Blockade of Programmed Death-1 Engagement Accelerates Graft-Versus-Host Disease Lethality by an IFN-γ-Dependent Mechanism[1]

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Acute graft-vs-host disease (GVHD) is influenced by pathways that can enhance or reduce lethality by providing positive or negative signals to donor T cells. To date, the only reported pathway to inhibit GVHD is the CTLA-4:B7 pathway. Because absence of the programmed death-1 (PD-1) pathway has been implicated in a predisposition to autoimmunity and hence a lack of negative signals, the effect of PD-1 pathway blockade on GVHD was explored using several distinct approaches. In each, GVHD lethality was markedly accelerated. Coblockade of CTLA-4 and PD-1 was additive in augmenting GVHD, indicating that these pathways are not fully redundant. Although neither perforin nor Fas ligand expression was required for GVHD enhancement, donor IFN-γ production was required for optimal GVHD acceleration in the absence of PD-1 ligation. These data indicate that PD-1 ligation down-regulates GVHD through modulation of IFN-γ production and suggest a novel therapeutic target for inhibiting GVHD lethality. ***The Journal of Immunology,*** **2003, 171: 1272–1277.**

Programmed death-1 (PD-1),[4] a recently described member of the B7:CD28 superfamily, has two known ligands, programmed death-1 ligand-1 (PD-L1) and programmed death-1 ligand-2 (PD-L2) (1–7). PD-1 is induced on mature peripheral T cells, B cells, and myeloid cells upon activation (1, 8) PD-1 has an immunoreceptor tyrosine-based inhibitory motif in its cytoplasmic tail, suggesting an inhibitory function of PD-1 signaling (9, 10). B6 PD-$1^{-/-}$ mice develop splenomegaly, a lupus-like glomerulonephritis, and destructive arthritis with aging (4). BALB/c PD-$1^{-/-}$ mice develop a host of tissue-specific and systemic autoimmunities (11). One of us (4) studied the effects of crossing B6 PD-$1^{-/-}$ mice with 2C TCR-transgenic mice that express a transgene reactive with H2-L^d. When 2C PD-$1^{-/-}$ mice were crossed with H2-L^d mice, the $H2^{b/d}$ progeny developed a GVHD-like syndrome. The 2C PD-$1^{-/-}$ $CD8^+$ T cells showed markedly increased proliferation to $H2^d$-allogeneic T cells, suggesting that potentially autoreactive peripheral T cells can become activated in the absence of PD-1 (4).

PD-L1, also termed B7-H1, is a member of the B7 family (6). PD-L1 is expressed on endothelium, thymic epithelium, and some activated T cells. PD-L2, also termed B7-DC, is expressed in pancreas, lung, and liver. Both PD-L1 and PD-L2 are expressed on monocytes, dendritic cells, and keratinocytes. Notably, with the exception of the pancreas, these organs are targets of GVHD. In addition, PD-L1 and PD-L2 are expressed in heart and skeletal muscle. IFN-γ up-regulates PD-L1 and to a lesser extent PD-L2 expression in monocytes (12), dendritic cells, and keratinocytes and PD-L1 expression in endothelium.

Although PD-$1^{-/-}$ mice are prone to autoimmunity, the effects of PD-1 signaling on T cell responses remain controversial. In vitro studies have attributed either a stimulatory (5, 7) or an inhibitory (6, 11, 13) function to this pathway. Little is known as to how PD-1/PD-L regulates T cell responses in vivo. To further define the function of the PD-1 pathway in vivo and to specifically analyze the effects of PD-1/PD-L interaction on regulating graft-vs-host disease (GVHD), studies were performed using irradiated recipients of allogeneic donor cells. Our studies demonstrate that PD-1 blockade potently enhances T cell alloresponses in vitro and in vivo. The effects of PD-1/PD1-L blockade were not dependent on the expression of perforin or Fas ligand (FasL) but were largely dependent upon IFN-γ production. These data have important implications for solid organ and hemopoietic stem cell transplantation.

Materials and Methods

Mice

C57BL/6 (termed B6) ($H2^b$) mice were purchased from the National Institutes of Health. B10.BR/SgSnJ ($H2^k$), B6.C-$H2^{bm12}$/KhEg (termed bm12), B6.C-$H2^{bm1}$/KhEg (termed bm1), B6.129S7-$Ifng^{tm1Ts}$ (termed B6 IFN-$\gamma^{-/-}$), C57BL/6-Pfp^{tm1Sdz} (termed B6 $perf^{-/-}$), and B6Smn.C3-$Tnfsf6^{gld}$ (termed B6 gld) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). bm12 and bm1 mice have mutant MHC class II and class I alleles, respectively, that differ from those of B6 mice. Mice were bred and housed in a specific pathogen-free facility in microisolator cages. PD-$1^{-/-}$ mice were generated as described and backcrossed over 11 generations with B6 mice (14). Donors and recipients were used at 8–12 wk of age.

