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ORIGINAL ARTICLE

Overexpression of program death-1 in T cells has mild impact on allograft survival

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Summary

Program death-1 (PD-1), an inhibitory receptor upregulated on T cells upon TCR stimulation, has been shown to attenuate a number of immune responses *in vivo*, including acute allograft rejection. We tested whether constitutive expression of PD-1 would further inhibit allograft rejection. To this end, we generated transgenic mice expressing T-cell-restricted PD-1 under the control of the *Lck* proximal promoter and *CD2* locus control. PD-1 transgenic (PD-1-Tg) mice did not develop gross abnormalities of thymic development and displayed normal numbers of thymocyte subsets and peripheral T cells. *In vitro*, PD-1-Tg T cells had reduced proliferative and cytokine secretion capacity upon TCR stimulation and cross-linking of PD-1 resulted in diminished phosphorylation of protein kinase C-θ and Akt, as well as increased activation of the phosphate and tensin homolog. However, only T-cell responses to minor but not major mismatches were reduced *in vitro*. Similarly, PD-1-Tg mice exhibited prolonged survival of cardiac allografts only in mice transplanted with heart allografts expressing multiple minor mismatches and treated with suboptimal doses of cyclosporine A. We conclude that genetic engineering of T cells to express PD-1 constitutively has only a mild impact on allograft survival.

Introduction

Acute allograft rejection is dependent on T cells. In addition to receiving signals upon binding of TCR to its cognate antigen, T cells engage multiple positive and negative costimulatory molecules that determine the quality of the T-cell response [1]. The best characterized positive costimulatory receptor on T cells is CD28, which binds CD80 and CD86, mostly expressed on APCs and activated T cells, and whose concurrent engagement during TCR ligation is necessary for T-cell activation. Three negative costimulatory receptors have been identified to date, namely cytotoxic T-lymphocyte attenuator-4 (CTLA-4), program death-1 (PD-1) and B and T-lymphocyte attenuator (BTLA). All three receptors are members of the CD28 immunoglobulin-like super-family, are induced upon T-cell activation and are thought to inhibit T-cell responses after transplantation. Using a mouse model of cardiac transplantation, we have previously shown that engagement of CTLA-4 normally inhibits an ongoing alloresponse, as its disruption accelerates acute rejection [2]. Blockade of BTLA but not PD-1 has been reported to trigger rejection of cardiac grafts expressing the partial MHC mismatch H-2^{bm12} that are otherwise permanently accepted by C57BL/6 recipients [3]. In contrast, targeting of PD-1 but not of BTLA resulted in accelerated rejection of fully mismatched cardiac allografts, suggesting that PD-1 suppresses stronger and more chronic responses than BTLA [3]. Similarly, blockade of PD-L1 restored skin allograft rejection after co-transfer of Tregs and conventional T cells to RAG-deficient mice [4].

Different approaches have been used to harness the therapeutic potential of these inhibitory receptors. We have previously targeted CTLA-4 using alloantigen-bearing cells engineered to express a membrane-bound anti-CTLA-4 scFv [5]. When transplanted subcutaneously, these cells inhibited T-cell alloresponses and triggered T-cell anergy [5]. The inhibitory function of CTLA-4 can also be induced by treatment with anti-CD45RB that exerts its immunosuppressive function by inducing

upregulation of CTLA-4 on T cells [6–8]. Testing the effects of forced expression of CTLA-4 has been performed via the generation of transgenic mice that overexpress CTLA-4 on T cells [9–11]. We have shown that transgenic expression of CTLA-4 on T cells is sufficient to prevent the lymphoproliferative disease and autoimmune hemolytic anemia that otherwise arise in IL-2-deficient mice, although it had no effect on the development of inflammatory bowel disease that may be mediated by other mechanisms [12].

Overexpression of PD-L1 on target tissues has been used to enhance PD-1 engagement and T-cell inhibition. For instance, transduction of cardiac allografts with adenoviral vectors encoding for PD-L1 resulted in prolonged survival of allografts in rats, especially if also treated with subtherapeutic doses of cyclosporine (CsA) [13]. However, we had found that transgenic expression of PD-L1 on pancreatic β cell resulted in acceleration rather than suppression of islet allograft rejection [14]. Although the molecular mechanism of this faster rejection process remains to be uncovered, it has been suggested that PD-L1 can bind a receptor other than PD-1, with possible positive rather than negative costimulatory effects [15].

