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PD-1:PD-L inhibitory pathway affects both CD4$^+$ and CD8$^+$ T cells and is overcome by IL-2

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Programmed death-1 (PD-1) is an immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing receptor expressed upon T cell activation. PD-1$^{-/-}$ animals develop autoimmune diseases, suggesting an inhibitory role for PD-1 in immune responses. Members of the B7 family, PD-L1 and PD-L2, are ligands for PD-1. This study examines the functional consequences of PD-1:PD-L engagement on murine CD4 and CD8 T cells and shows that these interactions result in inhibition of proliferation and cytokine production. T cells stimulated with anti-CD3/PD-L1.Fc-coated beads display dramatically decreased proliferation and IL-2 production, while CSFE analysis shows fewer cells cycling and a slower division rate. Costimulation with soluble anti-CD28 mAb can overcome PD-1-mediated inhibition by augmenting IL-2 production. However, PD-1:PD-L interactions inhibit IL-2 production even in the presence of costimulation and, thus, after prolonged activation, the PD-1:PD-L inhibitory pathway dominates. Exogenous IL-2 is able to overcome PD-L1-mediated inhibition at all times, indicating that cells maintain IL-2 responsiveness. Experiments using TCR transgenic CD4$^+$ or CD8$^+$ T cells stimulated with antigen-presenting cells expressing PD-L1 show that both T cell subsets are susceptible to this inhibitory pathway. However, CD8$^+$ T cells may be more sensitive to modulation by the PD-1:PD-L pathway because of their intrinsic inability to produce significant levels of IL-2.

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1 Introduction

T cell activation and the generation of protective immunity are the result of a balance between positive versus negative signals. Negative signals through cell surface molecules such as CTLA-4, CD95, CD5, CD31, LAIR, Ly49A, NKG2A inhibit T cell activation or induce apoptosis [1]. In the absence of proper control, T cell activation can lead to compromised self tolerance and disease, as illustrated by mutant mouse strains that develop fatal lymphoproliferative autoimmune disorders as a result of defects in negative regulatory molecules [2–5].

Likewise, PD-1$^{-/-}$ mice develop lymphoproliferative/autoimmune diseases, demonstrating a role for this molecule in lymphocyte deactivation and tolerance [6, 7]. Aged, PD-1$^{-/-}$ C57BL/6 mice develop a lupus-like disease and arthritis [6]; within 30 weeks, PD-1$^{-/-}$ BALB/c mice develop a fatal disease targeting the heart [7]. Like other negative regulators, PD-1 has tyrosines in ITIM-like motifs that may recruit phosphatases [8, 9]. PD-1 is expressed on activated T cells, B cells and monocytes and likely regulates these cell types [10]. These results emphasize the importance of PD-1 in down-modulating immune responses and in maintaining peripheral tolerance.

PD-1 ligands, PD-L1 and PD-L2, are 38% identical and are members of the B7 family [9, 11]. PD-L1 mRNA is expressed constitutively in heart, lung, kidney, liver and spleen [9] and is up-regulated by IFN-γ in endothelial cells, keratinocytes, dendritic cells and several tumor cell lines ([11] and unpublished observations). PD-L2 mRNA is constitutively expressed in the liver and at lower levels in lung and spleen. These results and the phenotype of the PD-1$^{-/-}$ mouse suggest a role for the PD-1:PD-L inhibitory pathway in peripheral tissues.

Previously, we reported that stimulation of wild type, but not PD-1$^{-/-}$, T cells in the presence of PD-L1.Fc or PD-

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Abbreviations: ITIM: Immunoreceptor tyrosine-based inhibitory motif **PD-1:** Programmed death-1 **PD-L:** Programmed death ligand

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L2.Fc inhibits proliferation [9, 11]. Here, we explore the modulatory effect of CD28 costimulation on PD-1-mediated inhibition of unseparated and CD4+ versus CD8+ T cells. We show that PD-1 engagement by PD-L1 or PD-L2 inhibits proliferation as a result of compromised IL-2 production. Initially, CD28 costimulation can overcome PD-1-mediated inhibition by inducing IL-2 production; however, later, the inhibitory pathway dominates. Both CD4+ and CD8+ T cells are inhibited by PD-1:PD-L interactions. For CD4+ T cells, inhibition can be overcome by costimulation via IL-2 induction. In contrast, CD8+ T cell inhibition is not reversed by costimulation but can be overcome by exogenous IL-2. Thus, we propose that CD8+ T cells may be more sensitive to the PD-1:PD-L inhibitory pathway because of their intrinsic inability to produce significant levels of IL-2.

2 Results

2.1 PD-1 expression PD-1 upon T cell activation

PD-1 has been reported to be a T cell activation antigen [10]. PD-1 expression was analyzed to determine the kinetics of cell surface expression under the conditions used in the functional assays. Purified lymph node (LN) T cells were stimulated with anti-CD3 mAb-coated beads and control fusion protein (ctrl.Fc), and at the indicated times, cells were harvested and stained for FACS analysis (Fig. 1A). After 24 h, PD-1 was up-regulated on 27% of T cells. As the assay progressed, the percentage of PD-1+ cells increased on subsequent days. There was no significant difference in PD-1 expression between CD4+ and CD8+ T cells (data not shown). Unlike antigen-stimulated T cells, PD-1 expression was not transient here due to the continuous presence of stimulatory beads. From these results, we conclude that using our stimulation conditions, PD-1 expression is strongly up-regulated upon T cell activation.

