FACS™Lysing Solution 1X (BD Biosciences) for 10 min at RT before flow cytometry. Intracellular staining was performed on cells fixed with Cytofix/Cytoperm™ (BD Biosciences) per manufacturer's instructions. Flow cytometry was performed (LSR FORTESSA II, BD Biosciences) with analysis using FlowJo software, version 9.7.6 (Tree Star, Inc.). Gates were set using Fluorescence Minus One (FMO) controls.

T cell-repertoire diversity

TRECs and polyclonal Vβ T-cell receptor 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 PBMC was used to amplify 24 TCR Vβ families to classify TCR repertoire by spectratyping.

T-cell exhaustion score

Z-scores relative to the mean of the control group for each IR (PD-1, 2B4, CD160) were calculated by the 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 naïve $CD8^+$ T-cell populations were obtained for RNA sequencing from 9 patients with *IL2RG* and 1 with *JAK3* deficient SCID, who had received HCT from MMRDs (3 patient samples from the initial cohort and 6 additional samples; see Supplement for methods, Table B and Supplemental Table E2 in the Online Repository).

Statistics

Statistical significance was determined by Wilcoxon-Mann-Whitney or Kruskal-Wallis test to compare groups for variables which did not follow a normal distribution. Analyses were performed using ABI Prism 6 software with data displayed as mean ± SEM. Correlation coefficients for normal variables were calculated by Pearson correlation. Univariate and multivariable analyses were conducted using linear regression to examine whether genotype, HCT product, donor type, or conditioning regimen were associated with exhaustion. Significance was set as *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001.

RESULTS

Patient and transplant characteristics

Of 69 patients from whom blood samples were received, 61 were analyzed. Two samples from patients with recent chronic GvHD were excluded, as were 6 with shipping or processing problems (Supplemental Table E3 in the Online Repository). The median patient age at HCT was 5.8 months (Table 1), 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) post HCT. Patients were grouped by genotype and phenotypic profile (Table 1), 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, one 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 the patients received mismatched related donor (MMRD) grafts, and only 33% received either reduced intensity or myeloablative conditioning. Post-transplant autoimmunity, acute GvHD or resolved chronic GvHD had occurred in 16%, 26% and 13% of patients, respectively (Supplemental Table E1 in the Online Repository).

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

Low $CD4^+$ T-cell counts in the first 2 years following 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 based on 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 vs. normal median 1628 cells/mm^3, range 1100–5306), and 29 (48%) with low $CD4^+$ counts (median 283 $CD4^+$ cells/mm^3, range 44–474 vs. normal median 885 cells/mm^3, range 528–2700) (Figure 1A). We evaluated the relative composition of various T-cell subsets, including naïve (T_{Naive}, $CD45RA^+/CCR7^+$), central memory (T_{CM}, $CD45RA^-/CCR7^+$), effector memory (T_{EM}, $CD45RA^-/CCR7^-$), and effector memory RA (T_{EMRA}, $CD45RA^+/CCR7^-$) T cells. The subjects with low $CD4^+$ T-cell counts had far fewer naïve $CD8^+$ and $CD4^+$ T cells than subjects with normal $CD4^+$ T-cell numbers post-HCT, with a shift favoring T_{EM} cells (Figure 1B–C). Similarly, proportions and absolute counts of recent thymic emigrants (RTEs, $CD4^+/CD45RA^+/CD31^+$) as well as T-cell receptor excision circles (TRECs) were lower in the low $CD4^+$ T-cell group (Figure 1D–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 T-cell (TCR) peaks in spectratype analysis (Figure 1F), indicating that better T-cell reconstitution was correlated with higher TCR diversity.

Poor T-cell reconstitution was associated with increased expression of inhibitory receptors and an activated T-cell state.

Presence of T-cell IRs (PD1, 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 (Figure 2A); 2B4 and CD160 expression were inversely correlated with the number of both $CD4^+$ T cells and naïve $CD4^+$ T cells (Figure E1 in the Online Repository). 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 (Figure 2B). Again, changes in the expression of activation markers correlated with $CD4^+$ and RTE counts (Figure E1 in the Online Repository). $CD8^+$ T-cell perforin expression was significantly more frequent in patients with low $CD4^+$ T cells, a phenomenon observed with terminal exhaustion (36) (Figure 2C). The pattern of expression of IRs and activation markers on $CD4^+$ T cells largely resembled that seen on $CD8^+$ T cells (Figure 2D–E).

Since 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 same receptors on naïve $CD45RA^+$ $CCR7^+$ $CD8^+$ T cells to circumvent potential bias. Strikingly, with the exception of TIGIT, all IRs were highly expressed on naïve $CD8^+$ 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 two subsets of patients following HCT (Figure 2F). Similarly, activation markers remained distinct on naïve $CD8^+$ T cells, with more cells expressing HLA-DR and fewer expressing CD27 in the low $CD4^+$ T-cell group (Figure 2G). 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 (Figure E2 in the Online Repository). Taken together, in our cohort, poor immune reconstitution following HCT coincided with a dysregulated pattern of expression of inhibitory and activation molecules, consistent with a state of aberrant T-cell activation and exhaustion.