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[4] Abbreviations used in this paper: PD-1, programmed death-1; BM, bone marrow; BMT, bone marrow transplantation; GVHD, graft-vs-host disease; FasL, Fas ligand; PD-L1, programmed death-1 ligand-1; PD-L2, programmed death-1 ligand-2; WT, wild type.

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GVHD generation

B10.BR recipients were irradiated with 8.0 Gy of total body irradiation on day −1. Donor bone marrow (BM) (20×10^6), depleted of T cells using anti-Thy-1.2 plus rabbit complement treatment, was infused on day 0 (15). To induce GVHD, donor splenocytes from various strains were infused at the doses indicated. To determine the effects of the PD-1/PD-L pathway on $CD4^+$ or $CD8^+$ T cell-mediated GVHD lethality, bm12 or bm1 recipients were sublethally irradiated (6.0 Gy of total body irradiation) and given $CD4^+$ or $CD8^+$ T cells, respectively, from B6 or B6 PD-$1^{-/-}$ donors. Mice were weighed twice weekly and monitored daily for survival and clinical evidence of GVHD (ruffled fur, cachexia, alopecia, diarrhea).

mAb and fusion protein administration

The hamster anti-murine PD-1, clone J43 (3), and anti-murine CTLA-4, clone C10-4F10--1, mAbs have been described. Control hamster IgG was purchased (Rockland Laboratories, Gilbertsville, PA). The generation and production of murine PD-L1.Ig protein, which consists of the extracellular domains of murine PD-L1 linked to the hinge-CH2-CH3 domains of a mutated murine IgG2a to reduce FcR and complement binding, have been described (6). Murine anti-human CTLA4 mAb (mouse IgG2a) was used as a control. mAbs and fusion proteins were administered at 200 μg/dose i.p. from days −1 to +5 and then three times weekly through day +28.

GVHD target tissue analysis

GVHD organs were snap-frozen in liquid nitrogen and stored at 80°C. Frozen sections were cut 4 μm thick and fixed for 5 min in acetone. Cryosections were stained using biotinylated mAbs (anti-CD4-, anti-CD8-, anti-$H2^b$- and anti-$H2^k$) (BD PharMingen, San Diego, CA), avidin-biotin blocking reagents, ABC-peroxidase conjugate, and diaminobenzidine chromogenic substrate (Vector Laboratories, Burlingame, CA) and $CD4^+$ and $CD8^+$ T cells

