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Programmed Death-1 Controls T Cell Survival by Regulating Oxidative Metabolism

Victor Tkachyev,* Stefanie Goodell,† Anthony W. Opipari,‡ Ling-Yang Hao,§ Luigi Franchi,§ Gary D. Glick,§ James L. M. Ferrara,* and Craig A. Byersdorfer†

The coinhibitory receptor programmed death-1 (PD-1) maintains immune homeostasis by negatively regulating T cell function and survival. Blockade of PD-1 increases the severity of graft-versus-host disease (GVHD), but the interplay between PD-1 inhibition and T cell metabolism is not well studied. We found that both murine and human alloreactive T cells concomitantly upregulated PD-1 expression and increased levels of reactive oxygen species (ROS) following allogeneic bone marrow transplantation. This PD-1Hi ROSHi phenotype was specific to alloreactive T cells and was not observed in syngeneic T cells during homeostatic proliferation. Blockade of PD-1 signaling decreased both mitochondrial H\textsubscript{2}O\textsubscript{2} and total cellular ROS levels, and PD-1-driven increases in ROS were dependent upon the oxidation of fatty acids, because treatment with etomoxir nullified changes in ROS levels following PD-1 blockade. Downstream of PD-1, elevated ROS levels impaired T cell survival in a process reversed by antioxidants. Furthermore, PD-1 blockade of PD-1 signaling decreased both mitochondrial H\textsubscript{2}O\textsubscript{2} and total cellular ROS levels, and PD-1–driven increases in ROS that underlies the potential for subsequent metabolic inhibition, an important consideration given the increasing use of anti–PD-1 therapies in the clinic. The Journal of Immunology, 2015, 194: 5789–5800.

A ctivation of T cells represents an intricate combination of pro- and antisignaling inputs, and cells must integrate inputs from multiple coreceptors to initiate and maintain an immune response (1, 2). The coinhibitory receptor programmed death-1 (PD-1) is a member of the CD28 superfamily and works in concert with its ligands, PD-L1 and PD-L2, to negatively regulate T cell function, including proliferation, cytokine secretion, and survival (3). PD-1 signaling is essential for maintaining lymphocyte homeostasis by preventing immune-mediated damage and inducing T cell exhaustion to chronically exposed Ags in infectious and tumor models (4–8). PD-1 is also upregulated after acute activation, where it helps to dampen the initial T cell response to robust stimulation (9).

PD-1 was first discovered as a marker of apoptosis (10), and recent applications have used PD-1 blockade to enhance T cell responses in a number of therapeutic areas (11–13). Of particular interest, blockade of the PD-1 pathway is being used to increase antitumor immunity in patients with advanced-stage cancers (4, 11, 13). However, augmenting T cell responses via PD-1 inhibition may have unintended consequences, including devastating immune reactions to routine infections (4, 5, 14, 15) and an increased prevalence of autoimmunity (6, 7, 16, 17). In graft-versus-host disease (GVHD), it is well known that an absence of PD-1 signaling results in increased IFN-γ production and lethal immunopathology (18), likely through increased alloreactive T cell expansion and heightened Th1 differentiation (19). Recently, it was suggested that PD-1 also facilitates changes in alloreactive T cell metabolism (20). However, the detailed mechanisms driving these metabolic changes in alloreactive cells remain incompletely understood. In addition, how PD-1 blockade affects a cell’s later ability to respond to subsequent metabolic modulation has not been explored.

In T cells, reactive oxygen species (ROS) are generated as a by-product of mitochondrial respiration, which is tightly coupled to a cell’s metabolic status (21, 22). During GVHD, T cells increase mitochondrial respiration, fatty acid oxidation (FAO), and ROS production (23, 24). Increased ROS levels produced during GVHD render T cells susceptible to inhibitory modulation of the F\textsubscript{1}F\textsubscript{0}-ATP-synthase complex (23) and also mediate T cell apoptosis (25, 26). Based upon these data, we hypothesized that PD-1 modulates apoptosis in alloreactive T cells by influencing the generation of ROS through control of oxidative metabolism. To test this hy-
mismatched BM transplantation (BMT), B6D2F1 mice were conditioned according to the manufacturer’s instructions. For the B6 into F1 MHC-without T cells, 24 h later (day 0). Unless stated otherwise, donor cells were negatively selected using the bead-based Pan T Cell Isolation Kit II (Miltenyi Biotec) and labeled with CellTrace Violet. NSG mice were injected with 10^7 human PBMCs, and each recipient mouse received PBMCs from a single donor, similar to previously published studies (29, 30). In some experiments, NSG mice were conditioned on day 1 with 240 cGy eB (137Cs source). Splenocytes were harvested between days 12 and 14, stained for human CD3, CD8, and PD-1, and assessed for ROS levels.

Mouse MLR
A total of 5 × 10^5 OT-I T cells or 10^6 B6-Ly5.2 or PD-1–KO T cells were negatively-selected from naive animals, labeled with CellTrace Violet, and cultured for 72 h with 3 × 10^5 C57BL/6 OT-I and B6D2F1 T cells, respectively. MLRs were performed in 96-well flat-bottom plates (Corning) in DMEM (HyClone) supplemented with 10% FBS (GemCell), l-glutamine, nonessential amino acids, sodium pyruvate, and penicillin/streptomycin (all from Life Technologies).

Human MLR
PBMCs from healthy volunteers were enriched by gradient centrifugation using lymphocyte separation medium (Corning). T cells were negatively selected using the bead-based Pan T Cell Isolation Kit II (Miltenyi Biotec) and labeled with CellTrace Violet. Non–T cell fractions of PBMCs were eluted off the column and treated with 25 μg/ml mitomycin C for 45 min at 37°C. T cells were cocultured with non-T BM cells from unrelated donors in AIM-V culture media (Life Technologies) for 6 d. Anti-human PD–1 blocking Abs (clone j116; Bio X Cell) or anti-PD-L1-blocking Abs (clone 19E2A3; BioLegend) were added at a final concentration of 20 or 10 μg/ml, respectively, on days 0, 3, and 6 of culture.

Anti-PD–1/PD-L1 Ab treatment in vivo
For B6-L2 blockade, transplant recipients received 200 μg clone 10E9G2 (or Rat IgG2 [clone LTE-2]) and for PD-1 blockade they received 250 μg mouse clone RMP1-14 (control = Rat IgG2 [clone 2A3]) on days 0, 3, and 6 post-BMT. For PD-L2 blockade, animals received clone TY25 (control = Rat IgG2 [2A3]), 200 μg/dose, on a similar dosing schedule. All blocking Abs were purchased from Bio X Cell.

LYC-31138 treatment
For BMT survival experiments, F344-ATPase modulator LYC-31138 was dissolved in vehicle (5% solubilizer/20% labrafil/75% carboxymethylcellulose [0.5% in PBS]) and administered by oral gavage, beginning on day 5 post-BMT, at 30 mg/kg every other day for a total of nine doses. LYC-31138 treatment following dendritic cell (DC) immunization was begun on day 1 and continued for a total of four doses. As a control for all LYC-31138 experiments, mice were administered vehicle only (5% solubilizer/20% labrafil/75% carboxymethylcellulose) on the same dosing schedule. To measure apoptosis and ROS following LYC-31138 treatment, a single treatment was administered, and splenocytes were harvested 2.5 h later (when the drug had reached maximal serum concentration).

