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Negative role of inducible PD-1 on survival of activated dendritic cells

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ABSTRACT

PD-1 is a well-established negative regulator of T cell responses by inhibiting proliferation and cytokine production of T cells via interaction with its ligands, B7-H1 (PD-L1) and B7-DC (PD-L2), expressed on non-T cells. Recently, PD-1 was found to be expressed in innate cells, including activated DCs, and plays roles in suppressing production of inflammatory cytokines. In this study, we demonstrate that PD-1 KO DCs exhibited prolonged longevity compared with WT DCs in the dLNs after transfer of DCs into hind footpads. Interestingly, upon LPS stimulation, WT DCs increased the expression of PD-1 and started to undergo apoptosis. DCs, in spleen of LPS-injected PD-1 KO mice, were more resistant to LPS-mediated apoptosis in vivo than WT controls. Moreover, treatment of blocking anti-PD-1 mAb during DC maturation resulted in enhanced DC survival, suggesting that PD-1: PD-L interactions are involved in DC apoptosis. As a result, PD-1-deficient DCs augmented T cell responses in terms of antigen-specific IFN-γ production and proliferation of CD4 and CD8 T cells to a greater degree than WT DCs. Moreover, PD-1 KO DCs exhibited increased MAPK1 and CD40–CD40L signaling, suggesting a possible mechanism for enhanced DC survival in the absence of PD-1 expression. Taken together, our findings further extend the function of PD-1, which plays an important role in apoptosis of activated DCs and provides important implications for PD-1-mediated immune regulation. *J. Leukoc. Biol.* 95: 621–629; 2014.

Introduction

DCs are professional APCs that are critical for regulation of immune tolerance, as well as immune activation. Upon encountering the foreign antigens with microbial signals, DCs become matured and activated and carry these antigens to the regional LNs, where they stimulate the naive T cells to induce active immune responses. In contrast, under steady-state conditions, DCs in peripheral tissues constitutively uptake self-antigens without being matured and thus, induce peripheral tolerance [1, 2]. It has been shown that immature DCs, characterized by low expression levels of MHC II and costimulatory molecules, inhibit effector functions of T cells and protect autoimmune encephalitis [3–5], demonstrating that the maturation status of DCs is crucial for their ability to induce tolerogenic immune responses.

In addition to DC maturation status, DC homeostasis, controlled by apoptosis, plays an important role in the immune regulation. As a result of the short lifespan of DCs, prolonged survival of DCs was required to enhance the antigen-specific immune responses during their interactions with naive T cells in the LNs. However, once DCs present antigens to T cells, they need to be eliminated in the LNs to limit the antigen availability to T cells, thus avoiding the aberrant T cell activation. Defects in DC apoptosis by deletion of proapoptotic genes, such as B cell lymphoma-2 interacting mediator of cell death and Bcl-2 or death receptor Fas could also disrupt the DC homeostasis and trigger autoimmune diseases [6–8]. In addition, transfer of excessive amount of DCs induces the development of autoimmune diseases [9, 10]. The mechanisms of how DC apoptosis contributes to the immune regulation are not yet fully understood, but recent studies have shown that apoptotic DCs taken up by viable DCs might induce immune tolerance via generation of regulatory T cells [11, 12].

Abbreviations: AKt=RAC-alpha serine/threonine kinase, Annexin-V=Annexin A5, B6=C57BL/6, Bcl-2/Bcl-xL=B cell lymphoma 2/extra-large, CD40L/CD95L=CD40/CD95 ligand, CMTMR=5-(and-6)-{[(4-chloromethyl) benzoyl] amino} tetramethylrhodamine, DDAO=7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one), dLN=draining LN, KO=knockout, NRF=National Research Foundation of Korea, OT-I/II=MHC I-restricted OVA-specific TCR transgenic CD8 (I)/CD4 (II), p-ERK1/2=phospho-ERK1/2, PD-1=programmed cell death 1, PD-L=programmed cell death ligand, RAG-1=recombination-activating gene 1

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PD-1, a member of Ig superfamily, is inducibly expressed on T and B cells upon activation, which delivers inhibitory signals in lymphocytes by interacting with its ligands, B7-H1 (PD-L1) and B7-DC (PD-L2) [13]. PD-1 has significant suppressive effects on cytokine production, such as IFN-γ, TNF-α, and IL-2, in T cells by inhibiting PI3K activity [14]. In addition, withdrawal of IL-2 by PD-1 ligation leads to T cell death [15], and blockade of PD-1 remarkably regresses tumor growth during cancer therapy via augmentation of tumor- specific T cell responses, implying the crucial role of PD-1 in immune tolerance [16–18]. It was reported that PD-1 is also induced in non-T and B cells, such as DCs and macrophages, upon TLR ligand-mediated stimulation, and its ligation negatively regulates cytokine productions from these cells, including TNF-α, IL-6, IL-12, and MIP-1α [19, 20].