As transgenic expression of CTLA-4 on T cells was so potent at inhibiting T-cell responses, we investigated the inhibitory potential of similar constitutive expression of PD-1 on T cells. To this end, we generated two lines of transgenic mice that constitutively expressed PD-1 selectively on T cells. PD-1 transgenic (PD-1-Tg) mice have been generated previously by Keir *et al.* and two separate lines of mice were found to have distinct patterns of expression of PD-1 on thymocyte subsets [16]. One founder displayed expression of PD-1 on double negative, double positive and single positive thymocytes resulting in impaired positive selection of T cells. In contrast, the other founder displayed much lower levels of PD-1 expression and only on single positive thymocytes and did not incur detectable defects in T-cell development. Our two lines of PD-1-Tg mice did not display PD-1 expression in immature thymocytes, but demonstrated expression on peripheral $CD4^+$ and $CD8^+$ T cells. Surprisingly, overexpression of PD-1 in T cells did not result in delayed rejection of fully mismatched cardiac allografts and only prolonged survival of grafts bearing minor mismatches if recipient mice were treated with suboptimal doses of CsA, suggesting that this approach may not bring substantial therapeutic benefits.

Materials and methods

Mice

C57BL/6 (B6; H-2^b) and 129/J (H-2^b) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). PD-1 deficient (H-2^b) mice [17,18] were a gift from Dr Honjo (Kyoto University, Japan). PD-1-Tg mice (H-2^b) were generated in our laboratory. Mouse PD-1 cDNA was initially cloned by RT-PCR into pUC19, then inserted into the AscI site of the mammalian expression vector plck.E2 (a generous gift from Jeffrey Leiden when at the University of Chicago), which contains the proximal *lck* promoter, the human growth hormone polyadenylation site, and locus control region elements from the human CD2 gene. mPD-1 was sequenced and the construct was excised and gel purified prior to microinjection into C57BL/6 mouse embryos by the University of Chicago Cancer Research Center Transgenic Mouse Facility. PstI-digested tail DNA from mice was hybridized to a radiolabeled 0.9-kb PD-1-specific probe. Two positive founders were identified by Southern blot and used to generate two lines of PD-1-Tg mice, line 24 (L24) and L15. Wildtype (WT) mice refer to nonTg control littermates. Animals were kept under specific pathogen-free conditions and used in agreement with our Institutional Animal Care and Use Committee, according to the National Institutes of Health guidelines for animal use.

Flow cytometric analysis

Lymphocytes were isolated from thymi, spleens and lymph nodes (LNs, axillary, brachial and inguinal) and processed into single cell suspensions. Cells were stained with APC-coupled anti-CD4 (L3T4), PE-Cy7-coupled anti-CD8 (Ly2), and PE-coupled anti-PD-1 (J43) mAbs (all from BD Pharmingen; San Diego, CA, USA) and analyzed by flow cytometry (BD LSR-II; BD Biosciences; San Diego, CA).

Expression of endogenous versus transgenic PD-1

T cells were isolated from the spleen and LNs (axillary, brachial and inguinal) of WT and PD-1-Tg mice (line 24) following staining with anti-PD-1 mAb. PD-1-Tg T cells were sorted into PD-1^{high} and PD-1^{low}, whereas all WT T cells were isolated. mRNA was extracted and reverse transcribed and primers were designed to amplify the 3'untranslated region of endogenous (P1) or transgenic (P2) PD-1. The following primers were used: forward primer (for P1 and P2): CCT GTC CCT AGT GTG GCC TA; reverse primer (P1): GCT ACA GTT CAG CCA AGG CT; reverse primer (P2): TCT GTT GCC CTC TGG TTT CT.

Cytokine analysis

Splenocytes from PD-1-Tg and nonTg littermates were suspended in complete DMEM [supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), strepto-

mycin (100 μg/ml), HEPES, 2-ME (50 μM), and additional amino acids] and plated on flat-bottom 96-well plates (2×10^5/well) in the presence of soluble anti-CD3 mAb (10 μg/ml). For some experiments T cells were also enriched by negative selection over magnetic beads according to the instructions of the manufacturer (StemCell Technologies, Vancouver, BC, Canada) and were stimulated with plate-bound anti-CD3 at the indicated concentrations and plate-bound anti-CD28 (1 μg/ml). Supernatants were harvested at 24 h, and the concentration of IL-2 and IFN-γ in each sample was detected by ELISA using Ab pairs as instructed by the manufacturer (BD Pharmingen). Absorbance was detected in a 96-well spectrophotometer (μQuant; Bio-Tek Instruments, Winooski, VT, USA), and data were analyzed using KC4 software (Bio-Tek Instruments) in comparison to a standard curve generated using recombinant cytokines at known concentrations.