Manganese(III) tetrakis[5,10,15,20-benzoic acid]porphyrin chloride and N-acetyl cysteine treatment
Manganese(III) tetrakis[5,10,15,20-benzoic acid]porphyrin chloride (MnTBAP) was dissolved in 0.1 N NaOH as 28-mM stock, filtered through 0.1-μm membranes, and finally diluted in sterile PBS to be given at 10 mg/kg. In recipient mice received a single dose of MnTBAP on day 7 post-BMT (for apoptosis measurements) or five daily doses of MnTBAP starting on day 8 (for clinical scoring). As a control, recipients received sterile PBS. For in vitro antioxidant treatment, MnTBAP or N-acetyl cysteine was added to MLR cell media at 24 h to a final concentration of 100 μM and 10 mM, respectively.

Flow cytometry
Flow cytometry was performed according to previous reports following generation of a single-cell suspension (24). Unless otherwise noted, all Ab and streptavidin staining was carried out on 10^6 cells for 15 min at 4°C in PBS with 2% FBS. To measure total or mitochondrial ROS, cells were stained for surface markers, loaded with 20 μM MitoSOX Red (Molecular Probes) or 10 μM MitoPyr1 (Tocris) in HBSS containing 2.5 mM probenecid (Molecular Probes), and incubated for 30 min at 37°C. Following incubation, cells were washed twice, kept ice-cold in HBSS/probenecid, and analyzed immediately. To measure superoxide production, Ab-stained cells were incubated with 2.5 μM dihydroethidium (Molecular Probes) in HBSS for 30 min at 37°C, followed by two washes and immediate analysis. For measurement of mitochondrial

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potential ($\Delta$$_{\text{NADH}}$). Ab-stained cells were placed in 40 nM tetra-methylrhodamine-methyl ester (TMRM) suspended in phenol red-free DMEM supplemented with 5% FBS, t-glutamine, sodium pyruvate, and nonessential amino acids. Cells were incubated with TMRM for 30 min at 37°C. Staining was quenched by placing samples on ice, and $\Delta$$_{\text{NADH}}$ was immediately assessed. To measure total and respiring mitochondrial mass, cells were stained for cell surface markers and incubated with 50 nM MitoTracker Red and/or 5 nM MitoTracker Deep Red (both from Molecular Probes) in PBS with 10% FBS for 30 min at 37°C. After incubation, cells were washed twice and kept on ice in PBS with 10% FBS until analysis. Apoptosis was measured using Annexin V-allophycocyanin in 1× Annexin V staining buffer, according to the manufacturer’s instructions (BD Biosciences). For intracellular IFN-γ and TNF-α staining, splenocytes were incubated for 6 h on 96-well plates coated with 2.5 μg/ml CD3/CD28 Abs (eBiosience) in the presence of 1 μM brefeldin A. Cells were stained for membrane Ags, fixed and permeabilized using a FoxP3 Fixation/Permeabilization Kit (eBioscience), and stained with anti-cytokine Abs for 40 min at 4°C. For granzyme B (GzmB) and GLUT1 detection, spleen cells were stained for surface Ags, fixed/permeabilized, and stained with PE-conjugated anti-GzmB Abs or unlabeled rabbit anti-glut-1 mouse GLUT1 Ab, followed by allophycocyanin-conjugated goat anti-rabbit IgG Fab fragment. Flow cytometry data were acquired using a FACSCanto II flow cytometer (BD Biosciences) and analyzed using FlowJo software, version 7.6.1 (TreeStar).

Oxygen consumption rate and extracellular acidification rate measurement

Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) values were measured using a Seahorse XF24 metabolic analyzer. Briefly, donor T cells were purified from B6 into F1 recipients on day 7 post-BMT using bead-based negative selection. Cell suspensions were pretreated with 100 μM Etomoxir (or PBS control) for 15 min, followed by plating (7–8 × 10^5 cells/well) onto XF24 plates (Seahorse Biosciences) coated with 0.5 mg/ml poly-t-lysine (Sigma). Cells were maintained in XF medium (Seahorse Biosciences) supplemented with 1 mM sodium pyruvate (Sigma), 11 mM glucose (Sigma), and 1% FBS. A total of 100 μl cells was spun down onto poly-t-lysine–coated plates, and 530 μl XF medium was added to each well, followed by incubation for 30 min in a CO$_2$-free incubator at 37°C. The Seahorse analyzer was run under the manufacturer’s protocol, with oligomycin (1 μM), FCCP (1 μM), and antimycin A (1 μM) injected through ports A, B, and C respectively.

Statistical analysis

Graphing and statistical analysis were performed using GraphPad Prism version 5.01 for Windows (San Diego, CA, www.graphpad.com). Bar graphs show mean ± SEM. The unpaired Student t test with two tails or the multiple t test with Holm–Sidak correction (for multiple comparisons) was used for statistical interpretation, unless noted otherwise. The paired Student t test was used to analyze human MLR data, and the Mantel–Cox log-rank test was used for survival curve analysis. The p values < 0.05 were considered statistically significant.

All investigations were conducted according to the Declaration of Helsinki principles. All animal and human studies were approved by the appropriate institutional review boards. Written informed consent was received from human participants prior to their inclusion in the study.

Results

T cells increase PD-1 expression during GVHD

We first studied PD-1 expression in T cells during GVHD. In initial experiments, B6 BM and B6-Ly5.2 (CD45.1+) T cells were transferred to lethally irradiated B6D2F1 recipients, as described in Materials and Methods. T cells from syngeneic transplants (B6-Ly5.2 T cells into B6 recipients) served as non-GVHD controls. Donor T cells (CD45.1+, TCR-β+) were recovered on day 7 post-BMT and analyzed for PD-1 expression by flow cytometry. Well-divided, CD8+ allospecific T cells upregulated PD-1 expression, but syngeneic CD8+ T cells did not (Fig. 1A, 1B). PD-1Hi T cells were exclusively among the well-divided cells, and the percentage of PD-1Hi cells increased from days 4 to 8 post-BMT and then plateaued (Fig. 1B). CD4+ T cells increased PD-1 levels similarly posttransplant (Supplemental Fig. 1A). In contrast to the exhausted phenotype displayed by some PD-1Lo cells (4, 8), allo-reactive PD-1Hi T cells upregulated IFN-γ production and increased GzmB expression (Supplemental Fig. 1B).