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

The sustained and simultaneous expression of multiple IRs is a key hallmark of T-cell exhaustion (9), and co-expression 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 (Figure 3A). 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 <0.0001) and in naïve $CD8^+$ T cells (89.790 vs. 9.517, P <0.0001) (Figure 3B). In addition, exhaustion scores were negatively correlated with absolute $CD4^+$ T-cell counts (R^2=0.2806, P <0.0001) (Figure 3C). Similarly, thymic output was markedly decreased with increasing exhaustion scores, as evidenced by lower RTEs and TRECs (Figure 3D–E). Finally, TCR diversity by spectratyping also showed an inverse correlation with exhaustion scores (Figure 3F). We thus observed a strong inverse relationship between the level of exhaustion and the presence of newly formed T cells following 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 immunosuppression alone (None/IS) had higher exhaustion scores than those who received either reduced intensity or myeloablative conditioning (RIC/MAC) (6.695 vs −1.160, P=0.0003) (Figure 4A). 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 2, Figure 4B–D). Similarly, neither the autoimmunity nor chronic, but resolved GvHD after HCT were correlated with exhaustion scores (Figure E3A–B in the Online Repository). However, patients with donor myeloid chimerism <5% had higher exhaustion scores than patients with full donor myeloid chimerism >80% (5.412 vs −0.4727, P=0.0463) (Figure 4E). Interestingly, patients who did not receive conditioning also showed skewed T-cell differentiation away from naïve T cells and a restricted TCR repertoire, similar to patients with low $CD4^+$ T cells (Figure E4A–D in the Online Repository). IRs and activation marker expression were also more frequent in these patients (Figure E4E–F in the Online Repository).

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 prior to 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 based on their $CD4^+$ T-cell

counts (Figure 5A). Within this subgroup analysis, unconditioned patients with poor $CD4^+$ T-cell recovery had very reduced $CD4^+$ and $CD8^+$ naïve T-cell counts (Figure 5B–C), with poor thymic output and limited TCR repertoire (Figure E5A–C in the Online Repository). Strikingly, the exhaustion scores of these poorly reconstituted, unconditioned patients were much higher both on total $CD8^+$ T cells and on naïve $CD8^+$ T cells (Figure 5D–E), with only 10 out of 28 None/IS patients (36%) with low $CD4^+$ T cells having a normal $CD8^+$ T-cell exhaustion score compared to 100% of patients with normal $CD4^+$ counts (Figure 5D). Individual IRs and activation markers also differed in patients with low $CD4^+$ T cells vs. those with normal $CD4^+$ T cells (Figure E5D–E in the Online Repository). Similar to the global cohort, the magnitude of exhaustion inversely correlated with the levels of total $CD4^+$ T cells, RTEs, TRECs and TCR polyclonality (Figure E5F–I in the Online Repository). 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 following unconditioned transplants, 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 (Supplementary Table E4 in the Online Repository). 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 post-HCT and exhaustion scores (R^2=0.3538, P <0.0001, Figure 5F, upper panel). In contrast, time since HCT did not affect the exhaustion score of conditioned patients (Figure 5F, lower panel). 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 (Figure 5G). In unconditioned patients with low $CD4^+$ T-cell recovery, time after HCT favored exhaustion (R^2=0.2976, P=0.0027, Figure 5H, upper panel), while it did not in those with normal $CD4^+$ T-cell counts (Figure 5H, lower panel), 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 naïve $CD8^+$ T cells in 9 patients (Supplemental Table E2 in the Online Repository) with *IL2RG* or *JAK3* genotype who received MMRD HCTs. Three patients were conditioned while 6 were not. At sample collection (median 9.25 years post-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 vs. normal $CD4^+$ T-cell counts (Figure 6A). At a less stringent FDR of 20%, the number of DE genes rose to 486 (Supplementary Table E5 in the Online Repository). Gene set enrichment analyses (GSEA) showed a striking enrichment for exhaustion signature genes (38) among

DE genes between individuals with low $CD4^+$ T cells (Figure 6B). Specifically, individuals with low $CD4^+$ T-cell counts showed increased expression of many genes known to be up-regulated 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 (Figure 6C) (39–42). Conversely, expression of genes associated with naïve T cells (e.g. *TCF7*, *SELL*, *LEF1*, *CCR7*) were down-regulated in individuals with low $CD4^+$ cells.(39–42) In accordance with observed protein marker expression, we found similar exhaustion signatures in the naïve $CD8^+$ T cells of unconditioned individuals with low $CD4^+$ T cells (Figure E6 in the Online Repository, FDR=2.8×10^{-4}, and Supplementary Table E7 in the Online Repository), 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 >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

This evaluation is the first to our knowledge of T-cell exhaustion following 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, 43–46, 47, 48–50). Further, in post-HCT leukemic patients the exhausted T cells are 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 PD1, three members from distinct families of IRs, as we noticed high expression of 2B4 and CD160 in patients with low $CD4^+$ T cells, and since PD1 is the most well studied IR acting 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 since low total and naïve $CD4^+$ T cells predict poor outcomes following 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 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, e.g., 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, since 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. With few patients in our cohort having 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-cells 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 transplant harbored exhausted total and naïve $CD8^+$ 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 HSC 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 naïve $CD8^+$ T cells early after HCT. Furthermore, very few samples were available from RIC/MAC recipients >15 years post-HCT; thus, we cannot exclude that such individuals might develop exhaustion later on, and that conditioning may merely delay exhaustion, rather than permanently prevent 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 the frequency of infections, 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 due to 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 post-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, and possibly lead to consideration of further interventions such as repeat HCT or gene therapy.

CONCLUSION

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 being 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 achieved normal $CD4^+$ T-cell numbers did not exhibit T-cell exhaustion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

CMV	cytomegalovirus
DE	differently expressed
EBV	Epstein-Barr virus
GSEA	gene set enrichment analyses
GvHD	graft-versus-host disease
HCT	hematopoietic cell transplant
HSC	hematopoietic stem cells
IR	inhibitory receptor
mAbs	monoclonal antibodies

MAC	myeloablative conditioning
MMRD	mismatched related donor
PIDTC	Primary Immune Deficiency Treatment Consortium
RIC	reduced intensity conditioning
RTE	recent thymic emigrants
SCID	severe combined immunodeficiency
TRECs	T-cell receptor excision circles
UCB	umbilical-cord blood

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CLINICAL IMPLICATIONS

Hematopoietic cell transplantation for severe combined immunodeficiency may require conditioning to guarantee durable production of new T cells, preventing development of $CD4^+$ T-cell lymphopenia and $CD8^+$ T-cell exhaustion.