2.2 PD-1 ligation by PD-L1 or PD-L2 inhibits proliferation

To assess the effect of mPD-1:PD-L1 or PD-L2 interactions on proliferation, T cells were stimulated using beads coated with anti-CD3 mAb and increasing amounts of mPD-L1.Fc or mPD-L2.Fc fusion proteins (Fig. 1B). T cells stimulated with anti-CD3/PD-L1.Fc or anti-CD3/PD-L2.Fc beads proliferate less than anti-CD3/ctrl.Fc-stimulated cells in a dose-dependent manner. Subsequent experiments were done with the maximal dose of PD-L1.Fc fusion protein (2 μg/10^7 beads). These results suggest that ligation of PD-1 by either of its ligands inhibits the ability of T cells to proliferate in response to TCR signals.

As shown in Fig. 1A, PD-1 expression is not detected on naive T cells, but is found as early as 24 h after stimula-

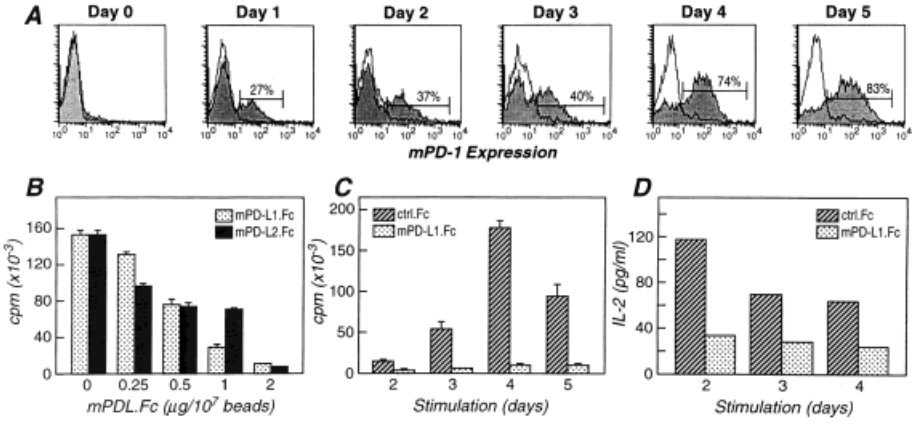

Fig. 1. PD-1 expression is up-regulated upon T cell stimulation and mediates inhibition of proliferation and IL-2 production when ligated by PD-L1.Fc or PD-L2.Fc. (A) Expression of PD-1. BALB/c LN T cells were stimulated with anti-CD3/ctrl.Fc beads and at the indicated time stained using anti-mPD-1-biotin mAb (filled) or hamster Ig-biotin (unfilled). Live-gated histograms of a representative experiment (*n*=3) is shown. (B) PD-L1.Fc and PD-L2.Fc dose-dependent inhibition of T cell proliferation. T cells were stimulated using beads coated with a constant level of anti-CD3 (3 μg/10^7 beads) and increasing concentrations of PD-L1.Fc or PD-L2.Fc fusion proteins. Proliferation was measured on day 3. Data are representative (*n*=4). (C) Kinetics of PD-L1.Fc-mediated inhibition. T cells were stimulated with anti-CD3/ctrl.Fc or anti-CD3/PD-L1.Fc beads and proliferation was measured. A representative experiment is shown (*n*=20). (D) PD-1:PD-L1.Fc engagement inhibits IL-2 production. T cells were stimulated as above, supernatants were harvested and assayed for IL-2 by ELISA. Shown is representative experiment (*n*=3).

tion and cell surface expression continues to increase over time. The kinetics of inhibition was examined as a measure of functional PD-1 expression (Fig. 1C). Minimal proliferation is detected 24 h after stimulation (data not shown). At other time points, activation with anti-CD3/PD-L1.Fc beads inhibits T cell proliferation relative to cells stimulated with anti-CD3/ctrl.Fc beads. Similar kinetics and inhibition were observed using anti-CD3/PD-L2.Fc beads (data not shown). These results establish a correlation between the kinetics of PD-1 expression and inhibition of T cell proliferation upon activation in the presence of PD-L1 or PD-L2.

2.3 PD-1:PD-L1 interactions inhibit IL-2 production

Because PD-1:PD-L1 interactions impair proliferation, we examined the effect of PD-1 engagement on production of the autocrine growth factor IL-2 (Fig. 1D). T cells were stimulated with beads as above and supernatants were collected for IL-2 analysis. Relative to anti-CD3/ctrl.Fc-stimulated cells, IL-2 production by anti-CD3/PD-L1-stimulated cells was inhibited 60–70%. IL-2 levels detected on days 3 and 4 from the anti-CD3/ctrl.Fc cultures are likely underestimated due to consumption by the proliferating cells. Thus, PD-1:PD-L1 ligation decreases IL-2 production, suggesting a mechanism for the observed inhibition of proliferation.

