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# Programmed Death 1 Expression on CD4+ T Cells Predicts Mortality after Allogeneic Stem Cell Transplantation

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Excessive or persistent programmed death 1 (PD-1) expression on virus- or tumor-specific T cells during chronic viral infection or malignancy has been associated with impaired immune control. To assess the role of the PD-1 pathway in allogeneic stem cell transplantation (SCT), we examined PD-1 expression and maturation phenotype on T cells from 42 patients early (day 55 to 85) after cord blood (CB), matched unrelated donor, and matched related donor transplantation. Expression of PD-1 on CD4+ T cells was significantly elevated in all transplantation types, with the highest level observed in CB subjects. Elevated PD-1 expression on CD4+ T cells early after transplantation was observed in nonsurvivors (median, 40.2%; range, 15.1 to 86.1) compared with survivors (median, 23.6%; range, 8.4 to 55.2; $P = .001$), indicating its association with increased risk for mortality, especially with CB transplantations, where PD-1 was increased in nonsurvivors (median, 64.6%; range, 36.5 to 86.1) compared with survivors (median, 34.1%; range, 15.9 to 55.2; $P = .01$). Furthermore, T cell subset analysis revealed that PD-1 expression was further elevated on CD4+ T central memory in nonsurvivors (median, 49.8%; range, 15.1 to 83.4) compared with survivors (median, 24.8%; range, 8.9 to 71.3; $P = .002$) and on T effector memory cells in nonsurvivors (median, 69.1%; range, 24.7 to 92.6) compared with survivors (median, 43.7%; range, 13.9 to 96.5; $P = .0003$). Our findings suggest that elevation of PD-1 expression on CD4+ T cells is associated with mortality in CB and possibly all SCT recipients.



## INTRODUCTION

T cell inhibitory receptors are critical regulators of T cell function [1-3]. In physiologic immune responses, T cell inhibitory receptors up-regulate in parallel with activating receptors to prevent an overwhelming immune response. Excessive or persistent T cell inhibitory receptor expression, however, has been associated with several pathologic states. During chronic viral infections such as human immunodeficiency virus or hepatitis C virus, elevated expression of inhibitory receptors on virus-specific T cells demarks "exhausted" cells, which are functionally compromised and poorly able to control infection [4,5]. In numerous malignancies, up-regulation of inhibitory receptors on tumor-specific T cells, as well as up-regulation of inhibitory receptor ligands on tumor cells themselves, contribute to tumor immune evasion [3,6,7]. Furthermore, the inhibitory programmed death 1 (PD-1) receptor is up-regulated on CD4+ and CD8+ T cells in patients with concurrent cytomegalovirus (CMV) infection and acute graft-versus-host disease (GVHD) [8]. Upregulation of PD-1 may not result in enhanced graft-versus-tumor (GVT) but instead lead to impairment of GVT via T cell exhaustion [9,10].

PD-1 is among the most well-studied inhibitory receptors. PD-1 expression is induced when T cells become activated, and persistent antigen stimulation maintains PD-1 expression [11]. Upon engagement with 1 of its ligands, programmed death-1 ligand 1 or 2 (PD-L1 or PD-L2), PD-1 inhibits T cell–activating kinases through the phosphatase SHP2 pathway, which disrupts T cell–receptor signaling [12,13]. PD-L1 is expressed constitutively on hematopoietic and nonhematopoietic cells and up-regulates after activation [14], while PD-L2 expression is restricted primarily to dendritic cells, macrophages, and cultured bone marrow–derived mast cells [15]. Increased PD-1 expression on T cells has also been

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implicated as a mechanism for maintaining feto-maternal tolerance [16,17]. Most importantly, blockade of the PD-1/PD-L1 pathway has resulted in regression of solid tumor malignancies [18] and improved control of human immunodeficiency virus/simian immunodeficiency virus (SIV) infection in humanized mice/macaques [19,20] as well as murine lymphocytic choriomeningitis virus (LCMV) and *Plasmodium* infection [21,22].