CAPSULE SUMMARY

Total and naïve $CD8^+$ T cells from poorly reconstituted SCID patients demonstrated exhaustion and aberrant activation. T-cell exhaustion was related to $CD4^+$ T-cell lymphopenia and was more frequent in recipients of unconditioned HCT late after transplantation.

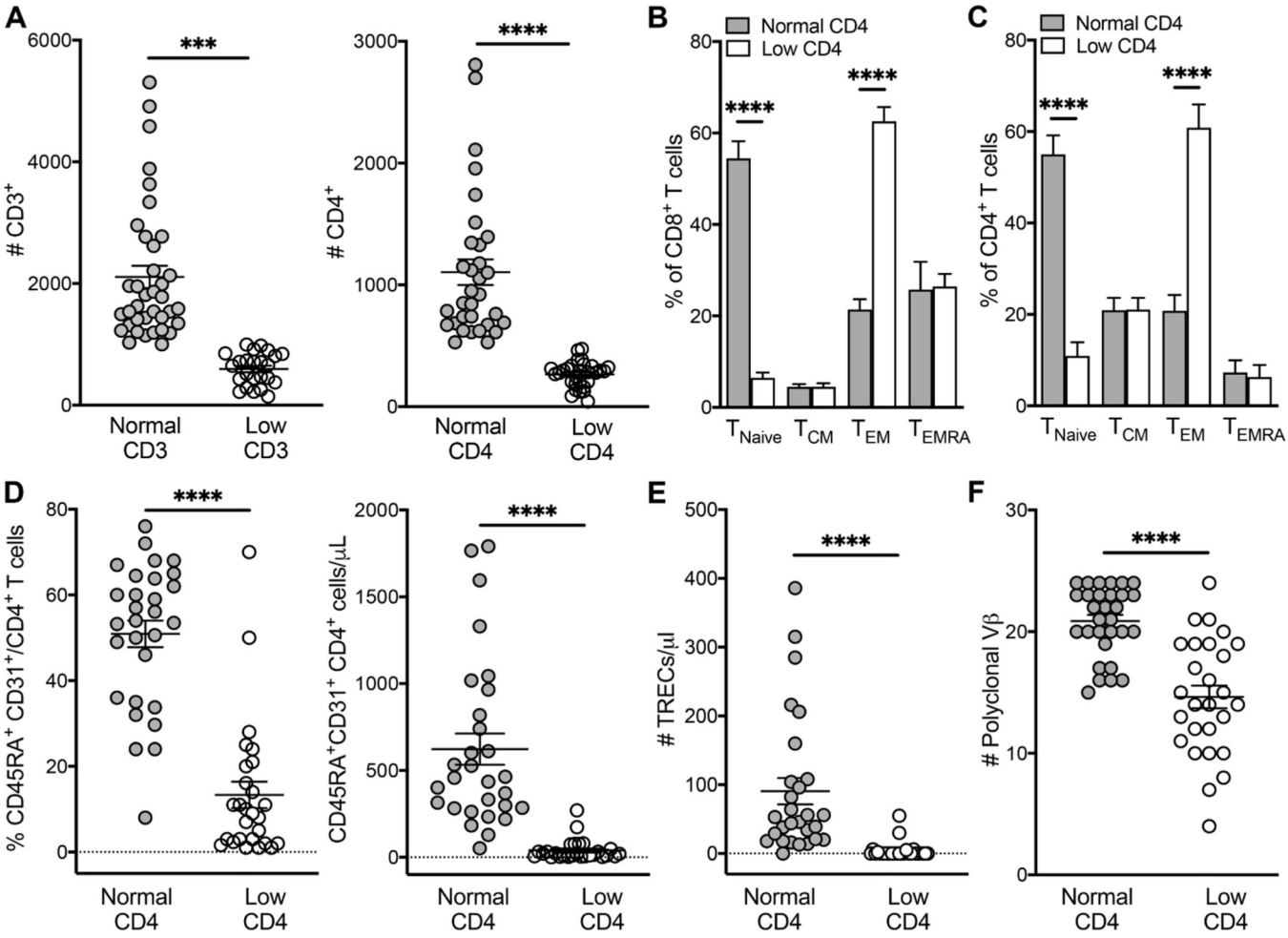

Figure 1. Low CD4+ counts post-HCT were correlated with few naïve T cells, reduced recent thymic emigrants and restricted T-cell diversity.

(A) Patients were separated into those with normal (n=37) and low (n=24) absolute $CD3^+$ T-cell counts (left panel) and those with normal (n=32) and low (n=29) absolute $CD4^+$ T-cell counts (right panel). (B-C) Frequency of $CD8^+$ (B) and $CD4^+$ (C) T-cell subsets in normal $CD4^+$ (n=32) and low $CD4^+$ (n=29) groups. T_{CM}=central memory T cells ($CD45RA^-/CCR7^+$); T_{Naive}=naïve T cells ($CD45RA^+/CCR7^+$); T_{EM}=effector memory T cells ($CD45RA^-/CCR7^+$); T_{EMRA}=effector memory RA T cells ($CD45RA^+/CCR7^-$). (D) Recent thymic emigrant (RTE) $CD4^+$ T-cell ($CD45RA^+$ $CD31^+/CD4^+$) frequency (left panel) and counts (right panel) among the normal $CD4^+$ (n=29) and low $CD4^+$ (n=27) groups. (E) T-cell receptor excision circles (TRECs) in normal $CD4^+$ (n=28) and low $CD4^+$ (n=27) groups. (F) Number of polyclonal Vβ T-Cell Receptor peaks in spectratype analysis of normal $CD4^+$ (n=30) and low $CD4^+$ (n=27) patients. Error bars indicate mean±SEM. Statistical significance was assessed by Wilcoxon-Mann-Whitney test. *P <0.05; **P <0.01; ***P <0.001; ****P <0.0001.