PD-1 levels correlate with levels of ROS

Alloreactive T cells increase superoxide levels during GVHD (23). Therefore, we asked whether levels of ROS tracked with PD-1 status by measuring CellROX staining in PD-1Lo versus PD-1Hi donor T cells. Well-divided PD-1Hi donor T cells had ROS levels > 3.5-fold higher than naive T cells at multiple times post-allogeneic BMT; PD-1Lo cells did not (Fig. 1C–E, Supplemental Fig. 1C). The superoxide-specific dye dihydroethidium demonstrated a similar increase specifically in PD-1Hi T cells (data not shown). We then tested the correlation between PD-1 and ROS in an mHc-mismatched model of GVHD by transplanting C3H.SW T cells into B6 recipients (details in Materials and Methods). PD-1Hi C3H.SW T cells from both CD4 and CD8 subsets also increased ROS levels in this second model (Supplemental Fig. 1D). As a control, >95% of syngeneic T cells undergoing lymphopenia-induced proliferation remained PD-1Lo and did not increase ROS levels (Fig. 1C, 1D). A small percentage of well-divided syngeneic T cells expressed PD-1 (Fig. 1A, upper left quadrant, 2.83%), and ROS levels were elevated in these PD-1Hi cells compared with well-divided PD-1Lo cells (Supplemental Fig. 1E). However, PD-1+ cells from syngeneic recipients expressed less PD-1 (based on median fluorescence intensity [MFI] values) and had lower CellROX staining than did PD-1+ T cells from allogeneic recipients (data not shown).

CellROX staining measures total cellular ROS levels. To test whether mitochondrial respiration also correlated with PD-1 expression, we measured mitochondrial membrane potential using TMRM and mitochondrial H$_2$O$_2$ levels with the organelle-specific probe MitoPY1 (31). Both parameters increased specifically in PD-1Hi T cells (Fig. 1F, 1G), suggesting a contribution from mitochondrial respiration toward total cellular ROS in these cells. We then asked whether ROS production served as a nonspecific marker of cellular activation or specifically tracked with PD-1Hi status. We assessed this by staining for multiple common activation markers (CD71, CD98, CD69, CD25, CD44, or CD11a) in ROSLo versus ROSHi T cells (32–37). Roughly 90% of ROSHi cells expressed PD-1, whereas only 30% of ROSLo cells did so, confirming an association between PD-1 and ROS (Supplemental Fig. 2A). None of the other activation markers correlated with ROS to this extent (Supplemental Fig. 2B–G). In addition, T cell differentiation status did not predict PD-1 status, because both PD-1Hi and PD-1Lo populations exhibited similar effector-like profiles (Supplemental Fig. 2H).

PD-1 controls ROS levels in allospecific T cells

We next explored whether PD-1 directly controlled ROS levels in allospecific T cells using two methods to inactivate PD-1 signaling. First, wild-type (WT) or PD-1–KO (27) T cells were transferred into B6D2F1 recipients, and ROS levels were quantitated on day 7 posttransplant. ROS levels decreased 2-fold in both CD8 and CD4 PD-1–KO cells, despite ROS levels being similar prior to transplant (Fig. 2A, 2B, Supplemental Fig. 3A). Mitochondrial H$_2$O$_2$ levels followed a similar pattern (Fig. 2C). PD-1–KO T cells also decreased ROS levels during an in vitro MLR, with decreases occurring at all cell divisions (Fig. 2D), indicating that PD-1 regulates ROS levels independent of cell division status.

To confirm the finding of decreased ROS levels in PD-1–KO cells, we next disrupted PD-1 signaling with blocking Abs against PD-1 or PD-L1. In vivo blockade of PD-L1 reduced ROS levels 2-fold in PD-1Hi T cells but did not affect ROS levels in PD-1Lo cells (Fig. 2E, 2F). Blocking Abs against PD-1 had a similar effect...
PD-1 blockade also lowered mitochondrial $H_2O_2$ levels in PD-1 Hi T cells (Fig. 2G). Blockade of PD-L2 did not alter ROS levels in alloreactive cells (data not shown).

Our data indicated that PD-1 inhibition decreased ROS levels in alloreactive cells. In contrast, Saha et al. (20) recently demonstrated that superoxide levels did not decrease in BALB/C T cells transplanted into PD-1-deficient B6 recipients. To explore this discrepancy, we repeated PD-1/ROS analysis in the BALB/C into B6 model (details in Materials and Methods). Similar to the findings of Saha et al. (20), ROS levels did not decrease in BALB/C T cells when transferred into irradiated PD-1-L1–KO recipients (Supplemental Fig. 3D). To determine whether one T cell metabolic phenotype was generalizable to most GVHD models, we analyzed ROS changes following PD-1 blockade in multiple additional MHC- and miHC-mismatched strain combinations (summarized in Table I). As an example, C3H.SW T cells transferred into PD-1L1-deficient mice (17) decreased both ROS and mitochondrial $H_2O_2$ levels identically to B6 T cells transferred into F1 recipients (Fig. 2H, 2I). Similar reductions in ROS levels following PD-1/PD-L1 blockade, using both genetic and Ab-driven inactivation, occurred in all additional models examined, including B6 T cells into BALB/C recipients, B6 T cells into C3H SW recipients, and C3H/HeJ T cells into B6 recipients. Genetic and pharmacologic blockade of PD-1 also drastically exacerbated GVHD severity, as seen in previous studies (18, 20), with PD-1–inhibited cells displaying an activated phenotype similar to that of PD-1–sufficient cells (data not shown).

To determine whether PD-1–driven changes in ROS occurred in human T cells, we performed MLRs using human PBMCs, followed by analysis of both PD-1 and ROS status. As seen in Fig. 3A, responding T cells from murine and human MLRs up-regulated PD-1, and PD-1 expression (PD-1Hi versus PD-1Lo) correlated with ROS level in both systems (Fig. 3B, Supplemental Fig. 3E). Interestingly, in human MLRs, a subset of PD-1Hi cells never divided. These PD-1 Hi cells also displayed higher ROS levels compared with nondividing PD-1 Lo cells (∼2-fold, $p < 0.001$).

**FIGURE 1.** Alloreactive T cells concomitantly increase PD-1 expression and cellular ROS levels. (A) B6-Ly5.2 donor T cells (CD45.1+) were labeled with CellTrace Violet dye (CellTrace) and transferred to irradiated B6D2F1 (allogeneic = GVHD) or syngeneic B6 (Syn) recipients, as detailed in Materials and Methods. T cells were recovered from spleens on day 7 post-BMT and stained for PD-1. Plots are gated on CD45.1+, TCR-b+ CD8a+ cells. (B) The percentage of PD-1Hi cells was quantitated in donor CD8 T cells on days 4, 8, and 12 following syngeneic (●) or allogeic (○) transplantation. (C) B6-Ly5.2 donor T cells were stained for PD-1 similar to (A), followed by assessment of total cellular ROS using CellROX Deep Red (CellROX). (D) CellROX MFI was quantitated in T cells recovered from multiple recipients and compared with MFI values in unmanipulated (naive) T cells, which was set to a relative value of 1. Gating and analysis for PD-1Hi/Lo were as shown in (C) ($n = 4–6$ mice/group). (E) ROS levels were measured in PD-1Lo and PD-1Hi as gated in (C) donor CD8 T cells on days 4, 8, and 12 post-BMT and expressed as the difference in CellROX MFI compared with naive T cells ($n = 5–7$ mice/group). ***(p < 0.001, ****p < 0.0001.****
To define the in vivo correlation between PD-1 and ROS in human T cells, PBMCs from healthy volunteers were labeled with CellTrace, transferred to immunodeficient NSG mice (xenogeneic model of GVHD detailed in Materials and Methods) (29, 30), and harvested between days 12 and 14. Human T cells activated in vivo upregulated PD-1 with increasing cell division (Fig. 3C), and PD-1 expression again correlated with ROS levels (Fig. 3D). Addition of PD-1– or PD-L1–blocking Abs to human MLR cultures reduced ROS levels similarly to changes seen in murine PD-1–KO T cells (Fig. 3E, 3F, Supplemental Fig. 3F). In summary, in multiple in vivo animal models, during both murine and human MLRs, and in the course of xenogeneic GVHD, alloreactive T cells upregulated PD-1 and, in all cases, higher PD-1 expression correlated with increased ROS levels. In addition, in every combination attempted save one, blockade of PD-1 decreased levels of ROS.