Given the critical role of donor T cells in mediating GVT, GVHD, and immune reconstitution after stem cell transplantation (SCT), understanding of T cell inhibitory receptors and their potential for manipulation in this setting may yield therapeutic opportunities. In murine models, PD-1 blockade has been demonstrated to enhance GVT [9,10,23], but it has also been shown to increase GVHD [24]. PD-L1 has been implicated as more important than PD-L2 in mediating GVHD reactions [25]. After human SCT, PD-1 expression on T cell populations has been observed to be elevated in patients with severe GVHD, with CMV disease [8], and in patients relapsing after SCT [26,27]. In vitro data suggest PD-1 blockade may improve GVT responses by enhancing antigen-specific $CD8^+$ T cell function [26].

Based on these findings, we hypothesized that increased frequencies of PD-1–expressing T cells after SCT may have significant impact on the clinical outcome. We investigated PD-1–expressing $CD4^+$ and $CD8^+$ T cell subsets after allogeneic transplantation with multiple donor sources and examined them in the context of relevant clinical parameters such as transplantation type, nonmyeloablative (NMAC) versus myeloablative (MAC) conditioning, acute GVHD, and survival. Our findings suggest that PD-1 expression, as well as T cell maturation, differs between the types of SCT. Most importantly, independent of cause of death, increased percentage of PD-1–expressing $CD4^+$ T cells, specifically in cord blood (CB) recipients, was associated with increased mortality after SCT. Taken together, these data suggest that PD-1 expression on $CD4^+$ T cells early after allogeneic SCT is a clinically relevant predictor for survival.

## METHODS

### *Study Population*

Peripheral blood mononuclear cells (PBMC) were collected from 42 patients who underwent transplantation between May 2010 and December 2012 and the patients were followed until last follow-up visit (up to 1953 days) or time of death. Blood was collected in Vacutainer tubes containing sodium heparin (BD Biosciences, San Jose, CA) between days 55 and 85 and after 1 year after SCT. Blood was collected from subjects who received CB (n = 13), matched unrelated (MURD) (n = 11), or matched related (MRD) (n = 18) transplantation. Blood from healthy subjects who did not undergo transplantation (n = 22) and unmanipulated CB units (n = 8) were used as controls. Controls were not matched for age or sex. Samples before SCT were not available for baseline staining. All subjects provided written informed consent as approved by Colorado Multiple Institutional Review Board. Patient characteristics, including age, gender, disease type, donor source, conditioning regimens, GVHD prophylaxis, GVHD status at time of assays, immunosuppression, disease status, donor chimerism, follow-up visit and cause of death are summarized in Table 1 and Table S1.

### *Immunofluorescent Staining*

PBMC were isolated from whole blood by density gradient centrifugation on ficoll and cryopreserved as previously described [28]. Cryopreserved PBMCs were thawed, washed with staining buffer (PBS containing 1% BSA) and $1.5 \times 10^6$ cells were surface stained with anti-CD3 PE-Texas Red (clone: UCHT1; Becton Coulter), anti-CD4 PerCP Cy5.5 (clone: OKT4; Biolegend), anti-CD8 Alexa Flour405 (clone: 3B5; Invitrogen), anti-CD45RA PE-Cy7 (clone: HI100; Biolegend), anti-CD27 APC-H7 (clone: M-T271; BD Biosciences), and anti-PD1 FITC (clone:MIH4; BD Pharmingen) mAbs for 30 minutes at 4ºC. Cells were washed with staining buffer. Cells were fixed with 1% paraformaldehyde and analyzed using a LSR-II flow cytometer (BD Immunocytometry Systems, San Jose, CA). Fluorescence minus 1 or isotype controls were used in all experiments.

**Table 1**
Patient Characteristics

| Characteristics | MRD | MURD | CB |
|---|---|---|---|
| n | 18 | 11 | 13 |
| MAC | 13 | 6 | 6 |
| GVHD prophylaxis: Tac/MTX | | | |
| NMAC | 5 | 5 | 7 |
| GVHD prophylaxis: Tac/MMF | | | |
| CSA/MMF | | | |
| CSA/MMF/Sir | | | |
| Acute GVHD | 8 | 9 | 8 |
| ≥ 90% Donor chimerism | 15 | 7 | 12 |

Tac indicates tacrolimus; MTX, methotrexate; MMF, mycophenolate mofetil; CSA, cyclosporine A; Sir, sirolimus.