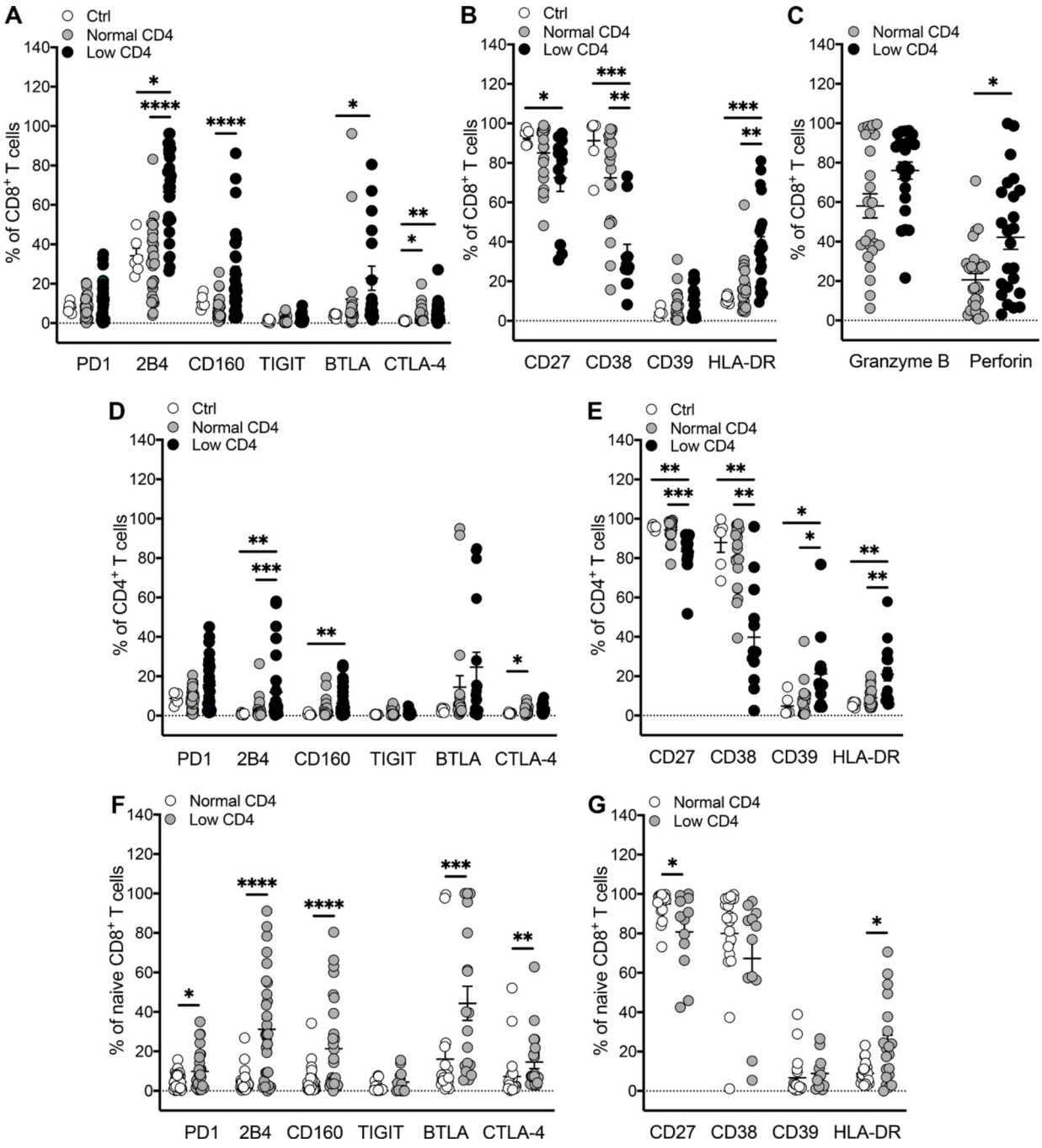

Figure 2. Poor T-cell reconstitution was associated with increased expression of inhibitory receptors (IR) and an activated T-cell state, even within naive cells.

(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 mean±SEM. Statistical significance was assessed by Kruskal-Wallis test (A, B, D, E) or Wilcoxon-Mann-Whitney (C, F, G). *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