Inhibiting FAO negates PD-1 regulation of ROS levels

We hypothesized that PD-1 increased ROS production in alloreactive T cells by driving enhanced oxidative respiration. In this hypothesis, actively respiring mitochondria would upregulate their transmembrane potential ($\Delta \psi_{\text{mt}}$), which would slow electron transfer between chain complexes, favoring electron leak and resulting in elevated superoxide formation (39). In support of this, PD-L1 blockade decreased $\Delta \psi_{\text{mt}}$ in allogeneic T cells, without significantly changing their mitochondrial mass (Supplemental Fig. 3G). Importantly, changes in ROS levels did not result from mitochondrial damage, because the percentage of cells with damaged mitochondria was similar between isotype and anti–PD-L1–treated animals (data not shown).

We showed previously that alloreactive T cells rely on FAO as a component of oxidative phosphorylation to meet their bioenergetic demands (23, 24). To test the relationship between oxidative metabolism and PD-1, we measured OCRs in day-7 allogeneic T cells following PD-L1 blockade. Consistent with a reduction in $\Delta \psi_{\text{mt}}$, anti–PD-L1–treated T cells had lower basal OCR (Fig. 4A, 4B). Pretreatment of alloreactive T cells with etomoxir, an inhibitor of FAO, reduced OCRs and nullified OCR differences between anti–PD-L1– and control–treated T cells (Fig. 4B, Supplemental Fig. 3H). Etomoxir treatment also decreased ROS and mitochondrial H2O2 levels and, most importantly, obviated the ability of anti–PD-L1 treatment to lower ROS levels further (Fig. 4C, 4D). PD-L1 blockade also increased the ECAR of alloreactive T cells but only when oxidative respiration was inhibited by oligomycin (Fig. 4E). This increased ECAR was likely facilitated by increased glucose transport, because GLUT1 levels rose following PD-1 pathway blockade (Fig. 4F). In total, these metabolic data...
demonstrate that, in alloreactive T cells, inhibition of FAO negates PD-1–driven changes in oxidative metabolism and ROS levels.

**PD-1 induces T cell apoptosis through a ROS-dependent mechanism**

Although PD-1 is implicated in immune homeostasis and peripheral tolerance (40), it was originally discovered as a marker of lymphocyte apoptosis (10). Therefore, we probed the relationships among PD-1, ROS, and cell death in alloreactive T cells. PD-1 expression directly correlated with Annexin V staining in alloreactive cells (Fig. 5A), and Annexin V + staining decreased after Ab blockade and genetic disruption of PD-1 (Fig. 5B, data not shown). Decreased alloreactive cell death following PD-1 blockade resulted in more well-divided T cells, a higher proportion of PD-1 Hi cells, and a dramatic exacerbation of GVHD (20). From these data, we posited that PD-1 potentiates apoptosis through modulation of ROS levels. To test this hypothesis, we treated MLR cultures, or day-7 alloreactive T cells placed ex vivo, with the antioxidant MnTBAP (41, 42), a compound known to protect lymphocytes from ROS-mediated cell death (26). Both MLR cultures and day-7 T cells incubated overnight with MnTBAP (or the structurally unrelated antioxidant N-acetyl cysteine) reduced their percentage of Annexin V + cells (Supplemental Fig. 4A–C).

We next confirmed the role of ROS in driving T cell apoptosis in vivo by administering MnTBAP to recipients during a miHCM-mismatched model of GVHD (C3H.SW into B6). To limit interference with initial priming, we waited to treat recipient mice until day 9 posttransplant with a single dose of MnTBAP (43). MnTBAP treatment reduced the percentage of Annexin V +, PD-1 Hi T cells, but it did not alter apoptosis in PD-1 Lo lymphocytes (Fig. 5C, Supplemental Fig. 4D). Cell death was similarly reduced following MnTBAP treatment in an MHC-mismatched model of GVHD (Supplemental Fig. 4E), and apoptosis of alloreactive T cells corre-

<table>
<thead>
<tr>
<th>GVHD Model</th>
<th>Method of PD-1/PD-L1 Blockade</th>
<th>PD-1 Correlated w/ROS Level</th>
<th>ROS Decreased w/PD-1 Blockade (% Decrease)</th>
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<tbody>
<tr>
<td>B6 into F1</td>
<td>Donor PD-1–KO T cells</td>
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<td>Anti–PD-L1 Abs</td>
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<td>Yes (52)</td>
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<td>Yes (50)</td>
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<tr>
<td>B6 into BALB/C</td>
<td>Donor PD-1–KO T cells</td>
<td>Yes</td>
<td>Yes (54)</td>
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<td>B6 into C3H.SW</td>
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<td>Yes</td>
<td>Yes (43)</td>
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<td>Anti–PD-L1 Abs</td>
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<td>BALB/C into B6</td>
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<td>Xenogeneic GVHD</td>
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**Table I. Influence of PD-1 blockade on ROS levels in multiple models of GVHD**

![Image](http://www.jimmunol.org/) Downloaded from http://www.jimmunol.org/ by guest on June 6, 2017

**FIGURE 3.** PD-1 controls ROS levels in human T cells. (A) Murine B6-Ly5.2 or purified human T cells were labeled with CellTrace and cocultured with F1 splenocytes (murine) or non-T PBMCs from unrelated donors (human). At harvest, cells were stained with cell surface markers, and PD-1 levels were quantitated. Murine plots are gated on CD45.1 +, TCR-β + CD8α + cells; human plots represent CD3 + CD8α + cells. (B) ROS levels were measured in well-divided murine or human PD-1 Lo (lower left quadrant) versus PD-1 Hi (upper left quadrant) CD8 + T cells, as outlined in (A). Graphs represent independent data from six murine and eight human samples. (C) Human PBMCs were labeled with CellTrace and injected into immunodeficient (NSG) mice in a model of xenogeneic GVHD. Cells were harvested between days 12 and 14, gated on human CD3 +, CD8 + cells, and stained for PD-1. (D) ROS levels were analyzed in human CellTrace + PD-1 Lo (lower left quadrant) versus PD-1 Hi (upper left quadrant) CD8 T cells during xenogeneic GVHD, as in (C) (n = 8 NSG recipients/group). (E) Human MLR cultures were treated with anti–PD-L1 Abs on days 0, 3, and 6, and ROS levels were measured in PD-1 Lo versus PD-1 Hi CD8 T cells 8 h after the final treatment, with gating for PD-1 Lo versus PD-1 Hi subsets similar to Fig. 3A. (F) Murine WT or PD-1–KO T cells were cultured with F1 splenocytes, followed by staining for ROS levels as in Fig. 3B (n = 5–6 mice/group). **p < 0.001, ***p < 0.0001.
lated inversely with GVHD severity following serial doses of MnTBAP (Fig. 5D). Notably, MnTBAP-treated animals had identical proportions of PD-1Hi T cells, with similar PD-1 MFI (Supplemental Fig. 4F), placing MnTBAP’s mechanism of action downstream of PD-1 but upstream of cell death. MnTBAP treatment had no effect in syngeneic recipients (data not shown).