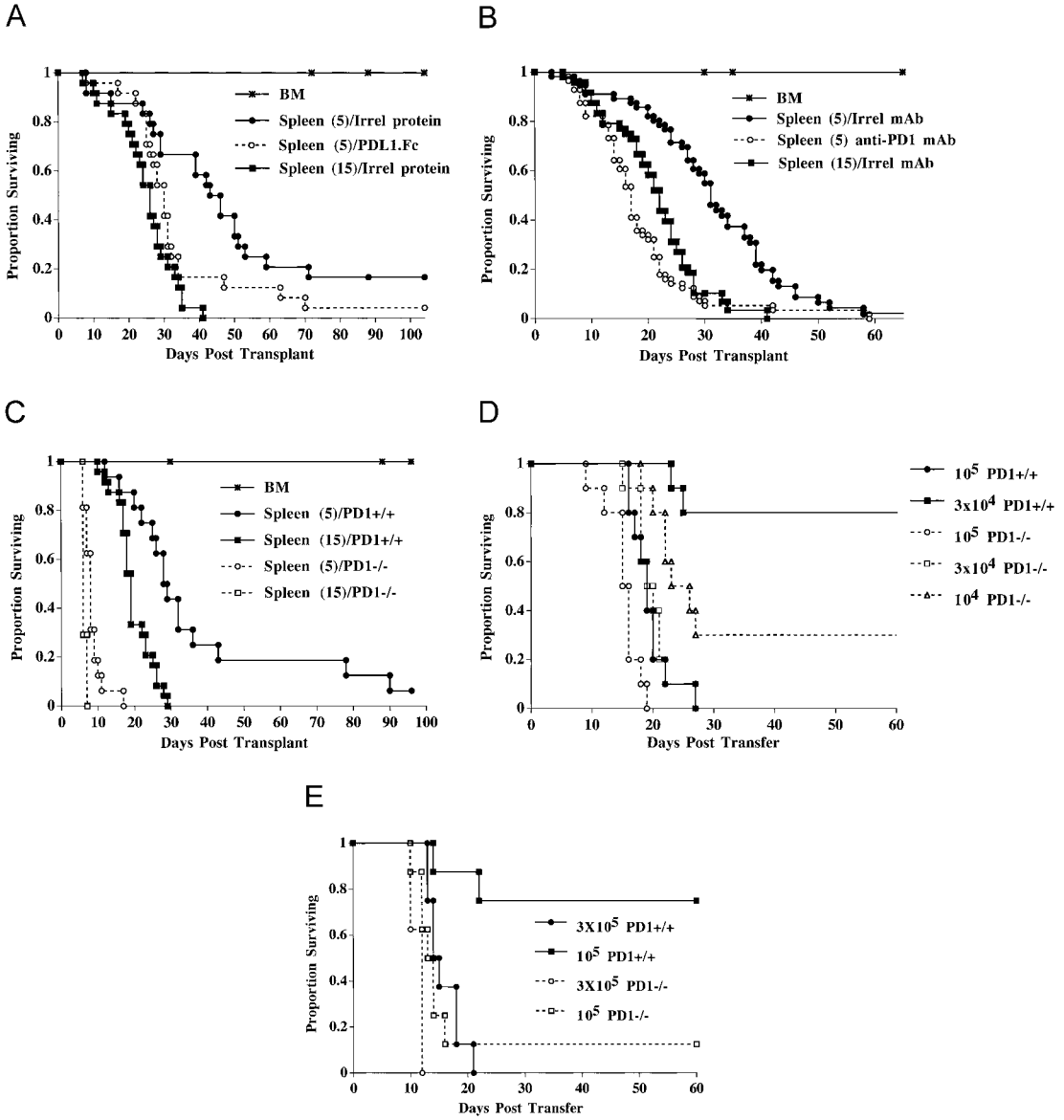

FIGURE 1. Infusion of PD-L1.Fc fusion protein, anti-PD-1 mAb, or PD-$1^{-/-}$ donor cells markedly accelerates GVHD mortality. *A* and *B,* Lethally irradiated B10.BR recipients were given B6 BM alone or with 5 or 15 million B6 splenocytes as indicated in parentheses $\times 10^6$. *A,* Cohorts were infused with irrelevant (Irrel) or PD-L1.Fc fusion protein ($p < 0.05$ for survival rate differences between cohorts receiving comparable splenocyte cell doses). n = 24/group. *B,* Cohorts were infused with irrelevant or anti-PD-1 mAb ($p < 0.005$ for survival rate differences between cohorts receiving comparable splenocyte cell doses). n = 48–56/group. *C,* Lethally irradiated B10.BR recipients were given B6 BM alone or with 5 or 15 million splenocytes (as indicated in parentheses $\times 10^6$) from B6 PD-$1^{+/+}$ or PD-$1^{-/-}$ mice ($p < 0.005$ for survival rates differences between cohorts receiving comparable splenocyte cell doses). n = 16–24/group. *D,* Sublethally irradiated bm12 recipients were given $CD4^+$ T cells at cell dosages indicated from B6 PD-$1^{+/+}$ or PD-$1^{-/-}$ mice ($p < 0.005$ for survival rate differences between cohorts receiving comparable T cell doses). n = 10/group. *E,* Sublethally irradiated bm1 recipients were given $CD8^+$ T cells at cell dosages indicated from B6 PD-$1^{+/+}$ or PD-$1^{-/-}$ mice ($p < 0.005$ for survival rate differences between cohorts receiving comparable T cell doses). n = 8/group. *x*-Axis, Days posttransplant or post-T cell transfer; *y*-axis, proportion surviving.

were enumerated (16). Representative sections from each block were stained with hematoxylin and eosin for assessment of GVHD using a published semiquantitative scoring system (17).

MLR cultures

Purified T cells from PD-$1^{-/-}$ and wild-type (WT) B6 mice (5×10^5 cells/well) were stimulated with 48-h LPS-activated and irradiated (2000 cGy) B10.BR splenocytes at various responder-stimulator ratios. Cultures were labeled with [^{3}H]thymidine (1 μCi) for the last 24 h of a 3-day assay.

Cytokine analysis

MLR supernatants or sera were obtained from cohorts of mice for cytokine analysis (IL-2, IL-4, IL-10, TNF-α, IFN-γ) using a cytometric bead assay (BD PharMingen) according to the manufacturer's specifications.