2.4 PD-1:PD-L1 interactions decrease cell divisions

To further characterize the activation and expansion of individual T cells stimulated with anti-CD3/ctrl.Fc beads or anti-CD3/PD-L1.Fc beads, CSFE dye was used to track cell division (Fig. 2). On day 2, cultures from either stimulation condition had completed as many as four rounds of cell division. However, relative to anti-CD3/PD-L1.Fc-stimulated cells, there were increased numbers of cells in the less fluorescent peaks in the anti-CD3/ctrl.Fc-stimulated cultures, indicating that more cells had undergone division. Anti-CD3/PD-L1.Fc stimulation resulted in increased percentages of undivided cells compared to anti-CD3/ctrl.Fc-stimulated cultures (47% *vs*. 32%). Differences in the percentage of non-dividing cells, as well as the number of divisions, become more pronounced on days 3 and 4. The kinetics of inhibition observed using CSFE analysis are in agreement with the kinetics of PD-1 expression and with the [^{3}H]thymidine proliferation assays (Fig. 1C). These results indicate that PD-1:PD-L1 interactions lead to decreased proliferation by limiting the number and the division rate of cells that enter cell cycle.

2.5 Costimulation overcomes PD-L1-mediated inhibition by inducing IL-2 production

Because many T cells are activated by professional APC-bearing costimulatory molecules, we analyzed the effect of costimulation on the PD-1:PD-L1 inhibitory pathway. T cells were stimulated with anti-CD3/ctrl.Fc or anti-CD3/mPD-L1.Fc beads in the presence or absence of anti-CD28 mAb (Fig. 3A). CD28 costimulation resulted in more rapid up-regulation of PD-1 expression (data not shown). Addition of soluble anti-CD28 mAb provides a costimulatory signal to T cells and overcomes PD-L1-mediated inhibition. Reversal of PD-L1 or PD-L2-mediated inhibition was dependent on anti-CD28 mAb concentration (data not shown). As CD28 costimulation is important for promoting IL-2 production and IL-2 production is defective in PD-L1.Fc-stimulated cells, we used a neutralizing anti-IL-2 mAb to test whether anti-CD28 costimulation overcomes the PD-1:PD-L1 inhibitory pathway by inducing IL-2. In the presence of anti-CD28 and anti-IL-2 mAb, inhibition is observed when

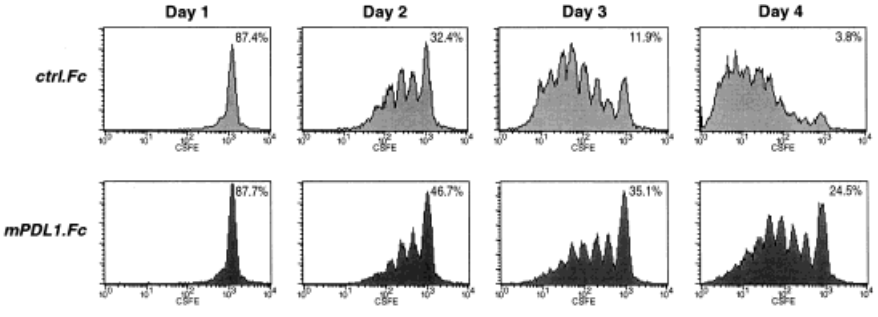

Fig. 2. PD-1:PD-L1.Fc interaction inhibits T cell proliferation as observed by CSFE staining. T cells were labeled with CSFE and stimulated with anti-CD3/ctrl.Fc or anti-CD3/PD-L1.Fc beads. Percentages indicate the fraction of cells in the non-dividing peak. Shown are live-gated histograms from a representative experiment (n=4).

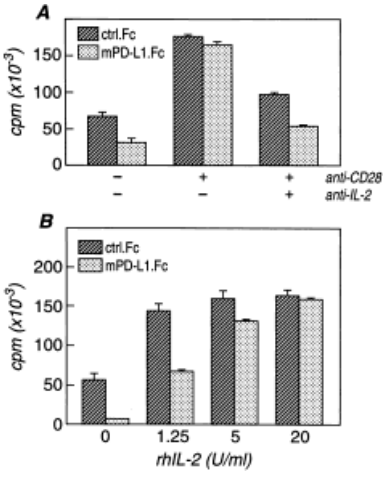

Fig. 3. Costimulation or exogenous IL-2 can overcome the PD-1:PD-L1 inhibitory pathway. (A) CD28 costimulation abrogates PD-1:PD-L1 inhibition of proliferation by inducing IL-2. T cells were stimulated with anti-CD3/ctrl.Fc or anti-CD3/PD-L1.Fc beads in the absence or presence of soluble anti-CD28 mAb (1 μg/ml) and anti-IL-2 mAb (10 μg/ml). Proliferation was assessed on day 2. Shown is a representative experiment (n=3). (B) Exogenous IL-2 reverses PD-1:PD-L1-mediated inhibition. T cells were stimulated with anti-CD3/ctrl.Fc or anti-CD3/PD-L1.Fc beads and the indicated amount of rhIL-2. Proliferation was measured on day 3. Similar results were observed at days 2, 4, and 5 (data not shown). A representative experiment (n=3) is shown.

cells are stimulated with anti-CD3/PD-L1.Fc (Fig. 3A), suggesting that CD28 costimulation overcomes the PD-1:PD-L1 inhibitory pathway by inducing IL-2.