Stable PD-1HiROSHi phenotype is specific to alloreactive T cells and requires exposure to Ag

To determine whether PD-1 regulation of ROS is unique to the GVHD environment, we compared PD-1 expression on alloreactive T cells during GVHD with T cells responding to cellular immunization. To model GVHD, OT-I T cells (responsive to the SIINFEKL peptide of OVA) were transferred into irradiated recipients expressing ubiquitous OVA (CAG-OVA). This model results in classic characteristics of acute GVHD, including hunched posture, fur ruffling, decreased mobility, weight loss, and 40% lethality by day 30 (data not shown). For cellular immunization, the same OT-I T cells were injected into syngeneic hosts (B6-Ly5.2), followed by i.v. immunization with purified CAG-OVA DCs (24). OT-I T cells transferred into an Ag-driven GVHD environment had robustly upregulated PD-1 levels by day 7 posttransplant; OT-I cells following DC immunization did not (Fig. 6A). Consistent with a lack of PD-1 upregulation, OT-I cells on day 7 following cellular immunization did not increase their ROS levels (Fig. 6B, 6C). Absent PD-1 expression was not the result of poor T cell proliferation or aberrant differentiation, because T cells postimmunization divided more than eight times by day 7 (Fig. 6A), acquired an effector-like phenotype (CD44 HiCD62LLo), upregulated GzmB expression, and had equivalent IFN-γ upon restim-

FIGURE 4. Inhibition of FAO negates PD-1–driven changes in ROS levels. (A) B6 into F1 recipients were treated with anti–PD-L1–blocking Abs (or Rat IgG control), as in Fig. 2C. On day 7 posttransplant, CD45.1+ donor T cells were isolated, and OCRs were quantitated using a Seahorse XF24 metabolic analyzer. (B) Baseline OCR values were measured in alloreactive T cells from multiple recipients, with data combined from two independent experiments. Primary data as shown in Fig. 4A (n = 8–9 mice/group). In some cases, T cells were treated with 100 μM etomoxir for 15 min prior to analysis (PBS as negative control). Day-7 PD-1Hi donor T cells from B6 into F1 transplants were treated with etomoxir for 15 min and then evaluated for changes in total cellular ROS (C) or mitochondrial H2O2 levels (D) (using MitoPY1) (n = 8–9 mice/group). (E) Cells were recovered as in Fig. 4A, and ECAR was measured using Seahorse XF24. (F) Following B6 into F1 BMT, day-7 donor CellTraceLo CD4+ or CD8+ T cells were stained for GLUT1 expression level by flow cytometry. **p < 0.01, ***p < 0.001.

FIGURE 5. ROS levels mediate PD-1–induced apoptosis. (A) The percentage of AnnexinV+ cells was quantitated in CellTraceLo PD-1Lo and CellTraceLo PD-1Hi donor T cells 7 d after B6 into F1 BMT. (B) Similar to (A), Annexin V staining was assessed in PD-1Hi CD8 T cells, with or without blockade with anti–PD-L1 Abs (Rat IgG as control). (C) C3H.SW into B6 recipients were treated with the antioxidant MnTBAP on day 9 post-BMT, and Annexin V positivity was assessed in PD-1Hi CD8 T cells the following day. Data represent the percentage of AnnexinV+ cells in PD-1Lo or PD-1Hi CD8 T cell subsets, averaged from n = 5 mice/group. (D) C3H.SW into B6 recipient mice were treated with five doses of MnTBAP, and clinical scores were assessed 1 wk after the final treatment. In all cases, gating for PD-1Lo versus PD-1Hi cells was performed similarly to Fig. 1C. *p < 0.05, ***p < 0.001.
ululation (data not shown). However, lack of PD-1 upregulation following immunization contrasted with PD-1 upregulation observed during acute viral infection (44) or following in vitro stimulation of T cells with anti-CD3 Ab (14). To investigate this discrepancy, we tested the expression of PD-1 on OT-I cells at an earlier time after immunization. On day 3 after DC injection, ∼70% of OT-I T cells adopted a PD-1<sup>HI</sup> phenotype (Fig. 6D), and PD-1 expression correlated with elevated ROS levels at this earlier point (Fig. 6E). Thus, cellular immunization results in limited PD-1 expression, with a concomitant increase in ROS levels, consistent with the transient increase in PD-1 observed during an acute viral response (44).

To test whether the inflammatory milieu of GVHD, combined with a lymphopenic environment, might be sufficient to drive adoption of a PD-1<sup>HI</sup>ROS<sup>HI</sup> phenotype, we cotransferred CD45.1/CD45.2 OT-I T cells and CD45.1<sup>+</sup> (B6-Ly5.2) T cells into irradiated CAG-OVA recipients or B6 syngeneic controls. In the GVHD environment (CAG-OVA recipients), OT-I T cells increased PD-1 expression and upregulated ROS levels; B6-Ly5.2 syngeneic T cells recovered from the same animals did not (Fig. 6F, 6G). No differences in PD-1 expression or ROS status were observed in OT-I or B6-Ly5.2 T cells transferred to syngeneic B6 mice. Thus, cell proliferation alone, even in the context of an inflammatory or lymphopenic milieu, is insufficient to drive adoption of a PD-1<sup>HI</sup>ROS<sup>HI</sup> phenotype.

**PD-1 is required for ROS-dependent metabolic inhibition**

Compounds that modulate the F<sub>1</sub>F<sub>0</sub>-ATP synthase complex have potential application as novel therapies for immune-mediated disease (23). These compounds work, in part, by inducing apoptosis of robustly activated, pathogenic lymphocytes in a manner dependent upon ROS generation (45, 46). Given the increased ROS levels in PD-1<sup>HI</sup> T cells, we hypothesized that PD-1<sup>HI</sup> T cells would show preferential susceptibility to F<sub>1</sub>F<sub>0</sub>-ATPase modulators. To test this idea, irradiated B6D2F1 recipients received either WT or PD-1–KO T cells, followed by a single treatment with the F<sub>1</sub>F<sub>0</sub>-ATPase modulator LYC-31138 on day 7 post-BMT. LYC-31138 treatment increased both ROS levels and Annexin<sup>V</sup><sup>+</sup> staining in WT PD-1<sup>HI</sup> T cells but did not change ROS levels or Annexin<sup>V</sup><sup>+</sup> staining in PD-1–KO T cells (Fig. 7A, 7B). In addition, LYC-31138-driven apoptosis was obviated by pretreatment with anti-PD-L1 Abs (Fig. 7C). We next transplanted C3H.SW T cells into WT or PD-L1–KO recipients in a miHC-mismatched model of GVHD, followed by administration of LYC-31138. In this model, PD-1<sup>HI</sup> T cells decreased nearly 2-fold in WT recipients following LYC-31138 treatment (Fig. 7D) but did not change in number in recipients lacking PD-L1. Consistent with a decrease in donor T cell numbers, LYC-31138 treatment specifically improved survival in WT recipients (Fig. 7E) but not in PD-L1–KO recipients in whom PD-1 signaling was abolished (Fig. 7F). As expected, donor T cell proliferation, cell number, and survival were not changed by LYC-31138 treatment during syngeneic transplantation or following cellular immunization (data not shown).