Statistical analysis

The Kaplan-Meier product-limit method was used to calculate survival rates. Differences between groups were determined using log rank analysis. Cytokine data were analyzed by Student's t test. Values of $p \leq 0.05$ were considered significant.

Results

Blockade of the PD-1/PD-L pathway enhances GVHD lethality

To determine whether targeting PD-1/PD-L interaction would affect GVHD-induced mortality, lethally irradiated B10.BR recipients were given B6 bone marrow plus supplemental splenocytes (0 or 5×10^6) and either irrelevant or PD-L1.Fc fusion protein consisting of the extracellular domains of murine PD-L1 linked to a mutated murine IgG2a. Recipients given PD-L1.Fc fusion protein had a significant acceleration in GVHD-induced mortality ($p < 0.02$), equivalent to the administration of a 3-fold higher spleen cell dose (Fig. 1*A*). In other studies, recipients were given either irrelevant or blocking anti-PD-1 mAb. Anti-PD-1 mAb infusion markedly accelerated GVHD lethality ($p < 10^{-6}$) which was significantly ($p = 0.03$) greater than the GVHD rate observed in recipients of a 3-fold higher splenocyte dose (Fig. 1*B*). Because PD-L1.Fc and anti-PD-1 mAb infusion could be functioning either to provide a positive or to preclude a negative costimulatory signal to donor T cells, experiments were performed comparing GVHD lethality of supplemental B6- PD-$1^{+/+}$ to B6-PD-$1^{-/-}$ splenocytes (5 or 15×10^6) (Fig. 1*C*). The GVHD lethality of B6-PD-$1^{-/-}$ splenocytes was markedly accelerated compared with B6 WT cells ($p < 0.0001$). The magnitude of GVHD acceleration was similar to that observed with either PD-L1.Fc or anti-PD-1 mAb. Together, these data suggest that PD-L1.Fc and anti-PD-1 mAb both blocked PD-1 ligation and inhibited a negative costimulatory signal to donor T cells.

To determine whether both $CD4^+$ and $CD8^+$ T cell-mediated GVHD was regulated by PD-1/PD-L interactions, purified $CD4^+$ or $CD8^+$ T cells from B6-PD-$1^{+/+}$ or B6-PD-$1^{-/-}$ donors were infused into sublethally irradiated bm12 or bm1, respectively. Based on T cell dose-response curves, GVHD lethality was augmented by ≥3-fold with PD-$1^{-/-}$ $CD4^+$ or $CD8^+$ T cells as compared with results obtained using B6 donors (Fig. 1, *D* and *E*). In aggregate, our studies provide conclusive evidence that prohibiting PD-1 engagement accelerated GVHD lethality.

The inhibitory effects of the PD-1/PD-L pathway on GVHD-induced mortality are not redundant with the CTLA-4:B7 pathway

The down-regulatory effects of the PD-1/PD-L pathway on GVHD parallel our previous observations for the CTLA-4/B7 pathway under these same conditions (15). To determine whether these pathways are fully redundant, B10.BR recipients were given B6-PD-$1^{+/+}$ BM, a low dose of splenocytes (5×10^6), and either irrelevant mAb or anti-PD-1, anti-CTLA-4, or both mAbs. Whereas anti-CTLA-4 mAb did not significantly accelerate GVHD lethality at this T cell dose ($p = 0.41$), anti-PD-1 mAb did, reducing the median survival time by 2 wk as compared with controls ($p = 0.002$). Importantly, the combined administration of both mAb was significantly more potent than either alone in accelerating GVHD lethality ($p < 0.04$; Fig. 2). These data indicate that the PD-1/PD-L, and CTLA-4/B7 pathways are not redundant and suggest a major regulatory role for the PD-1/PD-L pathway in GVHD modulation.

Neither perforin nor FasL is required for the accelerated GVHD lethality induced by anti-PD-1 mAb

On day 7 post-bone marrow transplant (BMT), tissues were obtained for GVHD scoring, using a semiquantitative scoring system (0→4) from representative recipients that received irrelevant or anti-PD-1 mAb and 5×10^6 splenocytes. In the liver, irrelevant mAb treated recipients had less severe injury than anti-PD-1 mAb-treated mice (2.5 vs 3.3, respectively; $p = 0.01$). Mean GVHD scores were not significantly different in colon, skin, or lung (not shown).