In vitro proliferation assays

Splenocytes ($1–2 \times 10^5$ per well) from PD-1 Tg and nonTg littermates mice were incubated with various concentrations of soluble anti-CD3 mAb in complete DMEM or with irradiated (2000 rads) splenocytes (4×10^5/well) from 129/J (H-2^d, minor mismatches) or BALB/c (H-2^d, major mismatches). For some experiments, T cells were also enriched by negative selection over magnetic beads according to the instructions of the manufacturer (StemCell Technologies) and stimulated with plate-bound anti-CD3 at the indicated concentrations and plate-bound anti-CD28 (1 μg/ml). Cell cultures were pulsed with [^{3}H]-Thymidine (1 μCi/well) for the last 8 h of a 72 h (for anti-CD3 stimulations) or 120 h (for allogeneic stimulations) culture and harvested for liquid scintillation counting.

Western Blot analysis

Wildtype and PD-1-Tg T cells enriched by negative selection over magnetic beads according to the manufacturer's instructions (StemCell Technologies) were incubated with anti-CD3 (5 μg/ml) with or without anti-PD-1 mAb (4 μg/ml; BD PharMingen) on ice for 30 min. The cells were washed twice in prewarmed complete RPMI1640, and then cross-linked with rabbit anti-hamster IgG (8 μg/ml) (Sigma; St Louis, MO, USA) for 5, 15 and 30 min. The stimulation was quenched by adding ice-cold 1× PBS, and solubilized in lysis buffer containing 1% Nonidet P-40, 20 mM Tris·HCl (pH 7.4), 100 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM sodium vanadate and protease inhibitor mixture (Roche Diagnostics; Indianapolis, IN, USA). The cell extracts were resolved on SDS-PAGE and transferred to nitrocellulose membranes (Hybond C Super; Amersham Biosciences, Little Chalfont, UK). Blots were blocked for 1 h at room temperature in PBS containing 2% BSA and 0.05% Tween-20. Membranes were incubated overnight with anti-phospho-tyrosine Ab (PY20) (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA), phospho-specific Abs against protein kinase C-θ (PKC-θ), Akt and phosphate and tensin homolog (PTEN) as well as against total Akt (Cell Signaling Technologies; Beverly, MA and Biosource International; San Diego, CA), then washed 3× in PBS containing 0.05% Tween-20, and detected using HRP-conjugated goat anti-rabbit IgG or rabbit anti-mouse IgG (Zymed Laboratories; San Francisco, CA, USA). After three washes in PBS containing 0.05% Tween-20, signals were revealed using the enhanced chemiluminescence detection system (Amersham) and visualized by autoradiography.

Heart transplantation

Minor mismatched abdominal cardiac transplantation was performed using a technique adapted from that originally described by Corry *et al.* [19]. 129/J (H-2^b) or BALB/c (H-2^d) cardiac allografts were transplanted in the abdominal cavity of C57BL/6 WT and PD-1-Tg mice (H-2^b) by anastomosing the aorta and pulmonary artery of the graft end-to-side to the recipient's aorta and vena cava respectively. Some mice were treated with (CsA, 200 μg i.p. q.i.d × 14 post-transplantation). The day of rejection was defined as the last day of a detectable heartbeat in the graft. Graft rejection was verified in selected cases by necropsy and pathological examination of H&E-stained graft sections.

Statistical analysis

Comparisons of mean values were performed using the Student's *t*-test or the Tukey test for multiple comparisons, when appropriate. Graft mean survival time and *P*-values were calculated using Kaplan–Meier/log rank test methods.

Results

T cells from PD-1-Tg mice constitutively express PD-1

To examine the inhibitory effect of constitutive expression of PD-1 on T cells, we generated PD-1-Tg mice in which expression of PD-1 is controlled by the proximal *Lck* promoter and the CD2 locus control region. Two founder lines were generated with slightly different patterns of expression. Although both lines had peripheral T cells with constitutive expression of PD-1, expression was more uniform on T cells from line 15 whereas T cells from line 24 were divided into those expressing PD-1^{high} and those expressing PD-1^{low} or undetectable (Fig. 1a).

It was possible that insertional positioning would result in activation of some T cells from line 24 and that the PD-1 protein detected by flow cytometry corresponded to the endogenous rather than the transgenic PD-1, as endogenous PD-1 is induced upon T-cell activation. To investigate that possibility, we FACS-sorted T cells from WT and PD-1-Tg line 24. PD-1-Tg T cells were sorted into PD-1^{high} and PD-1^{low}, mRNA was extracted and reverse transcribed and primers were designed to amplify the 3'untranslated region of endogenous (P1) or transgenic (P2) PD-1. As shown in Fig. 1b, WT T cells expressed endogenous but not transgenic PD-1 mRNA whereas both PD-1^{low} and PD-1^{high} transgenic T cells simultaneously expressed endogenous and transgenic PD-1. These results demonstrate that the transgenic PD-1 gene is expressed in the population of PD-1^{low}-Tg T cells, despite protein levels that are too low to be detected by flow cytometry.