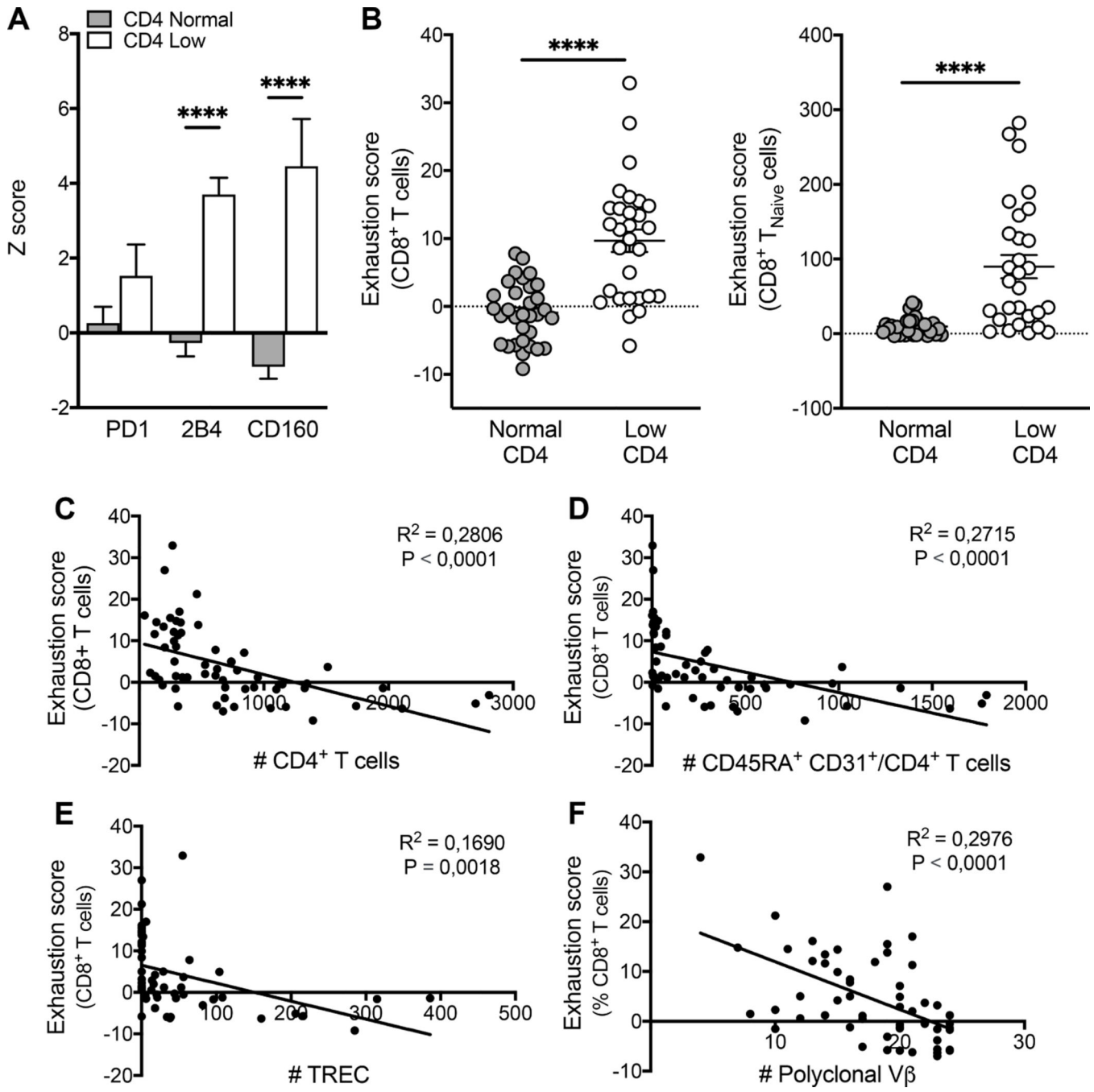

Figure 3. Exhaustion score was inversely correlated to quality of T-cell reconstitution.

(A) 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 PD1, 2B4 and CD160 IR on total $CD8^+$ T cells (left panel) or naive $CD8^+$ T cells (right panel). (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) T-cell receptor excision circles (TRECs) (n = 55) and (F) Polyclonal Vβ T-cell receptor peaks by spectratyping

(n=57). Error bars indicate mean±SEM. Statistical significance was assessed by Wilcoxon-Mann-Whitney (A-B). *P <0.05; **P <0.01; ***P <0.001; ****P <0.0001. Correlation was assessed by Pearson correlation coefficient (C-F). Coefficient R^2 and P-values are shown.