In the present study, we investigate the role of PD-1 in DC survival in vitro and in vivo. We demonstrated that PD-1 expressed on DCs plays a role in inducing apoptosis in activated DCs and that enhanced survival of DCs via PD-1 deficiency leads to improved antigen-specific CD4 and CD8 T cell responses. Thus, our results provide a novel function of PD-1 in the immune regulation by DC survival.

MATERIALS AND METHODS

Mice

Specific pathogen-free B6, B6 Thy1.1$^+$ OT-I, B6 Thy1.1$^+$ OT-II, B6 RAG-1 KO, and B6 CD45.1 mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). B6 PD-1 KO mice were obtained from Dr. Tasuku Honjo (Kyoto University, Japan) via Dr. Sang-Nae Cho (Yonsei University, Korea). Mice were bred and maintained in the animal facility at the Pohang University of Science and Technology Biotech Center (Pohang, Korea), and age- and sex-matched, 6- to 8-week-old mice were used for all experiments. All mouse experiments were performed in accordance with the U.S. National Institutes of Health guidelines, and protocols were approved by the Institutional Animal Care and Use Committee .

Isolation of DCs and T cells

Spleen fragments were digested at 37°C in 10% RPMI 1640 (10% FBS+2-ME), supplemented with 1.5 mg/ml collagenase D and 50 μg/ml DNase I (both from Roche Diagnostics, Indianapolis, IN, USA), for 45 min and then treated for an additional 5 min with 1 mM EDTA (pH 7.2). CD11c$^+$ DCs were isolated by using CD11c microbeads (Milteny Biotec, Auburn, CA, USA), according to the manufacturer's instructions. Purified cells ($\geq$93%) were CD11c-positive.

OT-I, OT-II, or CD5$^+$ cells were isolated by using CD8 and CD4 T cell isolation kits and CD5 microbeads (Milteny Biotec), according to the manufacturer's instructions.

In vitro culture of DCs

Cells/ml (2×10^6) of purified CD11c$^+$ WT (B6) and PD-1 KO DCs from naive mice were cultured without any stimuli for 24 h. During incubation, 10 μg/ml mAb to PD-1 (Clone 29F.1A12; a kind gift from Gordon J. Freeman at Havard Medical School, Boston, MA, USA) or rat IgG2a as an isotype control was treated to block the interaction of PD-1 with its ligands.

In vivo transfer of DCs and LPS injection

Purified DCs from WT and PD-1 KO mice were treated with 0.1 μg/ml LPS (*Escherichia coli* serotype 0111:B4; Invitrogen, Carlsbad, CA, USA) for 2 h, and then 1×10^6 DCs in 50 μl vol were injected into footpads of CD45.1 mice. At the indicated time-points after transfer, popliteal LNs were harvested for the analysis. For the study to block PD-1 expression on transferred DCs, DCs from CD45.1 mice were stimulated with LPS and then injected into footpads of RAG-1 KO (CD45.2) mice. Anti-PD-1 mAb (200 μg; Clone J43; Bio X Cell, West Lebanon, NH, USA) or the same amount of hamster IgG as an isotype control was injected simultaneously. For an ex vivo imaging experiment, WT and PD-1 KO DCs were labeled with 5 μM CMTMR and 10 μM DDAO (both Molecular Probes, Invitrogen), respectively. Fluorescence-labeled WT and PD-1 KO DCs were mixed with a 1:1 ratio, and then 2×10^6 of the cell mixture was injected s.c. into hind footpads of B6 mice. Forty-eight hours after transfer, DCs within the frozen sections of fixed popliteal LNs were visualized by a modified Zeiss Axio Observer.Z1 epifluorescence microscope with a 40× (Plan-Neofluar; NA=1.30) objective lens and a Roper Scientific CoolSNAP high-quality charge-coupled device camera and analyzed using MetaMorph (Molecular Devices, Downingtown, PA, USA).