Transgenic expression of PD-1 does not affect T-cell numbers in lymphoid organs

Transgenic expression of PD-1, when detectable in the thymus, has been shown to inhibit positive selection resulting in reduced numbers of single positive thymocytes. To determine if transgenic expression of PD-1 in our lines affected the number of thymocytes and peripheral T cells, single cell suspensions from thymi, spleen and LNs were analyzed by flow cytometry and percentages of T cells multiplied by the total number of live cells counted by Trypan blue exclusion to obtain the total number of T-cell subsets. As shown in Fig. 2, numbers of T-cell subsets in the thymus, spleen and LNs were comparable in WT, line 24 and line 15 mice, indicating that effects on positive selection, if any, do not affect T-cell numbers in lymphoid organs.

PD-1-Tg T cells have reduced T-cell function *in vitro*

To determine the functional effects of the transgenic expression of PD-1 on T cells, splenocytes from WT, L24 and L15 PD-1-Tg mice were stimulated with soluble anti-CD3. Percentages of T cells in the different spleens were comparable in all groups. Proliferation and production of IL-2 by PD-1-Tg T cells were significantly reduced when compared with WT T cells (Fig. 3a and b). In addition, there was a trend towards inhibition of IFN-γ production by PD-1-Tg T cells (Fig. 3b). Reduced function by PD-1-Tg T cells was not cause by an intrinsic defect of PD-1-Tg T cells because purified T cells stimulated with immobilized anti-CD3 and anti-CD28 displayed similar proliferation and cytokine production in all groups (Fig. 3c). These results indicate that the function of

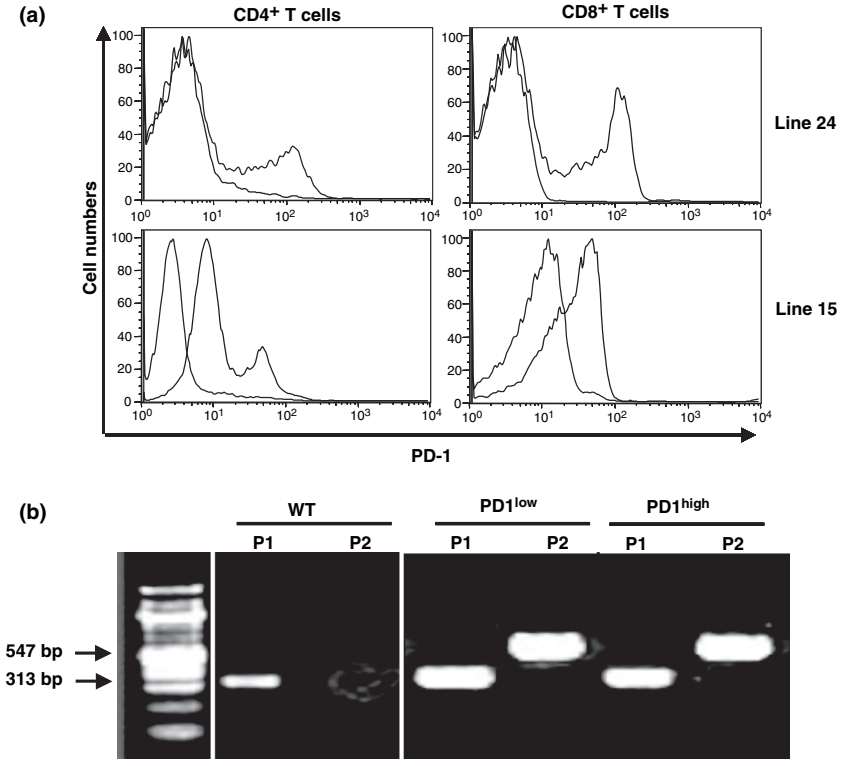

Figure 1 The PD-1 transgene (PD-1-Tg) is expressed in PD-1-Tg T cells. (a) Splenocytes were harvested from 8 to 12-week-old PD-1-Tg mice (L24 and L15) and nonTg littermates, stained with anti-CD4-APC-, anti-CD8-PE-Cy7- and anti-PD-1-PE mAbs and analyzed by flow cytometry. Events were gated on $CD4^+$ or $CD8^+$ cells and PE relative fluorescence intensity was displayed as a histogram. The lines correspond to isotope control staining and expression of program death-1 (PD-1) in wildtype and PD-1-Tg T cells. The plots are representative of at least four independent experiments. (b) T cells from PD-1-Tg mice (L24) and control littermates were stained with anti-PD1 mAb and PD-1^{low} and PD-1^{high} cells (or all T cells from control mice) were isolated by high speed flow cytometry sorting. cDNA was amplified using primers specific for endogenous PD-1 (P1) or from the transgenic PD-1 (P2) as described in Materials and methods.