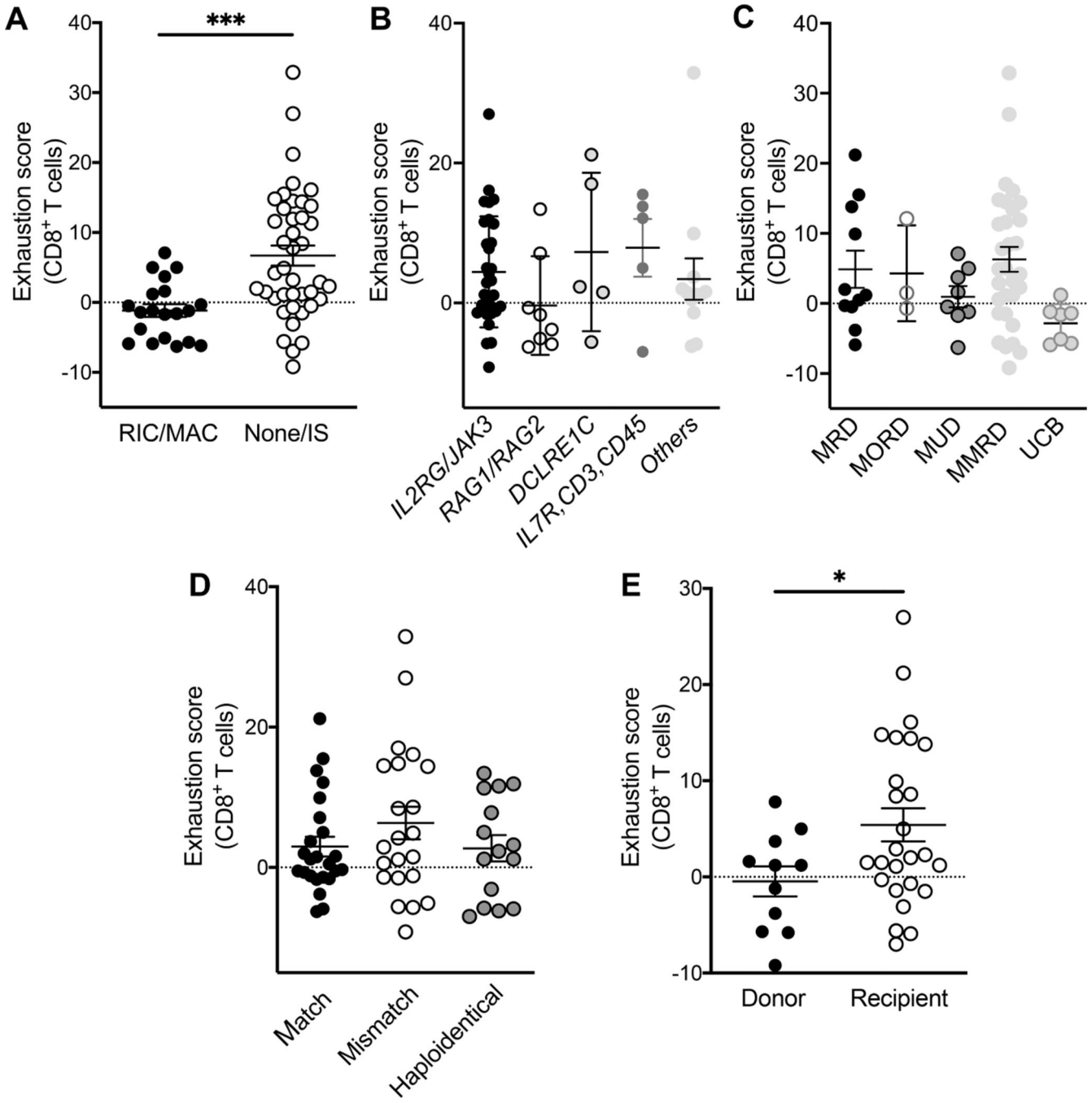

Figure 4. Elevated exhaustion score was associated with lack of HCT conditioning regimen and low donor myeloid engraftment.

(A) Exhaustion score of $CD8^+$ T cells in RIC/MAC (n=20) vs. non-conditioned (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 mean±SEM. Statistical significance was assessed by Wilcoxon-Mann-Whitney (A), Kruskal-Wallis (B-D) or Student t test (E). *P <0.05; **P <0.01; ***P <0.001; ****P <0.0001. cGvHD: chronic Graft-versus-host disease; *DCLRE1C*, DNA cross-link repair 1C; *IL2RG*, Interleukin-2 receptor gamma chain; *IL7R*, Interleukin-7 receptor alpha chain; IS: immunosuppression; *JAK3*, Janus kinase 3; MAC: myeloablative conditioning; MMRD, mismatched related donor; MORD, matched other related donor; MRD, matched related donor; MUD, phenotypic matched unrelated donor; *RAG*, Recombinase activating gene; RIC: reduced-intensity conditioning; UCB, unrelated umbilical cord blood.