In summary, our data demonstrate that PD-1 controls ROS levels and mitochondrial respiration in alloreactive T cells in a process that is dependent upon FAO, and that these increases in ROS directly impact T cell survival. In addition, PD-1 blockade, by lowering ROS levels, impairs the potential for subsequent ROS-driven metabolic modulation.

**Discussion**

PD-1 signaling plays an important role in immune homeostasis, both by dampening an initial T cell response to acute activation (9) and by inducing exhaustion in T cells undergoing chronic exposure to Ag (8). In this way, PD-1 acts as a safeguard against prolonged and potentially detrimental activation (5–7). During GVHD, PD-1 expression on alloreactive T cells limits the severity of disease (23). These compounds work, in part, by inducing apoptosis of robustly activated, pathogenic lymphocytes in a manner dependent upon ROS generation (45, 46). Given the increased ROS levels in PD-1<sup>HI</sup> T cells, we hypothesized that PD-1<sup>HI</sup> T cells would show preferential susceptibility to F<sub>1</sub>F<sub>0</sub>-ATPase modulators. To test this idea, irradiated B6D2F1 recipients received either WT or PD-1–KO T cells, followed by a single treatment with the F<sub>1</sub>F<sub>0</sub>-ATPase modulator LYC-31138 on day 7 post-BMT. LYC-31138 treatment increased both ROS levels and Annexin<sup>V</sup><sup>+</sup> staining in WT PD-1<sup>HI</sup> T cells but did not change ROS levels or Annexin<sup>V</sup><sup>+</sup> staining in PD-1–KO T cells (Fig. 7A, 7B). In addition, LYC-31138-driven apoptosis was obviated by pretreatment with anti-PD-L1 Abs (Fig. 7C). We next transplanted C3H.SW T cells into WT or PD-L1–KO recipients in a miHC-mismatched model of GVHD, followed by administration of LYC-31138. In this model, PD-1<sup>HI</sup> T cells decreased nearly 2-fold in WT recipients following LYC-31138 treatment (Fig. 7D) but did not change in number in recipients lacking PD-L1. Consistent with a decrease in donor T cell numbers, LYC-31138 treatment specifically improved survival in WT recipients (Fig. 7E) but not in PD-L1–KO recipients in whom PD-1 signaling was abolished (Fig. 7F). As expected, donor T cell proliferation, cell number, and survival were not changed by LYC-31138 treatment during syngeneic transplantation or following cellular immunization (data not shown).

In summary, our data demonstrate that PD-1 controls ROS levels and mitochondrial respiration in alloreactive T cells in a process that is dependent upon FAO, and that these increases in ROS directly impact T cell survival. In addition, PD-1 blockade, by lowering ROS levels, impairs the potential for subsequent ROS-driven metabolic modulation.
of GVHD, and lack of PD-1 signaling drives T cell expansion, increases IFN-γ production, and accelerates GVHD onset (18). PD-1 blockade also leads to metabolic changes in alloreactive cells (20), but the mechanisms underlying these metabolic changes have not been well studied, and the consequences of PD-1 blockade on subsequent metabolic modulation have not been explored.

In this study, we demonstrate that alloreactive T cells express high levels of PD-1 early posttransplant, with a phenotype similar to PD-1–expressing T cells recovered during acute viral infections (47, 48). Alloreactive PD-1Hi T cells also increase cellular ROS levels with a concomitant increase in T cell apoptosis. It is noteworthy that ROS levels do not correlate with expression of other common activation markers, including CD71 and CD98, which are directly linked to T cell metabolism (32). This suggests that there is something unique about the coordinated upregulation of PD-1 and ROS levels. Although ROS levels may correlate with additional members of the CD28 family, such as ICOS (49, 50), increased ROS are not simply generic markers of activation. Importantly, the PD-1Hi ROSHi phenotype is not seen in syngeneic T cells or in well-divided T cells 7 d after cellular immunization. Given the lower levels of fatty acid transport and metabolic coactivators observed in these nonalloreactive cells (24), it is not surprising that ROS levels were lower at the time points examined. For cellular immunization, we speculate that environmental influences dictate this lack of ROS, given that a limited amount of Ag is presented for a finite period of time in the context of nominal systemic inflammation. These limitations lead to only brief stimulation and, consequently, a transient upregulation of PD-1 with limited ROS production (51).

Saha et al. (20) recently demonstrated a role for PD-1 in controlling alloreactive T cell metabolism in a BALB/C into B6 GVHD model. In many respects, our findings complement their report. In both studies, blockade of PD-L1 (but not PD-L2) worsened GVHD and led to a more rapid onset of mortality. However, the response of ROS levels to PD-1 blockade was variable. ROS levels did not decrease in BALB/C T cells transferred to PD-L1–deficient hosts (20) (Supplemental Fig. 3E). In contrast, in all other strain combinations tested, using a variety of donor strains (B6, C3H/HeJ, and C3H.SW T cells), and a number of inactivation strategies, blockade of PD-1 decreased ROS levels and limited T cell apoptosis. We do not have a clear explanation for why BALB/C T cells behave differently than all other alloreactive cells examined, but we suspect that the impact of PD-1 on metabolic reprogramming may ultimately depend upon the genetic differences in the strain of the donor T cell (52, 53). Regardless of the reason for the difference, the weight of evidence in Table I demonstrates that, in a great majority of alloreactive T cells, PD-1 blockade decreases ROS levels, including in human T cells.