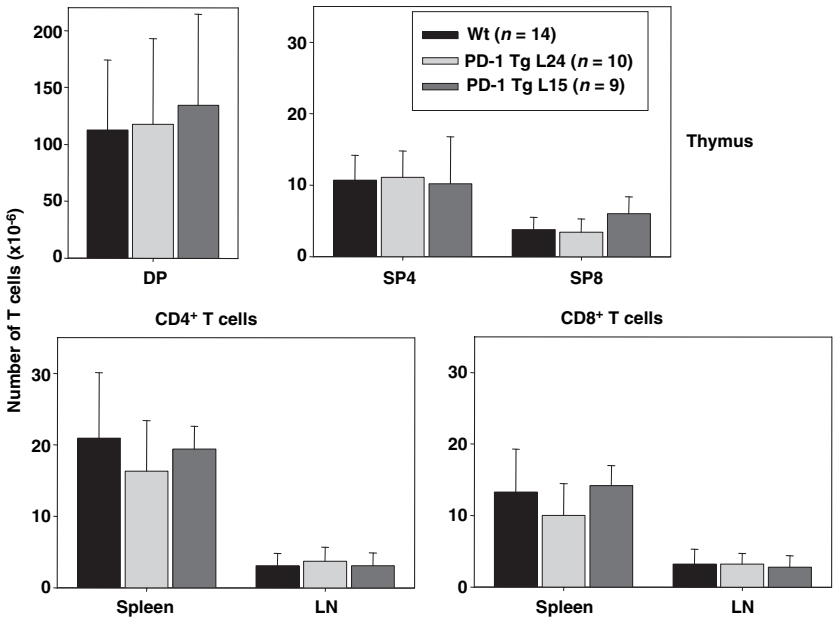

Figure 2 Over-expression of program death-1 in T cells does not affect T-cell numbers. Thymus, spleen and LN were harvested from 8 to 12-week-old PD-1-transgenic mice (L24 and L15) and non-Tg littermates. Lymphocytes were stained with anti-CD4-APC- and anti-CD8-PE-Cy7- mAbs and analyzed by flow cytometry. The percentage of T-cell subsets was multiplied by the total cell counts to obtain the number of DP, SP4 and SP8 T cells in the thymus, and the number of $CD4^+$ and $CD8^+$ T cells in the spleen and LN. The results are presented as the mean ± SD from the indicated numbers of mice.