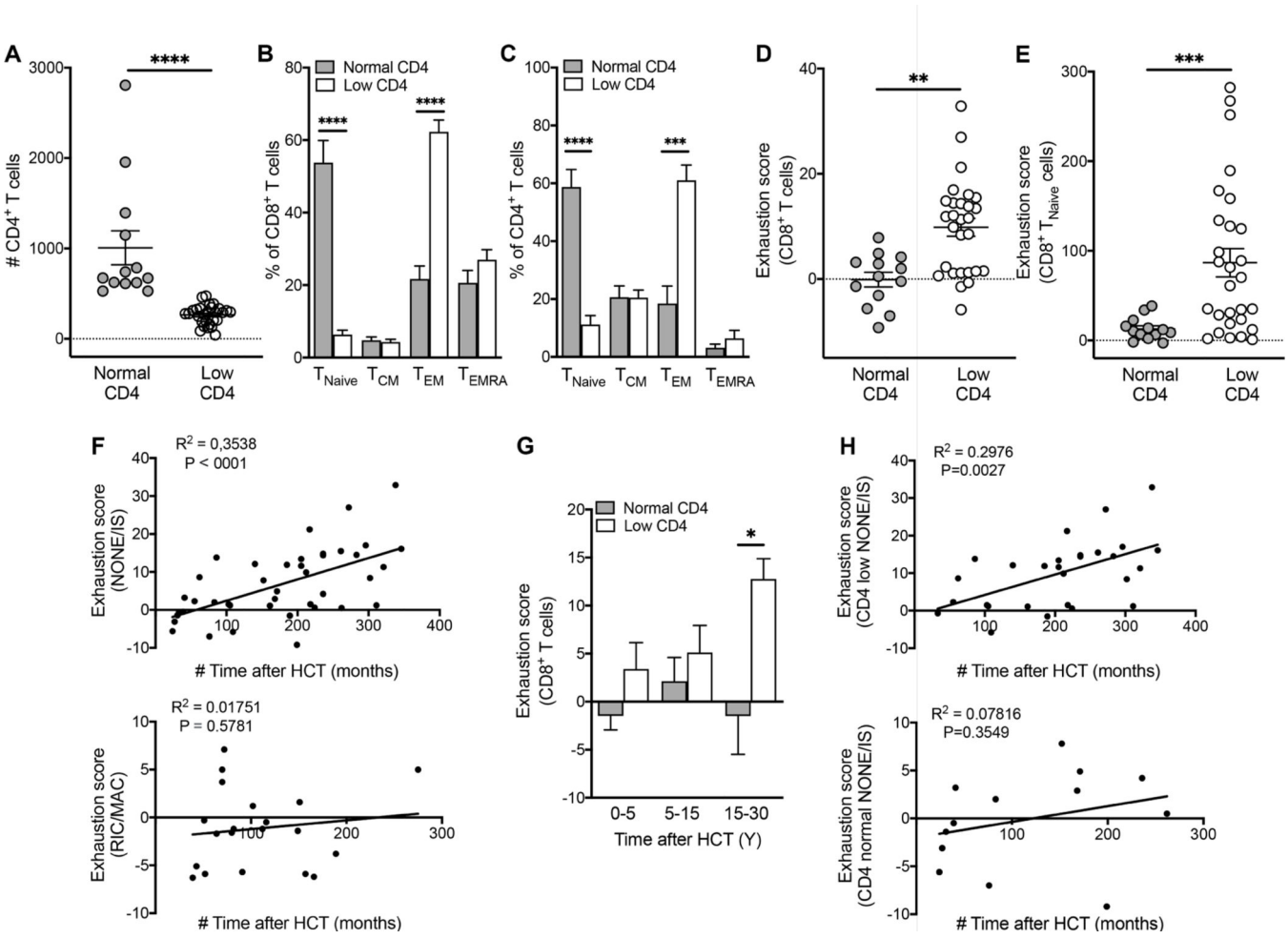

Figure 5. Unconditioned SCID patients with low CD4$^+$ counts had skewed T-cell differentiation and high exhaustion scores that correlated with their poor T-cell reconstitution.

(A) CD4$^+$ cell number in non-conditioned (None/IS) patients with normal (n=13) vs. low (n=28) CD4$^+$ T cells. (B-C) Percentage of CD8$^+$ (B) and CD4$^+$ (C) T-cell subsets in None/IS patients with normal (n=13) vs. low (n=28) CD4$^+$ T cells. T_{CM}=central memory T cells (CD45RA$^{-/}$CCR7$^+$); T_{Naive}=Naive T cells (CD45RA$^+$/CCR7$^+$); T_{EM}=effector memory T cells (CD45RA$^-$/CCR7$^-$); TEMRA=effector memory RA T cells (CD45RA$^+$/CCR7$^-$). (D-E) Global exhaustion score in None/IS patients with normal (n=13) vs. low (n=28) CD4$^+$ T cells. The exhaustion score is the sum of the Z scores of PD1, 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; upper panel) and RIC/MAC patients (n=20; lower panel). (G) Exhaustion score in None/IS patients with normal (n=13) vs. 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; upper panel) and normal CD4$^+$ counts (n=13; lower panel). Error bars indicate mean±SEM. Statistical significance was assessed by Wilcoxon-Mann-Whitney test (A-F). *P <0.05; **P <0.01;

***P <0.001; ****P <0.0001. Correlation was assessed by Pearson correlation coefficient (F, H). Coefficient R2 and P-values are shown.