FasL has been implicated in GVHD pathogenesis and especially hepatic GVHD (16, 18). To determine whether FasL expression was required for the accelerated GVHD lethality observed with anti-PD-1 mAb infusion, heavily irradiated B10.BR recipients were given B6 BM and supplemental splenocytes from FasL$^{-/-}$ (*gld/gld*) donors (Fig. 3*A*). Although GVHD lethality was delayed after the administration of FasL$^{-/-}$ donor splenocytes, anti-PD-1 mAb still accelerated GVHD. Because perforin-mediated cytolysis also has been implicated in GVHD pathogenesis, studies were performed using perforin$^{-/-}$ donor splenocytes (Fig. 3*B*). Similar to FasL$^{-/-}$ donor splenocytes, GVHD lethality was accelerated by anti-PD-1 mAb given to recipients of perforin$^{-/-}$ splenocytes. Thus, the mechanism(s) involved in PD-1-mediated GVHD regulation is not dependent on FasL or perforin.

Donor IFN-γ production is required for optimal GVHD acceleration induced by PD-1/PD-L blockade

Because two of the classical cytolytic effector pathways, perforin and FasL, did not appear to be essential for GVHD acceleration induced by PD-1/PD-L blockade, we explored the possibility that enhanced proliferative responses and/or the release of proinflammatory cytokines may provide an explanation for our findings. A

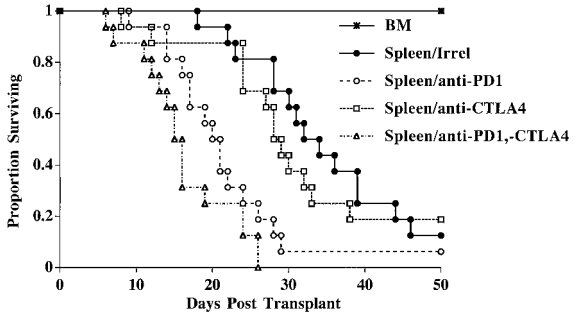

FIGURE 2. Effect of the PD-1/PD-L pathway on GVHD is not redundant with that of the CTLA-4/B7 pathway. Lethally irradiated B10.BR recipients were given B6 BM alone or with 5×10^6 B6 splenocytes and irrelevant (Irrel), anti-PD-1, and/or anti-CTLA-4 mAbs were administered. $n = 16$/group. x-axis, Days posttransplant; y-axis, proportion surviving. $p < 0.005$ for survival differences between cohorts receiving anti-PD-1 vs irrelevant mAb. $p < 0.05$ for survival rate differences between the cohort receiving anti-PD-1 and anti-CTLA-4 mAb and cohorts receiving either anti-PD-1 or anti-CTLA-4 mAb alone.

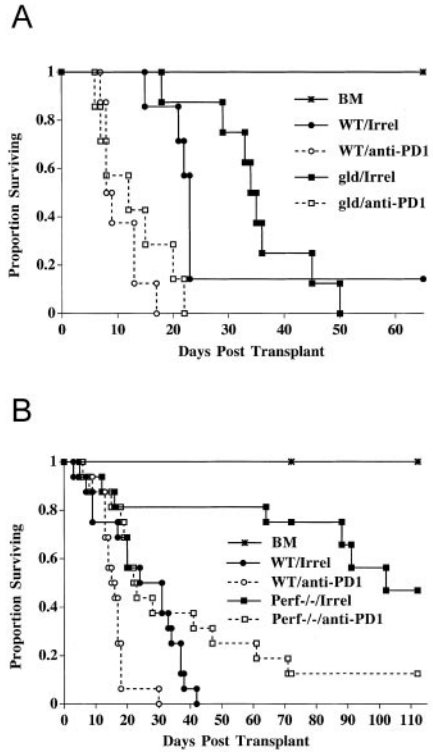

FIGURE 3. Accelerated GVHD lethality observed with PD-1/PD-1L blockade is not dependent on donor FasL or perforin expression. *A* and *B,* Lethally irradiated B10.BR recipients were given B6 BM alone or with B6 WT or B6 deletional mutant splenocytes as indicated, and irrelevant (Irrel) or anti-PD-1 mAb was infused. *A,* WT or *gld* (15×10^6 splenocytes/recipient; $n = 8$/group); *B,* WT or perf$^{-/-}$ (5×10^6 splenocytes/recipient; $n = 16$/group). *x*-Axis, Days posttransplant; *y*-axis, proportion surviving. $p < 0.005$ for survival rate differences between cohorts receiving comparable splenocyte doses and anti-PD-1 mAb regardless of the type (*gld, perf*$^{-/-}$, or WT) of donor splenocytes infused.