To demonstrate the pivotal role of IL-2 in overriding PD-1:PD-L1–mediated inhibition, increasing concentrations of rhIL-2 were added to cells stimulated with anti-CD3/ctrl.Fc or anti-CD3/PD-L1.Fc beads (Fig. 3B). IL-2 overcomes the PD-1:PD-L1 inhibitory pathway in a dose-dependent manner. Similar results were obtained with anti-CD3/PD-L2.Fc-coated beads (data not shown). Exogenous IL-2 provides an accessory signal resulting in augmented T cell proliferation to both anti-CD3/ctrl.Fc- and anti-CD3/PD-L1.Fc-stimulated cells, indicating that anti-CD3/PD-L1.Fc-stimulated cells are fully capable of responding to IL-2. These results suggest that the defective proliferation seen upon PD-1:PD-L engagement is in, part, the result of impaired IL-2 production and not impaired IL-2 responsiveness.

2.6 PD-1:PD-L1 inhibitory pathway dominates over costimulation by inhibiting IL-2 production

The ability of costimulation to overcome PD-1-negative signaling was examined at different time points (Fig. 4A). Over time, CD28 costimulation was less effective at reversing PD-1-mediated inhibition. This is not due to consumption or degradation of the mAb at later time points as there was still strong costimulation compared to cultures not receiving soluble mAb (data not shown). Likewise, increased concentrations of soluble anti-CD28 mAb could not restore proliferation in response to anti-CD3/PD-L1.Fc stimulation (data not shown), suggesting

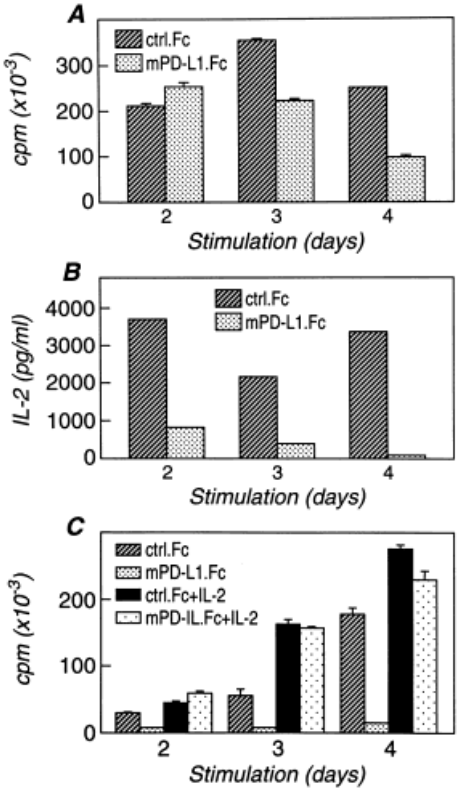

Fig. 4. Kinetics of costimulation- and IL-2-mediated reversal of the PD-1:PD-L1 inhibitory pathway. (A) CD28 costimulation reverses PD-1:PD-L1 inhibition. T cell proliferation was determined after stimulation with anti-CD3/ctrl.Fc or anti-CD3/PD-L1.Fc beads and soluble anti-CD28 mAb (1 μg/ml). A representative experiment (n=10) is shown. (B) IL-2 production is inhibited by PD-1:PD-L1 interactions, even in the presence of CD28 costimulation. T cells were stimulated as in (A), supernatants were harvested and assayed for IL-2 levels by ELISA. A representative experiment (n=3) is shown. (C) Exogenous IL-2 overcomes PD-1 inhibitory signals at all time points. T cells were stimulated with anti-CD3/ctrl.Fc or anti-CD3/PD-L1.Fc beads in the absence or presence of rhIL-2 (20 U/ml).

that the inability to overcome inhibition was not the result of sub-optimal antibody levels.

PD-1:PD-L1.Fc interactions inhibit IL-2 production in the absence of costimulation and neutralizing anti-IL-2 mAb abrogates the ability of CD28 costimulation to overcome the PD-1:PD-L1 inhibitory pathway (Figs. 1D and 3A). Therefore, we measured IL-2 titers to test for a correlation between the dominance of the PD-1:PD-L1 inhibitory pathway over costimulation and decreased IL-2 production (Fig. 4B). In the presence of anti-CD28, high titers of IL-2 were detected, even in the anti-CD3/PD-L1.Fc-stimulated cultures (compare Figs. 1D and 4B). However, stimulation with anti-CD3/PD-L1.Fc and anti-CD28 mAb inhibits IL-2 production relative to anti-CD3/ctrl.Fc stimulation. Levels of IL-2 decrease substantially as activation proceeds (840, 426, 118 pg/ml on days 2, 3 and 4), correlating with inability of CD28 costimulation to overcome the PD-1:PD-L inhibitory pathway. These results suggest there is a threshold level of IL-2 necessary to overcome PD-1:PD-L1-mediated proliferative inhibition, and costimulation is effective at overcoming inhibition only when it induces IL-2 levels higher than the threshold. However, the PD-1:PD-L pathway inhibits IL-2 production even in the presence of a strong costimulatory signal, suggesting that this inhibitory pathway can dominate over T cell activation.

In support of this, addition of exogenous IL-2 abrogates the inhibition of proliferation resulting from PD-1:PD-L1 interactions at all time points (Fig. 4C). Thus, IL-2, whether induced by costimulation or provided exogenously, overcomes proliferative inhibition mediated by PD-1:PD-L1 engagement. Similar results were observed with anti-CD3/PD-L2.Fc beads (data not shown). This, coupled with the observation that PD-1:PD-L engagement inhibits IL-2 production even in the presence of costimulation, suggests a central modulatory role for IL-2 in this inhibitory pathway.