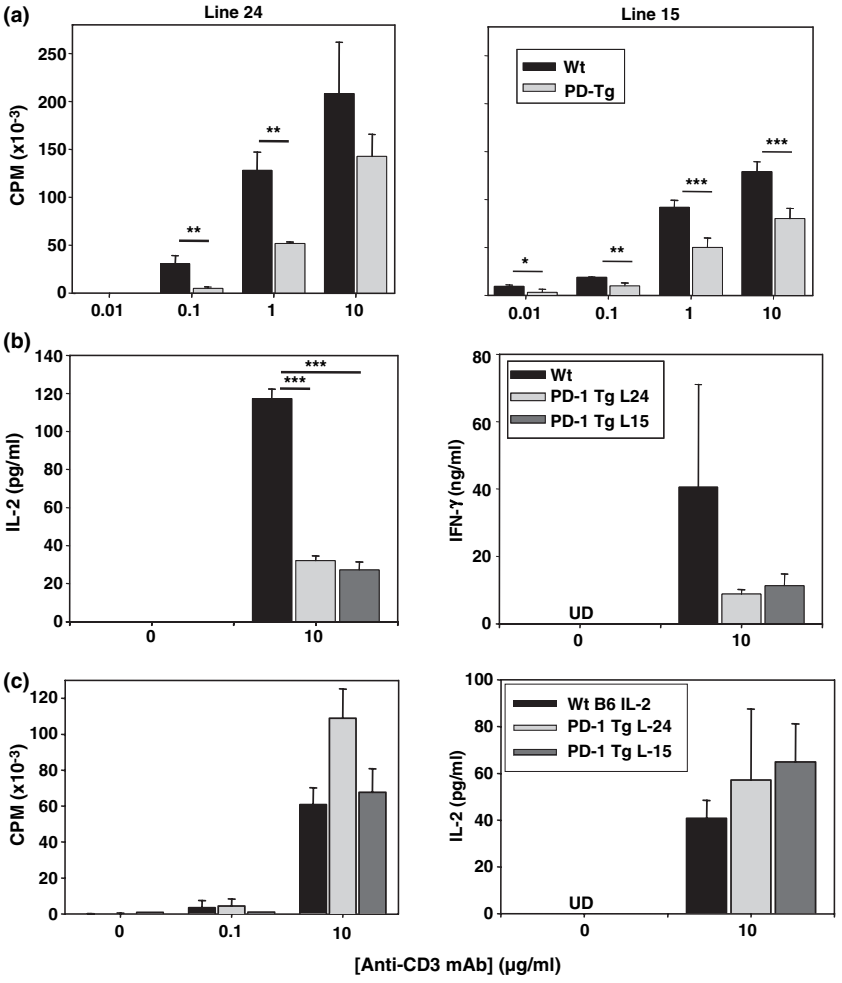

Figure 3 Over-expression of PD-1 in T cells impairs T-cell function. For A and B, splenocytes from 8 to 12-week-old PD-1-transgenic mice (L24 and L15) and nonTg littermates were stimulated with soluble anti-CD3 mAb at the indicated concentrations. (a) The cells were incubated for 72 h and pulsed with $[^3H]$-Thymidine for the last 8 h of the culture. (b) Supernatants were collected at 24 h and analyzed for IL-2 and IFN-γ production by ELISA. The plot represents the mean ± SD of triplicate determinations. (c) T cells were enriched by negative selection over magnetic beads and stimulated with immobilized anti-CD3 mAb at the indicated concentrations and anti-CD28 (1 μg/ml). $[^3H]$-Thymidine incorporation and IL-2 production were measured as in A and B. These results are representative of three independent experiments. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. UD, undetectable.

PD-1-Tg T cells is reduced when compared with that of WT T cells only when ligands of PD-1 expressed on nonT cells are present in the culture.

Cross-linking of the PD-1 transgene results in reduced activation of PKC-θ and Akt

To understand the potential molecular mechanisms that account for the reduced function of PD-1-Tg T cells in the presence of PD-1 ligands, we assessed the biochemical consequences of PD-1 ligation in WT and PD-1-Tg T cells. It has been reported that PD-1 signaling inhibits CD28-dependent activation of Akt, as a consequence of PI3K inhibition [20]. In addition, PD-1 ligation has been shown to reduce activation of PKC-θ, an essential component of T-cell activation [21]. We therefore compared the phosphorylation status of PKC-θ and Akt induced by anti-CD3 or anti-CD3/anti-PD-1 on WT and PD-1-Tg (line 24) T cells. PD-1 ligation greatly inhibited TCR-induced Akt and PKC-θ activation in PD-1 Tg but not WT T cells (Fig. 4).

Phosphate and tensin homolog negatively regulates cell proliferation and survival mediated by PI3K [22,23]. Therefore, we investigated whether PD-1 ligation led to hyper-activation of PTEN that may account for inhibition of Akt and PKC-θ. As shown in Fig. 4, TCR-induced activation of PTEN was significantly enhanced by PD-1 ligation, which closely correlated temporally with inhibition of Akt and PKC-θ activation. Taken together, our data suggest that transgenic PD-1 may inhibit T-cell activation via a PTEN/PDK1/Akt-PKC-θ pathway.

Transgenic expression of PD-1 in T cells promotes survival of minor mismatched but not major mismatched allografts

To determine if constitutive expression of PD-1 could reduce alloresponses and facilitate allograft survival, T-cell responses following mixed lymphocyte reactions were investigated. PD-1-Tg T cells showed reduced proliferation when stimulated with 129/J but not BALB/c splenocytes (Fig. 5a), indicating that only weak allogeneic

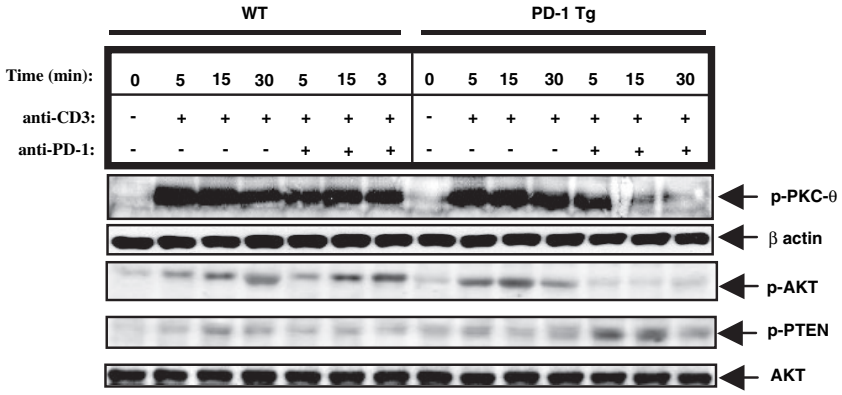

Figure 4 Program death-1 cross-linking attenuates TCR-mediated activation of protein kinase C-θ (PKC-θ) and Akt. C57BL/6 WT and PD-1-transgenic T cells were stimulated with anti-CD3 or anti-CD3 plus anti-PD-1 for 5, 15 and 30 min, and lysed. The cell lysates were immunoblotted with anti-phospho-PKC-θ, anti-phospho-Akt and anti-phospho-PTEN respectively. The membranes were reprobed with anti-Akt as a loading control.