### *T Cell Proliferation*

PBMC were labeled with CellTrace Violet (ThermoFisher) by incubating $10^7$ cells in a 1.5-μM solution of CellTrace Violet in HBSS for 15 minutes at 37ºC and were washed twice with RPMI containing 10% human AB serum to remove excess CellTrace Violet per the manufacturer's instructions. CellTrace Violet–labeled PBMCs were plated at $10^6$ cells/mL in a 48-well plate and were stimulated with 2 μg/mL CMV pp65 peptide, 5 μg/mL CMV lysate (Adriana Weinberg, University of Colorado AMC), phytohaemagglutinin, or they were unstimulated. Cells were cultured with or without anti–PD-L1 mAb (5 μg/mL; eBioscience, San Diego, CA) for 6 days at 37°C in a humidified 5% $CO_2$ atmosphere. On day 6, cells were washed with PBS containing 1% BSA and stained with anti-CD4 PerCP Cy5.5 (clone: OKT4; Biolegend), anti-CD8 FITC (clone: 3B5; Invitrogen), and anti-CD3 PE-Texas Red (clone: UCTH1; Beckman Coulter) for 30 minutes at 4ºC. Cells were washed, fixed, and analyzed as above.

### *Flow Cytometry*

Cells were analyzed using a LSR-II flow cytometer (BD Immunocytometry Systems) and between 1 and 3 million events were collected. Electronic compensation was performed with antibody capture beads (BD Biosciences) stained separately with individual mAbs used in the test samples. To ensure the accuracy and precision of the measurements taken from day to day, quality control was performed on the LSR-II daily using the Cytometer Setup and Tracking feature within BD FACSDiva software (BD Immunocytometry System, San Jose, CA). A manual quality control using rainbow beads was also performed daily to verify the laser delay and area scaling determined by the Cytometer Setup and Tracking feature. The data files were analyzed using Diva software (BD Biosciences). Lymphocytes were gated by their forward- and side-scatter profile. Biexponential scaling was used in all dot plots. The percentage of PD-1–expressing $CD4^+$ and $CD8^+$ T cells as well as T cell subsets naïve ($T_N$: $CD27^+CD45RA^+$), central memory ($T_{CM}$: $CD27^+CD45RA^-$), effector memory ($T_{EM}$: $CD27^-CD45RA^-$) and effector memory terminally differentiated ($T_{EMTD}$: $CD27^-CD45RA^+$) were examined. PD-1 expression was only analyzed on subpopulations of T cells with more than 100 events to ensure the measurement was robust.

### *Chimerism*

Human short tandem repeat (STR) analysis was completed by the Molecular Diagnostic Laboratory at the Children's Hospital Colorado using either $CD3^+$ T cells isolated by fluorescent activated cell sorter or whole bone marrow. PCR chimerism analysis was performed with the Beckman Coulter GeXP GenomeLab Human STR Primer Set (cat. no. A20100; AB Sciex, Framingham, MA) followed by fragment separation by capillary electrophoresis. Distinct STR profiles obtained by chimerism analysis were compared with analyses from samples obtained before transplantation to determine the percentage of cells that were donor derived versus recipient derived.

### *Statistical Analysis*

Statistical analysis was performed using GraphPad Prism (GraphPad, San Diego, CA). One-way ANOVA with Dunnet's adjustment for multiple comparisons was used to determine significance of differences between groups. A nonparametric *t*-test (Mann-Whitney U test) was used to compare 2 samples with non-normally distributed continuous variables. A Wilcoxon signed-rank test was used to compare continuous data points of same patients under different conditions. A Spearman's rank correlation coefficient was used to evaluate statistical dependence between 2 variables. *P* values of $< .05$ were considered statistically significant. Only data points with 3 or more samples in each group were compared.