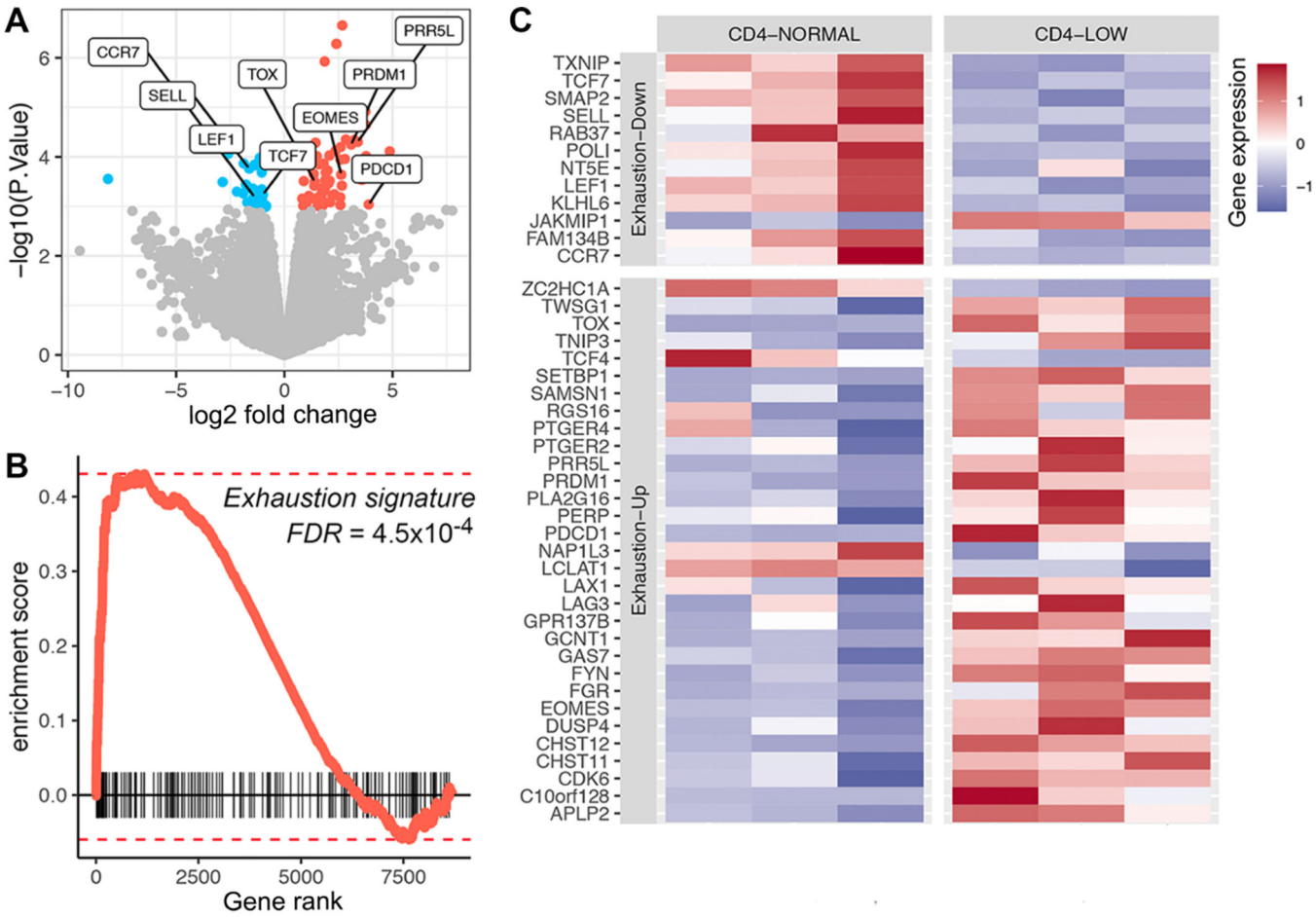

Figure 6. Unconditioned SCID patients with low CD4$^+$ counts showed a transcriptional signature of exhaustion.

(A) Volcano plot of genes differently expressed (DE) between CD8$^+$ T cells from individuals with normal vs. low CD4$^+$ T cells following unconditioned HCT. Labels indicate DE (False Discovery Rate (FDR)<10%) known to be associated with T-cell exhaustion. (B) Gene set enrichment analyses showing that genes known to be up-regulated in exhausted T cells were enriched among up-regulated 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 up-regulated to the most downregulated in patients with low vs. normal CD4$^+$ T cells. (C) Heatmap showing expression levels for exhaustion-associated genes that were differentially expressed between patients with normal vs. low CD4$^+$ T cells.

Table 1.

Patient and hematopoietic cell transplant characteristics

	Number (%) of patients*
Age at HCT	
≤3,5 m	25 (41)
>3,5 m	36 (59)
Median age at HCT (range, m)	5.8 (0.3–109.6)
Median time of blood sample after HCT (range, m) Genotype grouping, phenotype profile	124.8 (24–346)
IL2RG/JAK3, $T^-B^+NK^-$	31 (51)
RAG1/RAG2, $T^-B^-NK^+$	8 (13)
DCLRE1C, $T^-B^-NK^+$, radiation sensitive	5 (8)
IL-7R, CD3, CD45, $T^-B^+NK^+$	5 (8)
ADA, $T^-B^-NK^-$	3 (5)
RMRP, cartilage hair hypoplasia	3 (5)
ZAP-70	1 (2)
Unknown or not tested, *$T^-B^+NK^+$*	5 (8)
SCID therapy received prior to 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)
Umbilical-cord blood	7 (11)
Bone marrow plus umbilical-cord blood	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)

[*] 61 patients had samples analyzed.

[†] Only 2 patients received conditioning regimen (RIC, n=1; MAC, n=1)

[‡] ≤4/8 graft, n=13; >4/8 graft, n=18

[§] ≤4/8 graft, n=15

Abbreviations: m, months. *ADA*, Adenosine deaminase; *DCLRE1C*, DNA cross-link repair 1C; HCT, Hematopoietic cell transplantation; *IL2RG*, Interleukin-2 receptor gamma chain; *IL7R*, Interleukin-7 receptor alpha chain; IS, Immunosuppression; *JAK3*, Janus kinase 3; MAC, Myeloablative Conditioning; MMRD, Mismatched related donor; MMUD, Mismatched unrelated donor, MORD, Matched other related donor; MRD, Matched related donor; MUD, Matched unrelated donor; *RAG*, Recombinase activating gene; RIC, Reduced-Intensity Conditioning; *RMRP*, RNA component of mitochondrial RNA processing endoribonuclease; UCB, Umbilical-cord blood; *ZAP-70*, Zeta chain of T-cell receptor associated protein kinase 70kDa.

Table 2.

Univariate analysis of independent variables for exhaustion score

VARIABLE AND CATEGORIES	number of patients	Mean Z score	95% CI*	P Value
Genotype grouping, phenotype profile				0.261
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	
IL-7R, 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	
ZAP-70	1			
Unknown or not tested	5			
Product type				0.053
Bone marrow	42	5.61	2.85 – 8.37	
Peripheral blood stem cells	11	3.32	−2.59 – 9.22	
Umbilical-cord blood	7	−2.83	−5.38 - −0.28	
Bone marrow+UCB	1			
Graft type				p=0.063
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	p<0.001

In multivariable analysis only conditioning regimen was significantly associated with the exhaustion score (p <.001).

Abbreviations: *ADA*, Adenosine deaminase; *DCLRE1C*, DNA cross-link repair 1C; *JAK3*, Janus kinase 3; *IL2RG*, Interleukin-2 receptor gamma chain; *IL7R*, Interleukin-7 receptor alpha chain; IS, Immunosuppression; MAC, Myeloablative Conditioning; MMRD, Mismatched related donor; MMUD, Mismatched unrelated donor, MORD, Matched other related donor; MRD, Matched related donor; MUD, Matched unrelated donor; *RAG*, Recombinase activating gene; RIC, Reduced-Intensity Conditioning; *RMRP*, RNA component of mitochondrial RNA processing endoribonuclease; UCB, Umbilical-cord blood; *ZAP-70*, Zeta chain of T-cell receptor associated protein kinase 70kDa.