2.7 The PD-1:PD-L1 pathway regulates CD4$^+$ and CD8$^+$ T cells

Previous reports suggest that CD4$^+$ and CD8$^+$ T cells are differentially affected by particular activation and deactivation pathways [12–16]. Therefore, we examined the ability of these T cell subsets to be regulated by the PD-1:PD-L1 inhibitory pathway. T cells were labeled with CSFE and stimulated with anti-CD3/ctrl.Fc or anti-CD3/PD-L1.Fc beads, harvested at the indicated times and stained for CD4 and CD8 expression. The sensitivity of these subsets to PD-L1-mediated inhibition was determined by electronically gating on the CD4$^+$ or CD8$^+$ T cell populations and measuring CSFE fluorescence intensity

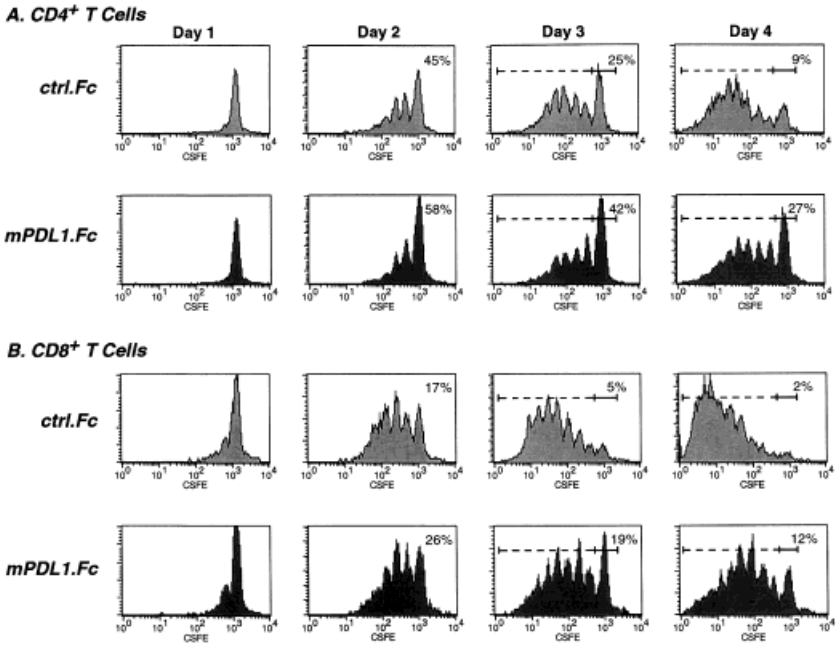

Fig. 5. CD4$^+$ and CD8$^+$ T cells in a mixed culture are susceptible to the PD-1 inhibitory pathway. T cells were labeled with CSFE and stimulated with anti-CD3/ctrl.Fc or anti-CD3/PD-L1.Fc beads. At the indicated time, cells were harvested, stained with anti-CD4-PE and anti-CD8-Cy-chrome, fixed and analyzed by flow cytometry. (A) CD4-gated CSFE histograms. (B) CD8-gated CSFE histograms. Percentages refer to fraction of cells in the non-dividing peak. Shown is a representative experiment (n=3).

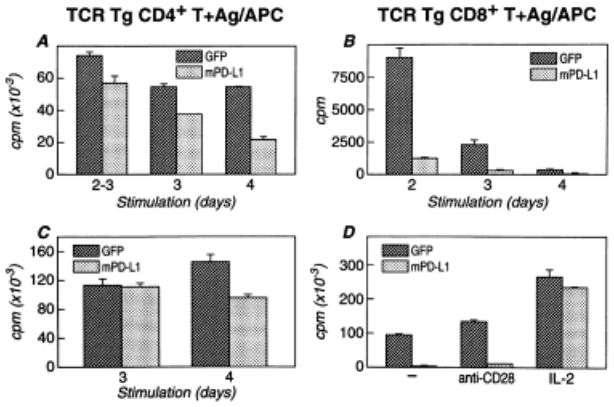

Fig. 6. Stimulation with PD-L1-expressing APC inhibits proliferation of CD4+ and CD8+ T cells. (A) 53HED CD4+ T cells were stimulated with COS-IE^k APC expressing GFP or mPD-L1 and PCCF peptide. Shown is a representative experiment (*n*=3). (B) 2C CD8+ T cells were stimulated with p2Ca peptide-pulsed RMAS/L^d APC expressing GFP or mPD-L1. Shown is a representative experiment (*n*=5). (C) In the same experiment shown in (A), 53HED CD4+ T cells were stimulated in the presence of soluble anti-CD28 mAb (1 μg/ml). Shown is a representative experiment (*n*=2). (D) 2C CD8+ T cells were stimulated as in (B) with or without anti-CD28 mAb (1 μg/ml) or 20 U/ml rmIL-2. Proliferation was measured on day 3. Shown is a representative experiment (*n*=3).

(Fig. 5A, B). After 2 days of stimulation, CD4+ and CD8+ cells were cycling and there was modest inhibition of proliferation in both subsets in the presence of PD-L1.Fc as assessed by the percentage of cells in the non-dividing peak and the decreased number of peaks. At days 3 and 4, CD4+ and CD8+ T cell division was markedly inhibited by PD-L1.Fc stimulation. CD8+ T cells seemed to be more sensitive to the PD-1:PD-L1 inhibitory pathway. By analyzing the geometric mean fluorescence intensity of dividing cells stimulated with anti-CD3/PD-L1.Fc *vs*. anti-CD3/ctrl.Fc, there is a larger fold difference between these two culture conditions for CD8+ (45.4 *vs*. 11.5) than CD4+ (65.6 *vs*. 31.7) T cells. These results suggest that CD4+ and CD8+ T cells are susceptible to inhibitory signals delivered through PD-1; however, at a given time point after stimulation, CD8+ T cells are more sensitive.