For in vivo LPS treatment, mice were injected i.v. with 50 μg LPS. Control mice were treated with the same volume of PBS.

mAb and flow cytometry

Cells were analyzed with a Gallios cytometer (Immunologics; Beckman Coulter, Brea, CA, USA). Before staining, cells were FcR-blocked with 1 μg purified anti-CD16/32 mAb (2.4G2; eBioscience, San Diego, CA, USA). The mAb used for staining were allophycocyanin- or eFluor450-conjugated CD4, allophycocyanin- or PE-Cy7-conjugated CD8α, eFluor450-conjugated Thy1.1, allophycocyanin-conjugated CD45.1, PE-conjugated CD45.2, FITC-conjugated CD11b, allophycocyanin- or eFluor450-conjugated anti-CD11c, FITC-conjugated plasmacytoid DC antigen-1, PE-conjugated B220, eFluor450-conjugated anti-MHC II, FITC-conjugated anti-B7-1, FITC-conjugated anti-B7-2, biotin-conjugated anti-B7-H1, FITC-conjugated Annexin-V and PI, PE-conjugated CD40, and PE-conjugated CD40L (all from eBioscience); purified ERK2, FITC-conjugated anti-mouse IgG1, and PE-conjugated p-ERK1/2 (BD Biosciences, San Diego, CA, USA); and PE-conjugated anti-PD-1 (Clone RMP1-30) and its isotype control (BioLegend, San Diego, CA, USA).

T cell simulation

DCs from WT and PD-1 KO mice were pulsed with 10 μg/ml $OVA_{257–264}$ (OT-I, SIINFEKL; synthesized by Peptron, Korea) or $OVA_{323–339}$ (OT-II, ISQAVHAAHAEINEAGR; AnaSpec, Fremont, CA, USA) for 2 h. For in vitro study, 1×10^5 OT-I or OT-II cells labeled with 1.25 μM CFSE were cultured with OVA peptide-pulsed DCs for 60 h at various DC:T ratios. For in vivo study, 3×10^5 OVA peptide-pulsed DCs (0 h) and 1.5×10^6 OT-I or OT-II cells (5 h), labeled with 5 μM CFSE, were transferred i.v. into Thy1.2$^+$ B6 recipients, and T cell proliferation was examined at 60 h after T cell transfer.

For in vitro PD-1 blockade experiment, 1×10^5 PD-1 KO CD5$^+$ cells (pan-T cells) were stimulated by 1×10^4 WT or PD-1 KO DCs with 1 μg/ml anti-CD3ϵ mAb (BD Biosciences) in the presence of 20 μg/ml anti-PD-1 mAb (J43). IFN-γ production was examined at 60 h.

mRNA purification and quantitative real-time PCR

Total RNA from CD11c$^+$ splenic DCs of WT or PD-1 KO mice were extracted by using TRIzol reagent (Invitrogen), and the cDNA was synthesized with random primers (Qiagen, Venlo, Netherlands). The expressions of apoptosis-related genes were analyzed by SYBR Green-based quantitative real-time PCR using RT^2 Profiler apoptosis PCR array (Qiagen). Gene expression was normalized to the expression of housekeeping genes (GAPDH, β-actin, and heat shock protein 90).

Statistics

Data are typically shown as mean ± SEM. The differences between the groups were assessed using two-tailed Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Enhanced recovery of PD-1-deficient DCs in the dLN after in vivo transfer

To investigate whether PD-1 plays a role in homeostasis of DCs, we stimulated WT or PD-1 KO DCs (CD45.2) with LPS in vitro and then injected them into hind footpads of CD45.1$^+$ recipient mice. The DCs recovered in dLNs were assessed by analyzing CD45.2$^+$ CD11c$^+$ populations. As shown in **Fig. 1A**, by 2 days after transfer, more fractions of PD-1 KO DCs were detected in dLNs compared with WT DCs. The number of WT or PD-1 KO DCs in dLNs peaked at 2 days after transfer and then decreased gradually and became undetectable on Day 7 (Fig. 1B). Interestingly, the average number of accumulated PD-1 KO DCs in the dLNs was significantly higher than that of WT DCs on Day 2. Consistently, accumulation of WT DCs was increased by injection of anti-PD-1 mAb (Supplemental Fig. 1). We observed that the expression of CCR7, an important chemokine receptor for migration of DCs to the dLN, was similar between two DCs (Fig. 1C), suggesting that increased recovery of PD-1 KO DCs in the dLN compared with WT DCs was not a result of the enhanced migration capacity of these DCs.