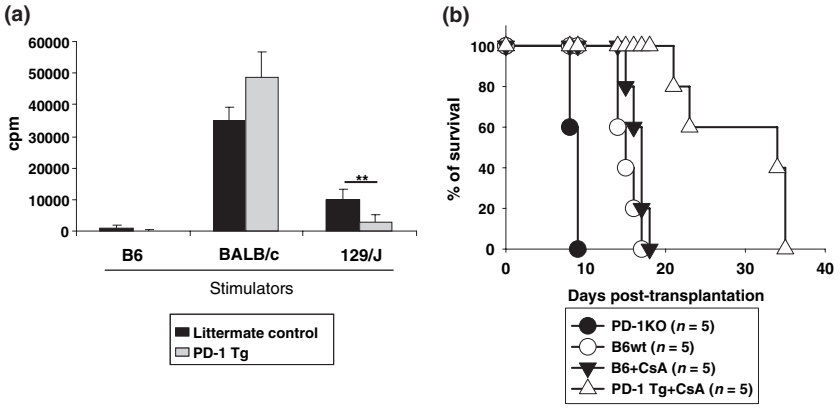

Figure 5 Minor impact of the program death-1 (PD-1) transgene on alloresponses. (a) Splenocytes from wildtype and PD-1-Tg (L24) mice were stimulated with irradiated syngeneic (B6), fully mismatched (BALB/c) or minor mismatched (129/J) splenocytes. ^{3}[H]-Thymidine incorporation was evaluated after 5 days of culture. $^{**}P < 0.01$. (b) 129/J hearts were transplanted into PD-1-Tg mice (L24), non-Tg littermates and PD-1-deficient (PD-1KO) mice in the presence or absence of treatment with cyclosporine-A (200 μg i.p., every day for 14 days), as indicated. Graft survival was assessed over time.

responses are inhibited by constitutive expression of PD-1. Prolongation of cardiac allograft survival was only observed when PD-1-Tg mice were transplanted with a graft bearing minor mismatches (129/J) in recipients treated with suboptimal doses of CsA ($P = 0.021$, Fig. 5c). Conversely, PD1-deficient mice showed accelerated rejection of 129/J allografts ($P = 0.031$, Fig. 5b). Together, these results demonstrate that both endogenous and transgenic expression of PD-1 on T cells result in weak attenuation of alloresponses.

Discussion

Several inhibitory molecules are induced upon T-cell activation that can terminate an ongoing T-cell response. Forcing expression of these inhibitors on T cells may be a therapeutic approach to reduce immune responses *in vivo* in settings of autoimmunity or transplantation. However, our study shows that transgenic expression of PD-1 on T cells results in a weak reduction of T-cell alloresponses. Ligation of the PD-1 transgene results in inhibition of PKC-θ and Akt, similarly to effects previously ascribed to engagement of endogenous PD-1, and correlates with enhanced PTEN activation.

PD-1 transgenic mice have been generated previously by Keir *et al.* and expression of the transgene was shown to affect thymic development in one line but not the other [16]. We independently generated PD-1-Tg founders and mice derived from both our founders resembled the second line of the published animals, as we did not detect significant effects on thymic development. It is likely that the site of integration and copy number affect whether the transgene is expressed in the thymus or not, as well as levels of expression on thymocytes and peripheral T cells. Although peripheral T-cell numbers were normal in our PD-1-Tg mice, we could not exclude more subtle effects of the transgene on the T-cell repertoire. Nevertheless, we did not detect any intrinsic functional defect in PD-1-Tg T cells when stimulated in the absence of PD-1 ligands.