2.8 Inhibition of antigen-specific CD4+ and CD8+ T cells by APC expressing PD-L1

The effects of PD-1:PD-L1.Fc interactions were examined using TCR transgenic (Tg) T cells and antigen/APC. APC lines expressing the restricting MHC II or MHC I molecules were engineered to express mPD-L1 and used to stimulate CD4+ or CD8+ T cells. Cell lines were chosen based on lack of endogenous PD-L1 expression (data not shown). CD4+ T cells purified from 53HED TCR Tg mice were stimulated with peptide PCCF-pulsed, irradiated COS-IE^k/GFP or COS-IE^k/mPD-L1 cells (Fig. 6A, C). In separate experiments, CD8+ T cells from 2C TCR Tg mice were stimulated with p2Ca-pulsed, irradiated RMAS-L^d/GFP or RMAS-L^d/mPD-L1 cells (Fig. 6B, D). In agreement with results of bead-based assays, proliferation of both CD4+ and CD8+ T cells was inhibited in the presence of PD-L1+ APC (Fig. 6A, B). PD-1:PD-L1 engagement results in dramatic inhibition of CD8+ T cells at all time points examined (86% on days 2 and 3). In contrast, proliferation of CD4+ T cells was more strongly inhibited at later time points (23%, 31% and 60% at days 2, 3 and 4, respectively). This supports the results of the bead stimulation assays, suggesting that CD8+ T cells are more sensitive to inhibition upon PD-1:PD-L1 engagement.

2.9 Costimulation reverses PD-1-mediated inhibition of CD4+, but not CD8+, T cell responses

Data from the bead stimulation assays suggested that CD28 costimulation overcomes PD-1-mediated inhibition by inducing IL-2 production (Fig. 3A). When CD28 costimulation failed to induce sufficient IL-2 production, PD-1 ligation resulted in T cell inhibition (Fig. 4B). Since CD4+ T cells produce high levels of IL-2 when costimulated through CD28 [17], but CD8+ T cells do not ([18] and unpublished observations), the ability of CD28 costimulation to overcome the PD-1:PD-L1 inhibitory pathway was compared between these T cell subsets. 53HED CD4+ T cells (Fig. 6C) or 2C CD8+ T cells (Fig. 6D) were tested for their ability to proliferate when stimulated with GFP- or mPD-L1-expressing APC in the presence of soluble anti-CD28 mAb. For CD4+ T cells, costimulation was effective at overcoming proliferative inhibition mediated by PD-1:PD-L1 interactions (compare Fig. 6A and C). This correlated with the induction of IL-2 production (data not shown). In contrast, CD28 costimulation did not overcome inhibition of CD8+ T cells and did not induce measurable IL-2 even at high concentrations of mAb or with protein A cross-linking (data not shown). 2C CD8+ T cells were responsive to anti-CD28 mAb as some costimulation was observed. Interestingly, addition of exogenous IL-2 effectively overcomes PD-1:PD-L1-mediated inhibition of CD8+ T cells. These results strongly suggest that CD8+ T cells are more susceptible to the PD-1:PD-L1 inhibitory pathway due to their intrinsic inability to produce IL-2.

3 Discussion

In this study we have examined the functional consequences of PD-1:PD-L engagement on mixed and purified $CD4^+$ and $CD8^+$ T cell populations and have evaluated the capacity of CD28 costimulation to modulate these responses. We show that engagement of PD-1 by its physiological ligands PD-L1 or PD-L2 results in inhibition of T cell proliferation and IL-2 production. In this study, we have concentrated on the responses of unactivated murine T cells and show inhibition of both $CD4^+$ and $CD8^+$ T cells using anti-CD3/PD-L.Fc-coated beads as well as APC expressing PD-L1. To the best of our knowledge, this is the first report analyzing the effects of the PD-1:PD-L inhibitory pathway in both T cell subsets. Interestingly, we found that, although IL-2 can override the PD-1:PD-L1-mediated inhibition of both unseparated and purified T cell populations, CD28 costimulation can only restore $CD4^+$ T cell proliferation. In addition, we show that the ability of CD28 costimulation to overcome the PD-1:PD-L1 inhibitory pathway is dependent on IL-2 induction. Although other reports in the literature suggest that interactions of T cells with PD-L1 or PD-L2 result in costimulation [19–21], the data presented here and in our previous work agree with the phenotype of the PD-$1^{-/-}$ mice and support an inhibitory function for PD-1 when it is engaged by PD-L1 and PD-L2.