For the in situ visualization of WT and PD-1 KO DCs in the dLN after DC transfer, we labeled WT and PD-1 KO DCs with cell-tracker dyes and then coinjected them into the same footpad of B6 recipients. Consistently, fewer CMTMR-labeled WT DCs were detected than DDAO-labeled PD-1 KO DCs in frozen sections of the dLN (**Fig. 2**). Similar results were also obtained when cell-tracker dyes were switched (data not shown), indicating that these dyes did not affect the viability of DCs. Taken together, these data suggest that PD-1 expression on DCs is involved in the negative regulation of DC survival in vivo.

PD-1 induced by DC activation is involved in DC apoptosis after LPS administration

When we compared in vitro, spontaneous apoptosis between WT and PD-1 KO DCs, PD-1 KO DCs were less apoptotic compared with WT DCs, by ~11% (Fig. 1D; Untreated). This result coincides with the PD-1-expressing DC populations, which are 10–20% in the same in vitro culture condition (data not shown). When WT DCs were treated with anti-PD-1 mAb, which block interactions of PD-1 with its ligands, such as B7-H1 and B7-DC, the apoptosis of WT DCs were abrogated to the level analogous with untreated PD-1 KO DCs (from

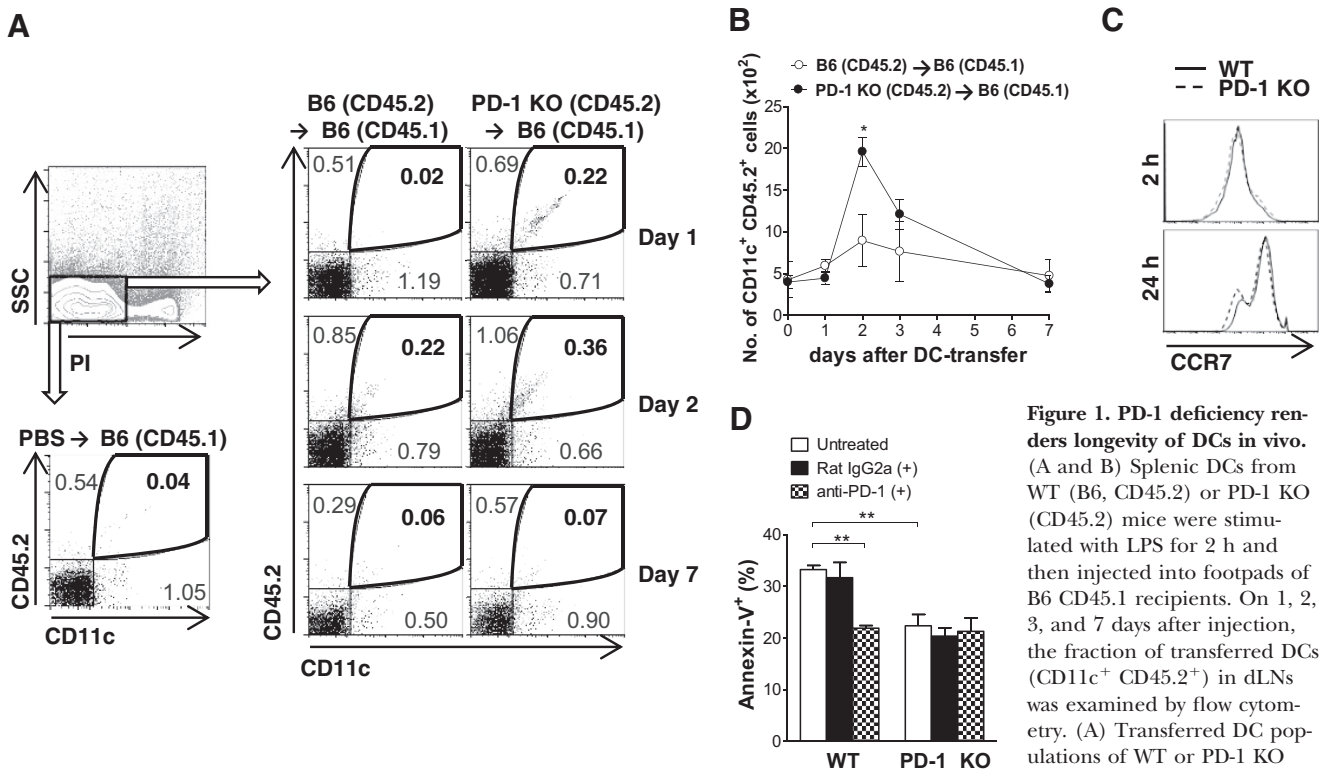

Figure 1. PD-1 deficiency renders longevity of DCs in vivo. (A and B) Splenic DCs from WT (B6, CD45.2) or PD-1 KO (CD45.2) mice were stimulated with LPS for 2 h and then injected into footpads of B6 CD45.1 recipients. On 1, 2, 3, and 7 days after injection, the fraction of transferred DCs (CD11c$^+$ CD45.2$^+$) in dLNs was examined by flow