**FIGURE 7.** PD-1–driven ROS are required for subsequent metabolic inhibition. WT or PD-1−/− T cells were transferred to F1 hosts, and recipient mice were treated with LYT-31318 (or vehicle control) on day 7 post-BMT. Changes in total ROS (A) or the percentage of AnnexinV+ cells (B) was quantitated in WT PD-1Lo, WT PD-1Hi, or CellTraceLo PD-1−/− CD8 T cells 150 min after LYT-31318 treatment (n ≥ 3 mice/group). PD-1 status gated as in Fig. 1C. (C) B6 into F1 recipient mice were treated with anti–PD-L1–blocking Abs (as in Fig. 2E), followed by LYT-31318 treatment (or vehicle control) on day 7 post-BMT, and the percentage of AnnexinV+ cells was quantitated 150 min after LYT-31318 treatment (n ≥ 3 mice/group). (D) C3H.SW T cells were transferred to WT or PD-1−/− deficient mice, and recipients were treated with four doses of LYT-31318 or vehicle control. Cells were harvested on day 12 post-BMT, and the absolute number of PD-1Hi or PD-1−/− donor, CD8+ T cells (Ly9.1+ TCR-β+) was quantitated. Gating for PD-1 subsets was performed similarly to Fig. 1C. Data were pooled from two identical experiments (n ≥ 6/group). (E) C3H.SW T cells were transferred to irradiated C3H.SW (syngeneic) or WT B6 recipients. Beginning on day 5 post-BMT, recipient mice received LYT-31318 (half-filled circles) or vehicle (•) every other day for a total of nine doses. Survival was monitored up to 70 d postransplant. (F) Similar to (E), only C3H.SW T cells were transferred to PD-L1−/− KO recipients, followed by LYT-31318 or vehicle treatment (n = 10–12 mice/allogeneic group). *p < 0.05.
In T cells, ROS can be generated by either NADPH oxidases (e.g., NADPH oxidase 2 [NOX2], dual-substrate oxidase 1 [DUOX1]) (54–56) or the action of respiring mitochondria, whose function is tightly coupled to a cell’s metabolic status (57–59). We observed no change in NOX or DUOX enzyme complexes in alloreactive T cells, and ROS levels were insensitive to both NOX2 and DUOX1 inhibition (data not shown). In contrast, mitochondrial respiration likely contributes to PD-1–driven changes in total cellular ROS because a significant correlation exists between total cellular ROS and mitochondrial H$_2$O$_2$ levels (Supplemental Fig. 3D). Furthermore, decreases in mitochondrial H$_2$O$_2$ mirrored decreases in ROS in PD-1–KO T cells (Fig. 2B, 2C), following PD-L1 blockade (Fig. 2E, 2F), in T cells recovered from PD-L1–deficient recipients (Fig. 2G, 2H), as well as following blockade of FAO with etomoxir (Fig. 4C, 4D). Importantly, PD-1 blockade does not completely prevent ROS generation, because ROS levels in PD-1–KO T cells remain 2-fold higher than in unmanipulated T cells (Fig. 2). In this context, blockade of PD-1 may simply reduce a portion of ROS (e.g., the ROS generated from FAO) while preserving ROS generation from other internal sources (Fig. 4), thus lessening apoptosis while still preserving the physiological levels of ROS critical for T cell activation (58). In addition, the fact that PD-1 blockade modulates ROS levels in PD-1LO cells, but not in adjacent PD-1HI cells, suggests that PD-1-dependent regulation of mitochondrial respiration is likely a cell-intrinsic process.

We reported previously that alloreactive T cells increase superoxide levels during GVHD as a result of increased mitochondrial respiration (23). In this study, we demonstrate that PD-1 regulates T cell oxygen consumption rates, mitochondrial H$_2$O$_2$ production, and total cellular ROS levels. Our data also suggest that PD-1–driven increases in ROS depend upon FAO, because ROS levels are not additionally susceptible to PD-1 blockade if cells are treated with etomoxir (Fig. 4). From these data, we speculate that PD-1 signaling drives T cells toward increased lipid metabolism. During GVHD, intensely activated T cells must choose between glucose and alternative substrates, such as fatty acids, to meet their bioenergetic demands. PD-1 signaling influences this choice by blocking glucose use through restricted Glut1 expression (20, 21), necessitating the upregulation of alternative energy sources, including fat metabolism. When PD-1 signaling is blocked, Glut1 inhibition is released (Fig. 4F), and T cells readily upregulate aerobic glycolysis, particularly when oxidation is compromised (Fig. 4E). In support of this proposition, PD-1 also modulates other key regulators of T cell glucose metabolism, including both Akt and mTOR signaling (21, 22).

Increased ROS mediate T cell death in numerous systems (25, 26, 55, 60), including through the process of activated T cell autodestruction (25). During activated T cell autodestruction, high ROS levels drive down the expression of antiapoptotic proteins (e.g., Bcl2), facilitating the release of intracellular mediators into the cytoplasm (e.g., cytochrome c) and ending in apoptotic cell death (61). In the current study, both in vitro and in vivo antioxidant treatment prevented apoptosis of PD-1LO T cells but they did not have the same effect on PD-1HI T cells, confirming that ROS are key to carrying out PD-1–induced cell death (Fig. 5). We postulate that, in alloreactive T cells, ROS levels generated from PD-1–driven increases in oxidative respiration trigger the release of intracellular mediators that initiate T cell apoptosis. We cannot rule out a role for Fas/FasL–induced apoptosis in the current study (62), but this mechanism seems less likely given that Fas ligand expression was not required for GVHD enhancement in previous studies of PD-1 inhibition (18).

Given the emerging clinical significance of PD-1 blockade as a novel immunotherapy (11, 13), it is extremely important to understand how disruption of PD-1 signaling affects subsequent T cell function. Given their increased oxidative status, we correctly reasoned that PD-1HI lymphocytes would be preferentially susceptible to F$_1$–F$_0$-ATPase modulation because of their high ROS levels (Fig. 7B). This suggests that augmentation of PD-1 signaling might sensitize cells to ROS-dependent interventions and enhance the efficacy of ROS-targeted therapies. Conversely, as seen in Fig. 7D–F, PD-1 blockade renders T cells resistant to future ROS-dependent metabolic inhibition (45, 46), a finding that may have significant clinical implications. For example, adjuvant therapies that depend upon increased ROS levels [e.g., glucocorticoids, methotrexate, and cyclophosphamide (63–65)] will likely exhibit reduced efficacy in T cells following PD-1 blockade. In these situations, the use of alternative ROS-independent therapies [e.g., targeted use of cyclosporine or mycophenolate (66–68)] might be preferred.

In summary, PD-1 controls disease severity in multiple GVHD models by dictating T cell sensitivity to apoptosis through modulation of mitochondrial respiration and subsequent generation of intracellular ROS. Blockade of PD-1 signaling decreases apoptosis and also lessens the susceptibility of T cells to future oxidative manipulation. It is easy to envision how these PD-1 signals might be further exploited to influence immune reactions in a bidirectional manner. Downregulation of PD-1 could preserve T cell survival by minimizing ROS generation and limiting ROS-driven apoptosis, an important attribute for in vivo immunotherapy. In contrast, augmentation of PD-1 signals might increase ROS-dependent apoptosis, thereby serving as a potent treatment for T cell–mediated immune disease, particularly in situations where intracellular ROS levels are already increased. Thus, constructing a detailed understanding of the metabolic effects downstream of PD-1 will remain an important area of investigation as the clinical use of anti–PD-1 therapies continues to grow.

Disclosures
A.W.O., L.-Y.H., L.F., and G.D.G. are employed by and have financial interest in Lycera, Inc., a private company that has licensed technology from the University of Michigan that may have some relationship to content in this report. The other authors have no financial conflicts of interest.