Interaction of PD-1 with PD-L1 or PD-L2 results in inhibition of T cell proliferation; these effects are dependent on the density of PD-L1 and correlate with the kinetics of PD-1 expression. Our analysis of CSFE-labeled cells shows that unseparated, $CD4^+$ and $CD8^+$ T cell populations stimulated in the presence of PD-L1 have an increased percentage of non-dividing cells and that those cells which do divide undergo fewer mitotic events. These findings are consistent with PD-1:PD-L1 interactions initiating cell cycle arrest ([9] and unpublished results), and indicate that the *in vitro* functional consequences of PD-1 engagement are similar to those of CTLA-4 [22, 23]. Further mathematical analysis of our CSFE data showed that T cells activated in the presence of anti-CD3/PD-L1.Fc or anti-CD3 alone, had the same time to first cell division. This is consistent with T cell activation being required for PD-1 expression. However, the rate of subsequent divisions is slowed when T cells are activated with anti-CD3/PD-L1.Fc. Whether PD-1 signals are directly affecting the rate of cell division or whether this is a consequence of limited IL-2 levels resulting from PD-1:PD-L is currently under investigation.

Our data suggest that IL-2 has a central role in the PD-1:PD-L inhibitory pathway. Costimulation augments IL-2 production and can thus overcome PD-1-mediated inhibition of T cell proliferation at early time points after T cell stimulation. Neutralization of IL-2 abrogates the ability of CD28 costimulation to reverse PD-1 proliferative inhibition, suggesting that an IL-2-dependent mechanism underlies the activity of CD28 signals in this pathway. The data presented in Fig. 4B suggest that IL-2 is produced when T cells are stimulated in the presence of anti-CD28 mAb and PD-L1, prior to PD-1 expression. However, once PD-1 expression is up-regulated and ligated by PD-L1, there is little further IL-2 production and consumption of the amount initially made. Similarly, CTLA-4 engagement, in the presence of CD28 costimulation, also leads to reduced proliferation and IL-2 production [22, 23]. However, unlike PD-1-mediated inhibition, CTLA-4-mediated negative regulation occurs at all time points [24]. CTLA-4 engagement results in inhibition of IL-2 transcription without affecting CD28-mediated stabilization of IL-2 [25]. Whether PD-1 engagement affects IL-2 transcription or IL-2 mRNA half-life is currently being investigated. Whatever the mechanism, our data suggest that activated T cells are susceptible to inhibition upon PD-1:PD-L interactions, particularly when IL-2 is limiting.

In contrast to IL-2 induced by costimulation, exogenous IL-2 is always able to overcome the PD-1:PD-L-mediated inhibition. These results suggest that this inhibitory pathway interferes with IL-2 production not IL-2 responsiveness. Preliminary data suggests that addition of other growth-promoting cytokines can also overcome T cell proliferative inhibition upon PD-1:PD-L interactions. Thus, the PD-1:PD-L inhibitory pathway may play a prominent inhibitory role in immune responses when cytokine levels are diminished or at tissue sites where the cytokine milieu is less favorable to proliferation.

We demonstrate that both $CD4^+$ and $CD8^+$ T cells are susceptible to inhibition via the PD-1:PD-L pathway. This is of interest as these T cell subsets have been shown to be differentially sensitive to several costimulatory and inhibitory pathways. For instance, primary and secondary responses of $CD4^+$ T cells are regulated by CTLA-4 [12] and $CTLA4^{-/-}$ animals develop a fatal lymphoproliferative disorder due primarily to the expansion of $CD4^+$ T cells [26]. The effects of CTLA-4 engagement on $CD8^+$ T cells are more subtle; primary $CD8^+$ T cells responses are unchanged, but secondary responses are enhanced in CTLA-$4^{-/-}$ mice [13, 14, 27]. $CD8^+$ T cells express and are sensitive to inhibition via KIR [16, 28, 29]. $CD4^+$ T cells are more responsive to CD40L:CD40 interactions [15]. $CD8^+$ T cells have previously been shown to be effected by the PD-1 pathway: PD-$1^{-/-}$ 2C TCR Tg $CD8^+$ T cells show enhanced responsiveness to antigen/APC *in vitro*; while *in vivo* 2C TCR Tg × PD-$1^{-/-}$ develop a chronic

graft-versus-host disease when bred to an H-2^{bxd} autoreactive background [6]. $CD4^+$ T cells have also been shown to be effected by the PD-1:PD-L1 inhibitory pathway [9, 11]. Here, for the first time, we have directly compared PD-1 function in $CD4^+$ and $CD8^+$ T cells. Our data show that PD-1:PD-L1-mediated inhibition of $CD4^+$ T cells is less dramatic than inhibition of $CD8^+$ T cells in cultures of unseparated T cells and using purified $CD4^+$ and $CD8^+$ T cells from TCR transgenic mice. This is not the result of differences in PD-1 expression (data not shown). However, $CD4^+$ T cells, unlike $CD8^+$ T cells, produce high titers of IL-2 when stimulated. As IL-2 can overcome PD-1:PD-L inhibitory pathway, this may explain the differential sensitivity of these subsets. Alternatively, $CD8^+$ T cells could be more sensitive to the reduced IL-2 levels produced upon PD-1:PD-L1 interactions. Also, $CD8^+$ T cells produce high levels of IFN-γ, which can up-regulate PD-L1 expression in a variety of cell types ([11] and unpublished observations). $CD8^+$ T cells, via their MHC I restriction, can interact with most cells in the body, not just professional APC, making it more likely that they would encounter PD-L1 in the absence of costimulatory signals and growth-promoting cytokine milieu. This strongly suggests that the PD-1 pathway may be more potent for $CD8^+$ T cell inhibition.