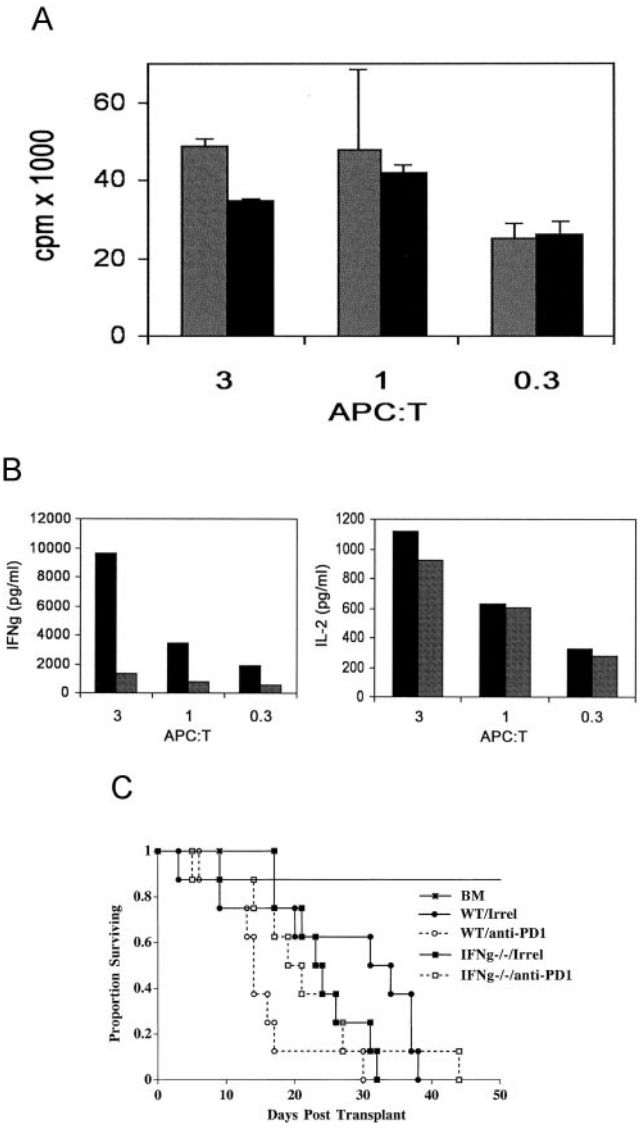

FIGURE 4. Blockade of the PD-1/PD-L pathway increases IFN-γ production, which is required for maximal GVHD acceleration. *A,* Results of day 3 primary MLR responses using B6 WT or PD-1$^{-/-}$ responder T cells and a variable ratio of B10.BR blasts as stimulators (termed APCs). A representative experiment of three performed is shown. ■, PD-1 knockout (KO; PD-1$^{-/-}$); ▨, WT. *B,* IFN-γ (*left*) and IL-2 (*right*) concentrations present in MLR culture supernatants from *A*. A representative experiment of two performed is shown. ■, PD-1KO (PD-1$^{-/-}$); ▨, WT. *C,* Survival post-BMT. Lethally irradiated B10.BR recipients were given B6 BM, B6 (5×10^6) or B6 IFN-$\gamma^{-/-}$ (1×10^6) splenocytes, and irrelevant (Irrel) or anti-PD-1 mAb was infused ($n = 8$/group). *x*-Axis, Days posttransplant; *y*-axis, proportion surviving. $p < 0.05$ in survival rate differences between the cohort of recipients of WT splenocytes receiving anti-PD-1 vs irrelevant mAb. In contrast, no significant differences in survival rates were observed between cohorts receiving IFN-$\gamma^{-/-}$ splenocytes.

previous report has shown that PD-1/PD-L ligation of naive CD4$^+$ and CD8$^+$ T cells led to decreased proliferation (13). In addition, 2C PD-1$^{-/-}$ T cells show enhanced proliferation on repeated stimulation with H2L^d-derived splenocytes (4). However, the effect of the PD-1 pathway in primary MLR responses has not been examined. Thus, primary MLR cultures were established using responder T cells from WT vs PD-1$^{-/-}$ B6 mice and LPS blasts from B10.BR mice as stimulators at various ratios. WT T cells isolated during the MLR culture were PD-1$^+$, whereas virtually all LPS blasts were PD-L1$^+$ (data not shown). Primary MLR cultures did not consistently reveal significantly higher proliferation of PD-1$^{-/-}$ vs WT T cells (Fig. 4*A*), suggesting that the PD-1/PD-L pathway does not play a major role in regulation of proliferation in primary MLR responses.