References


Supplementary Figures

Title: Programmed death-1 controls T cell survival by regulating oxidative metabolism

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Contents:

4 Supplemental Figures and Legends
Supplementary Figure 1. Donor CD4 T cells simultaneously increase PD-1 and ROS levels post allogeneic BMT. A, B6 Ly5.2 (CD45.1⁺) donor T cells were labeled with CellTrace and transferred to syngeneic (Syn) or allogeneic (GVHD) recipients. T cells were recovered on days 4, 8 and 12 post-BMT, stained for PD-1 and the percentage of PD-1⁺ cells (of total donor CD4 cells) was quantitated. Plots are gated on CD45.1⁺, TCR-β⁺, CD4⁺ cells, n =4-6 mice/group. B, For IFN-γ staining, day 7 T cells were cultured for 6 hours on anti-CD3/CD28-coated plates in media containing brefeldin A, then stained for surface markers, fixed, permeabilized, and stained for intracellular IFN-γ (left panel). T cells were similarly stained for intracellular Granzyme B, only without ex vivo stimulation (right panel). The percentage of IFN-γ⁺ and GzmB-positive cells was quantitated in donor CD45.1⁺ TCR-β⁺ CD8⁺ cells. C, Donor T cells were harvested as in Supplementary Figure 1A, followed by measurement of total cellular ROS. Changes in CellROX MFI in donor CD4 T cells from multiple recipients were compared to the value in un-manipulated (naïve) T cells (set to relative value of 1), n = 4-6 mice/group. D, CellTrace-labeled C3H.SW donor T cells (Ly9.1⁺) were transferred to irradiated B6 recipients (miHC-mismatched BMT) and recovered on day 10 post-transplant. Ly9.1⁺ TCR-β⁺ donor cells were then stained for CD4 versus CD8, PD-1, and ROS similar to Figure 1D. CellROX MFI differences were then quantitated in well-divided PD-1Lo and PD-1Hi T cells from multiple recipients, n = 3-5 mice/group. E, CellROX levels in divided syngeneic T cells were determined in PD-1Lo (lower left quadrant) versus PD-1Hi (upper left quadrant) cells as shown in Figure 1A. In the remainder of cases, gating for PD-1Lo and PD-1Hi subsets was done as shown in Figure 1C. **p < 0.01, ***p < 0.001
Supplementary Figure 2. In allogeneic T cells, ROS levels correlate specifically with PD-1 status. A-G, B6 Ly5.2 (CD45.1⁺) T cells were labeled with CellTrace and transferred to irradiated, B6D2F1 allogeneic recipients. On day 7 post-BMT, donor T cells were stained for activation markers, cell surface markers, and cellular ROS levels as in Supplementary Figure 1. The percentage of CD71⁺, CD98⁺, CD69⁺, CD25⁺, CD11aHi or CD44Hi cells was then quantified in ROS Lo versus ROSHi T cells from either CD8 (left) or CD4 (right) T cell subsets. H, The percentage of cells with naïve (CD44LoCD62LHi), central-memory (CD44HiCD62LHi) and effector-memory (CD44HiCD62LLo) phenotypes was quantified in PD-1 Lo versus PD-1Hi donor CD8 T cells. PD-1Lo versus PD-1Hi subsets were gated as shown in Figure 1C.
Supplementary Figure 3. PD-1 controls cellular ROS levels in murine and human allogeneic T cells. 

A, Wild-type (WT) or PD-1 knockout (PD-1KO) T cells were transferred to B6D2F1 recipients and changes in CellROX MFI measured day on 7 post-BMT in CD4+ WT PD-1Hi versus PD-1KO T cells, n = 3-5 mice/group. B, B6 into F1 recipient mice were treated with anti-PD-1 blocking antibodies (or Rat IgG control) and ROS levels measured on day 7 in donor PD-1Hi CD4 and CD8 T cells. Staining and gating similar to Figure 2D, n = 3-5 mice/group. C, B6 into B6D2F1 recipients were treated with anti-PD-L1 blocking antibodies, followed on day 7 by simultaneous measurement of total cellular ROS (CellROX) and mitochondrial H2O2 (MitoPY1) in donor, PD-1Hi, CD8 T cells. Linear regression between CellROX and MitoPy1 was then calculated, n = 3-5 mice/group. D, CFSE-labeled Balb/C T cells were transplanted into irradiated WT (solid) or PD-L1KO (hatched) recipients. Donor T cells were recovered on day 5 post-BMT and assessed for cellular ROS in PD-1Lo versus PD-1Hi T cells, n = 3-4/group. PD-1Lo versus PD-1Hi subset gating as performed similar to Figure 1C. E, Murine and human T cells were plated in MLRs (outlined in Figure 3) and harvested on day 4 (murine) or day 6 (human). PD-1 expression and ROS levels were then measured in PD-1Lo (left lower quadrant) versus PD-1Hi (left upper quadrant) CD4+ T cells. F, Human MLR cultures were treated with anti-PD-1 antibodies on days 0, 3, and 6. Eight hours after the final treatment, ROS levels were measured in CD4+, PD-1Hi cells; n = 8 independent human samples. G, B6 into F1 recipients were treated with anti-PD-L1 antibodies (or Rat IgG control) as in Figure 2C. On day 7 post-transplant, mitochondrial transmembrane potential was measured in donor CD8+ T cells. H, BMT and treatment as in Figure 4A (solid circles = anti-PD-L1 antibodies, open circles = Rat IgG control). On Day 7 post-BMT, donor T cells were isolated, pretreated with 100 μM Etomoxir for 15 minutes, and applied to Seahorse XF24 analyzer to measure oxygen consumption rates (OCR).

*p<0.05, **p<0.01, ***p<0.001
**Supplementary Figure 4. Antioxidants protect PD-1\textsuperscript{Hi} T cells from apoptosis.** A, OT-I T cells were stimulated *in vitro* with CAG-OVA splenocytes as detailed in Materials and Methods. Anti-oxidant compounds MnTBAP (100 μM) or NAC (10 mM) were added at 48 hours, cells harvested the following day, and donor T cells (CD45.2\textsuperscript{+}TCRβ\textsuperscript{+}CD8\textsuperscript{+}) stained for AnnexinV. B, The percentage of AnnexinV\textsuperscript{+} cells was quantified in TCRβ\textsuperscript{+}CD8\textsuperscript{+} OT-I cells from multiple replicates, n = 5 donors/group. C, Donor T cells from B6 into B6D2F1 recipients were purified on day 7 post-BMT and plated with B6D2F1 splenocytes for 24 hours. MnTBAP (100 μM) or NAC (10 mM) were added at the time of re-stimulation. After 24 hours, donor T cells were stained for surface markers and AnnexinV as in Supplementary Figure 4B. The percentage of AnnexinV\textsuperscript{+} cells was then quantitated in donor PD-1\textsuperscript{Hi} CD8 T cells. PD-1\textsuperscript{Hi} subset gated as in Figure 1C, n = 5 recipient mice/group. D, B6 into B6D2F1 recipients were treated with PBS or MnTBAP on day 7 post-BMT, donor cells recovered the following day, and donor CD4 or CD8 T cells (CD45.1\textsuperscript{+}, TCR-β\textsuperscript{+}) stained for PD-1 and AnnexinV. E, The percentage of AnnexinV\textsuperscript{+} cells in donor PD-1\textsuperscript{Lo} or PD-1\textsuperscript{Hi} CD4 T cells was quantitated from multiple recipients, per primary data in Supplementary Figure 4D. F, Cells were harvested and stained similar to Figure 5C. Graphs represent the percentage of PD-1\textsuperscript{Hi} T cells (left) or the MFI of PD-1 staining (right) on donor CD8 T cells from multiple recipients, n = 5 mice/group. *p < 0.05, **p < 0.01, ***p < 0.001