Inhibition of IL-2 production and proliferation in the presence of costimulation are defining characteristics of anergy [30–32]. Following these criteria, our data suggest that PD-1:PD-L1 interactions result in T cell anergy. Addition of exogenous IL-2 has been shown to reverse classical anergy [33] and likewise abrogates PD-1:PD-L-mediated inhibition. The breakdown of peripheral tolerance seen in the $PD\text{-}1^{-/-}$ mice supports a role for this pathway in the generation and/or maintenance of an unresponsive state [6, 7]. Further experiments are being conducted to determine if PD-1-mediated anergy is a transient or persistent state and if T cells stimulated in the presence of PD-L1 exhibit the biochemical hallmarks of anergy [34, 35]. Thus, therapeutic modulation of the PD-1:PD-L pathway may be useful in situations where tolerance is being abrogated (tumors) or generated (transplantation).

In summary, we show that engagement of the PD-1:PD-L inhibitory pathway can potently inhibit the proliferation and IL-2 production of murine T cells. PD-1:PD-L-mediated inhibition can be overcome in an IL-2-rich environment; however, cells that have been triggered through PD-1 are less able to produce IL-2 themselves. $CD4^+$ and $CD8^+$ T cells are sensitive to PD-1:PD-L interactions; however, $CD8^+$ T cells may be more susceptible to negative regulation by this pathway because of their intrinsic inability to produce IL-2. The potency of PD-1:PD-L-mediated inhibition *in vitro* and the lymphoproliferative disease in $PD\text{-}1^{-/-}$ animals support a critical role for this pathway in down-regulation of T cell function. It will be important to determine physiological settings where this pathway is dysregulated and how this contributes to disease.

4 Materials and methods

4.1 Reagents

Anti-CD3 (2C11), anti-IL-2, biotinylated-hamster Ig, anti-CD4-PE and anti-CD8-Cy-chrome mAb were purchased from BD-PharMingen (La Jolla, CA). Control.Fc, PD-L1.Fc and PD-L2.Fc were constructed and purified as described in [11]. Biotinylated-anti-mPD-1 [10], anti-CD28 (PV1.17.10.1) mAb and peptides (PCCF; KAERADLIAYLKQATAK and p2Ca; LSPFPFDL) were produced at Wyeth-Genetics Institute. rhIL-2 was purchased from Gibco (Rockville, MD). Strepavidin-PE was purchased from Southern Biotech (Birmingham, AL).

4.2 Mice

Female BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). 53HED TCR Tg mice [36], which recognize PCCF in the context of H-2 $I\text{-}E^k$, were bred at Taconic (Germantown, NY). The 2C TCR Tg mice [37], which recognize p2Ca peptide in the context of the H-2 L^d were bred at Charles River Laboratories (Wilmington, MA).

4.3 Preparation of beads

Tosyl-activated Dynabeads (Dynal, Lake Success, NY) were covalently coupled to the indicated antibody and fusion protein as previously described [38]. Coated beads were stored at 4°C and used within 10 days of preparation. For titration experiments ctrl.Fc was used to keep constant the total amount protein.

4.4 APC lines

APC were engineered to express mPD-L1. Briefly, retroviral vectors encoding IRES-GFP or mPD-L1-IRES-GFP were constructed using GFP-RV vector, (provided by Dr. K. Murphy, Washington University, [39]). Virus-containing supernatants were generated using the 293-VSVg packaging cell line [40] and used to spin-infect $COS\text{-}IE^k$ (provided by Dr. Ron Ger-main, NIH) and $RMAS\text{-}L^d$ [41] as described at http://www-leland.standford.edu/group/nolan/NL-phnxr.html. Stable lines were selected by flow cytometric sorting.

4.5 T cell preparation, proliferation and IL-2 assays

T cells were purified from LN of 8–12-week-old BALB/c mice with negative selection T cell columns (R&D Systems, Min-

neapolis, MN), resulting in >95% purity. For bead stimulation assays, T cells (0.5×10^5–1×10^5) were cultured at 2:1 beads:cells ratio in RPMI 1640-based T cell media. For antigen/APC assays, $CD4^+$ T cells were purified from the LN of 53HED TCR Tg mice using $CD4^+$ selection followed by murine CD4 DETACHaBEAD® (Dynal, Lake Success, NY). $CD4^+$ T cells (10^5) were mixed with irradiated (2,000 rad) COS-IE^k/GFP or COS-IE^k/PD-L1 cells (10^4) and 10 μM PCCF peptide. $CD8^+$ T cells were purified from the LN nodes of 2C TCR Tg mice using negative selection T cell columns. 2C T cells (5×10^4) were cultured with overnight peptide-pulsed, irradiated (10,000 rad) RMAS-L^d/GFP or RMAS-L^d/PD-L1 cells (5×10^4). Proliferation assays were done in flat-bottom 96-well plates and labeled with 1 μCi [^{3}H]thymidine for the last 8–12 h of culture. For IL-2 determination, supernatants were harvested from parallel wells and assayed by ELISA (limit of detection 40 pg/ml) (R&D Systems, Minneapolis, MN).

4.6 CSFE staining and analysis

Purified T cells were labeled with 1 μM CSFE (Molecular Probes, Eugene, OR) for 12 min at 37°C then washed twice with PBS and stimulated as described above. Fixation was done with 2% paraformaldehyde in PBS. Live-gated cells were then subjected to Modfit analysis (Verity Software House, Topsham, ME) for quantitation.

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