To determine whether the absence of PD-1 ligation led to higher levels of cytokine production, supernatants from MLR cultures and sera from post-BMT recipients were assayed for proinflammatory (IL-2; IFN-γ; TNF-α) and anti-inflammatory (IL-4; IL-5) cytokines (Fig. 4*B* and Table I). In primary MLR cultures, IFN-γ was substantially increased in cultures containing PD-1$^{-/-}$ T cells (Fig. 4*B*). On sera collected post-BMT, IFN-γ levels were significantly higher on days 4 and 6 post-BMT in anti-PD-1 mAb-treated recipients (Table I).

Because increased IFN-γ production was observed in vitro with PD-1$^{-/-}$ T cells and in vivo upon anti-PD-1 treatment, we next performed studies using B6 IFN-$\gamma^{-/-}$ or WT donor splenocytes administered to irrelevant or anti-PD-1 mAb-treated B10.BR recipients (Fig. 4*C*). As we have previously reported, IFN-$\gamma^{-/-}$ splenocytes result in substantial acceleration of GVHD mortality relevant to WT splenocytes (19), likely due to defective T cell apoptosis (20). Whereas recipients of WT splenocytes had a substantial acceleration in GVHD lethality induced by anti-PD-1 vs irrelevant mAb (median survival time, 14 vs 31 days; $p < 0.02$), there was no significant effect in recipients of IFN-$\gamma^{-/-}$ splenocytes (median survival time, 19 vs 24 days; $p = 0.42$). These data are consistent with a requirement of donor IFN-γ production in the GVHD-accelerating effect of anti-PD-1 mAb.

Table I. *Induction of proinflammatory cytokine release by anti-PD-1 mAb given to recipients early post-BMT*[a]

		Th1 (pg/ml)			Th2 (pg/ml)	
	Day	IL-2	IFN-γ	TNF-α	IL-4	IL-15
Irrelevant mAb	2	4 (1)	6 (1)	38 (2)	1 (1)	26 (3)
Anti-PD-1 mAb	2	5 (1)	7 (2)	33 (1)	1 (1)	53 (34)
Irrelevant mAb	4	3 (1)	60 (24)	56 (21)	0 (0)	20 (3)
Anti-PD-1 mAb	4	5 (1)	588 (147)[b]	57 (3)	0 (0)	25 (28)
Irrelevant mAb	6	4 (1)	441 (64)	152 (10)	1 (0)	29 (7)
Anti-PD-1 mAb	6	3 (0)	1327 (155)[b]	347 (52)[b]	1 (0)	7 (3)[b]

[a] Sera were obtained from cohorts of irradiated BMT recipients that were given either irrelevant or anti-PD-1 mAb as described in *Materials and Methods.* Four mice per group were individually analyzed at the indicated time periods post-BMT. Numbers in parentheses, mean ± SEM.

[b] $p < 0.05$ compared with irrelevant mAb controls.

Discussion

Immune responses are regulated by both positive and negative pathways with the net result determining outcome. In our studies, we used PD-L1.Fc protein (mutated murine IgG2a Fc portion), anti-PD-1 mAb and PD-$1^{-/-}$ donor T cells to conclusively demonstrate that the dominant in vivo effect of the PD-1/PD-L pathway is to down-regulate T cell responses. These data are consistent with studies demonstrating that B6 2C PD-$1^{-/-}$ and BALB/c PD-$1^{-/-}$ mice were susceptible to developing autoimmunity (4, 14, 21). Interestingly, Hancock et al. reported that PDL-1.Fc (human IgG1 Fc portion) inhibited cardiac allograft rejection in CD28$^{-/-}$ recipients or in WT recipients treated with immunosuppressive agents (22). A potential explanation for the different outcomes of our and Hancock's study may reside in the distinct Fc portions of the PD-L1.Fc proteins used. Fusion proteins with different Fc portions may lead to PD-1 ligation or blockade (human IgG1 vs a mutated murine IgG2a). However, our studies do not rule out the existence of a second receptor for PD-L proteins that may deliver a positive T cell-costimulatory signal, which is differentially affected by the two distinct PD-L1.Fc proteins (5, 7).

The effect of PD-1/PD-L interactions on down-regulating T cell responses in vivo has striking similarities to CTLA-4/B7 interactions. Both PD-$1^{-/-}$ and CTLA4$^{-/-}$ mice develop a lymphoproliferative syndrome with organ infiltration by activated T cells, although this process is more rapid in CTLA4$^{-/-}$ mice (4, 9, 10, 14). PD-1 engagement inhibits cell cycle progression, similar to CTLA-4 engagement (13, 23). A failure of cell cycle progression has been associated with multiple forms of anergy. Conversely, CTLA-4 engagement has been shown to promote tolerance induction (23, 24). Thus, it is possible that PD-1 ligation also may be required for optimal tolerance induction (4). In any event, our data along with the overlapping yet distinct tissue expression patterns of the CTLA-4/B7 and PD-1/PD-L pathways suggest differential effects of these pathways on regulating GVHD. We have observed that blockade of the PD-1/PD-L pathway was more potent than CTLA-4/B7 blockade for GVHD acceleration and that the combined blockade of both pathways was additive in accelerating GVHD. These latter data suggest that PD-1/PD-L and CTLA-4/B7 act independently to down-regulate GVHD lethality.