The intra-cellular portion of PD-1 comprises an ITIM and an ITSM motifs. The ITIM motif has been shown to associate with SHP-2, while the ITSM motif can associate with both the SHP-1 and SHP-2 proteins [21]. Interaction with these protein phosphatases is thought to down-regulate TCR-mediated signaling events necessary for T-cell activation. Engagement of PD-1 has been previously shown to result in reduced TCR/CD28-mediated phosphorylation of Akt by preventing CD28-mediated activation of PI3K in a manner dependent on PD-1's ITSM motif [20]. In our study, PD-1 ligation resulted in reduced Akt activation in a system devoid of CD28 cross-linking as T cells were stimulated with anti-CD3 in the absence of anti-CD28. Our results suggest that in addition to reducing CD28-mediated PI3 K activation, engagement of PD-1 can inhibit TCR-dependent PI3 K activation. Engagement of PD-1 has also been shown to reduce TCR-mediated activation of PKC-θ [21]. This effect may also be secondary to the inhibition by PD-1 of PI3K, as PI3K is thought to be involved in phosphorylation and activation of PKC-θ via the phosphatidylinositol-dependent kinase 1 [24]. Our results show that PD-1 cross-linking results in augmented PTEN phosphorylation. Because PTEN negatively regulates PI3K-mediated cell proliferation and survival [22,23], these data are consistent with the hypothesis that PD-1 inhibits PI3K activation and its downstream targets Akt and PKC-θ by enhancing PTEN activation. $PTEN^{+/-}$ or T-cell-specific $PTEN^{-/-}$ mice have increased spontaneous tumor incidence, lymphoid hyperplasia development and display autoimmune disorders [22,23]. By enhancing PTEN activity, PD-1 also has the potential to reduce autoimmunity *in vivo*.

In vivo, T cells that constitutively express PD-1 are likely to encounter PD-1 ligands both in secondary lymphoid organs and in target organs, as PD-L1 is constitutively expressed on T cells, B cells, dendritic cells and macrophages and is further upregulated upon cell activation [25]. In addition, nonhematopoietic cells, such as cardiac endothelial cells, also express PD-L1. In contrast, PD-L2 is mostly expressed on activated APCs. Thus, delayed cardiac allograft rejection in PD-1-Tg mice may be due to T-cell inhibition both at the priming phase in secondary lymphoid organs, as well as at the effector phase in the heart allograft itself.

Program death-1 has been shown to play a role in transplant responses. Genetic ablation of PD-1 induced accelerated rejection of fully mismatched but not of MHC class I- or MHC class II singly-mismatched cardiac allografts, perhaps because partial MHC mismatches did not elicit an immune response vigorous enough to result in upregulation of PD-1 mRNA [3]. Our results extend these observations by showing accelerated cardiac allograft rejection by PD-1-deficient mice in an MHC-matched model of multiple minor mismatches, confirming that endogenous PD-1 normally inhibits alloresponses *in vivo*. We speculate that peptides from the minor mismatches may be presented by both MHC class I and MHC class II and elicit a stronger alloresponse than bm1 or bm12 alloantigens, therefore revealing the PD-1-dependency of the alloresponse *in vivo*.

Endogenous PD-1 has also been used as a therapeutic target to reduce transplant responses. Administration of PD-L1.Ig or PD-L2.Ig that are thought to trigger PD-1 signaling *in vivo* was shown to prolong survival of fully mismatched cardiac allografts only under weak allostimu-

latory conditions such as in $CD28^{-/-}$ recipients or in WT mice in the presence but not in the absence of rapamycin [26]. Similarly, treatment with PD-L1.Ig resulted in islet allograft survival only when combined with anti-CD154 mAb [27]. Finally, adenoviral-mediated transduction of PD-L1 in cardiac allografts resulted in slightly prolonged survival of rat allografts in untreated animals, but more significant prolongation when low doses of CsA were administered [13]. Together, these results indicate that targeting of endogenous PD-1 is mildly effective in improving survival of allografts in the context of impaired T-cell responses. The greater success of these approaches when compared with over overexpression of PD-1 on T cells may be due to insufficient constitutive expression of the PD-1 ligands to ensure sufficient engagement of the PD-1 transgene.

Interestingly, some immunosuppressive therapies appear to work through the upregulation of endogenous PD-1, such as the combination of rapamycin with blockade of the MCP1/CCR2 pathway [28] or, unexpectedly, the genetic ablation of the negative regulator BTLA [3]. We tested another approach to target PD-1 therapeutically to inhibit transplant responses, by overexpressing PD-1 constitutively on T cells. However, overexpression of PD-1 on T cells prolonged the survival of minor mismatched cardiac allografts only in animals treated with suboptimal doses of CsA. This indicates that PD-1, even when present constitutively and not only after TCR stimulation, is not sufficient by itself to prevent alloresponses *in vivo*, although it can somewhat synergize with immunosuppressive regimens under weak immunostimulatory conditions.

Gene therapy has been used with some success to correct severe combined immunodeficiency in children [29] and more recently, by transducing T cells with melanoma-specific TCR, to promote cancer rejection [30]. Although more progress needs to be made to improve efficacy as well as to prevent complications caused by insertional positioning, our past [12] and current results indicate that it may be of greater therapeutic interest in the future to overexpress in T cells CTLA-4 than PD-1, in settings of autoimmunity or transplantation.

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Authorship

LC: performed research, wrote paper; YH: contributed biochemistry data; KWH: generated the mice; YW: performed transplantation; PZ: performed transplantation; MLA: designed research, wrote paper.

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