A potential mechanism for the accelerated GVHD lethality caused by PD-$1^{-/-}$ as compared with WT cells may be that the expansion or infiltration of donor T cells into GVHD target tissues was increased by blocking PD-1/PD-L interactions. However, no increased in vitro proliferation was observed in primary MLR responses with PD-$1^{-/-}$ T cells. Similarly, flow cytometric analysis of splenocytes and immunohistochemical staining of GVHD target organs (spleen, liver, lung, colon, skin) did not reveal differences in the number of $CD4^+$ or $CD8^+$ T cells assessed days 6–8 post-BMT (data not shown), indicating that differences in donor T cell infiltration likely were not responsible for the increased GVHD lethality induced by PD-1/PD-L blockade. Consistent with the immunohistochemistry results, in preliminary studies designed to examine the proliferative effects of anti-PD-1 mAb on donor T cells early post-BMT, the proportion of donor $CD4^+$ and $CD8^+$ T cells in the spleen or in the lymph node that had undergone 0–1 cell divisions, as measured by CFSE staining, was comparable in recipients treated with anti-PD-1 as compared with irrelevant mAb ($n = 3$/group) when assessed on day 3 post-BMT (data not shown). Thus, the marked acceleration in GVHD lethality induced by anti-PD-1 mAb does not appear to be due to a pronounced effect of PD-1/PD-L blockade on T cell proliferation.

Anti-PD-1 mAb accelerated GVHD lethality independently of FasL or perforin expression. As PD-1 ligation has been shown to decrease cytokine production in in vitro assays (11), we considered the possibility that the induction of inflammatory cytokine cascades may have been the responsible effector mechanism for the augmented GVHD lethality observed after PD-1/PD-L blockade. We observed an increase in IFN-γ production in MLR supernatants derived from PD-$1^{-/-}$ T cells. Systemic IFN-γ levels were augmented by PD-1/PD-L blockade and the accelerated GVHD lethality observed under these conditions was dependent on donor IFN-γ production. The role of IFN-γ levels in regulating GVHD has not been entirely resolved; sustained high IFN-γ production can result in more severe GVHD in some models (19, 25). However, the link between high IFN-γ and worse GVHD is not a universal finding, with some studies showing reduced GVHD associated with increased donor T cell apoptosis (20, 26, 27). Therefore, any propensity toward an increased division rate that might occur when the PD-1/PD-L pathway is blocked may be offset by an increased apoptosis rate caused by IFN-γ elevation. Early post-BMT, the dominant effect of IFN-γ may be the up-regulation of cell surface determinants (e.g., PD-L1 or PD-L2), host MHC or costimulatory molecules, or stimulation of host production of other inflammatory cytokines that function as GVHD effector mechanism (e.g., TNF-α or IL-1), as recently implicated by Reddy et al. (20). Serum TNF-α levels were significantly elevated in anti-PD-1 vs irrelevant mAb-treated recipients on day 6 post-BMT. The regulation of systemic IFN-γ production by the PD-1/PD-L pathway in our GVHD setting is consistent with the reduction in cardiac graft IFN-γ associated with the infusion of PD-L1.Fc which appears to have resulted in PD-1 ligation (22). Taken together, we conclude that the PD-1/PD-L pathway is a major regulator of

IFN-γ production in vivo, a finding that has implications for the biological role of this pathway in IFN-γ-responsive diseases.

In summary, the major finding of this study is that the PD-1 pathway is a negative regulator of both $CD4^+$ and $CD8^+$ T cell alloresponses. This conclusion was derived from three distinct approaches used to block the PD-1 pathway: PD-L1.Fc; anti-PD-1 mAb; and PD-$1^{-/-}$ donor cells. PD-1 pathway blockade was associated with a heightened release of proinflammatory cytokines, particularly IFN-γ, that likely contributed to GVHD lethality and was not dependent perforin/FasL-mediated cytolysis. Our data provide definitive evidence that PD-1 engagement diminishes alloreactive T cell responses after BMT. These data have important implications for our understanding of T cell alloresponse and GVHD pathophysiology. Ligation of the PD-1 receptor may represent a novel approach for preventing GVHD.

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