cytometry. (A) Transferred DC populations of WT or PD-1 KO mice among the PI-negative LN cells of the recipient mice were shown on 1, 2, and 7 day after DC transfer. SSC, Side-scatter. (B) Absolute cell numbers of transferred DCs in the dLN of recipients were analyzed at each time-point. The values on dots indicate mean ± SEM of three mice/group. Data are representative of four independent experiments with similar results. $^*P < 0.05$ versus WT controls by Student's *t*-test. (C) CCR7 expression in WT and PD-1 KO DCs after LPS stimulation. DCs treated with LPS for 2 h were washed more than three times and were incubated further for 24 h. Before (2 h) and after (24 h) the additional incubation, the expression of CCR7 on DCs was analyzed. Representative histograms from three independent experiments were shown. (D) Purified WT and PD-1 KO DCs were incubated with 10 μg/ml anti-PD-1 mAb or rat IgG2a as a control. At 24 h after incubation, binding of Annexin-V was analyzed by flow cytometry. The values on bars indicate percent (mean±SD) of Annexin-V-positive cells. Data are representative of more than three independent experiments with similar results. $^{**}P < 0.005$ by Student's *t*-test.

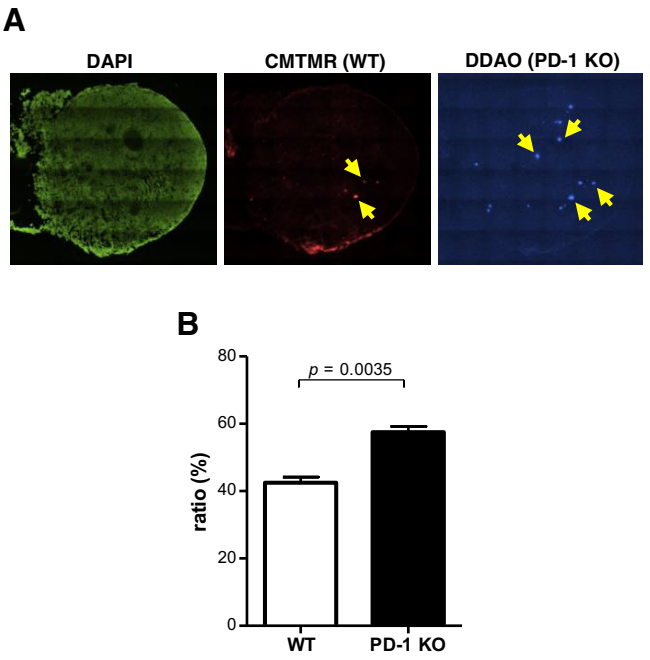

Figure 2. Enhanced DC recovery in the absence of PD-1. (A and B) LPS-stimulated DCs were labeled with 5 μM CMTMR (WT) or 10 μM DDAO (PD-1 KO), mixed with a 1:1 ratio, and injected into footpads of B6 mice. At 48 h after injection, transferred DCs within the frozen sections of fixed dLNs were visualized by the MetaMorph system. (A) Representative image of more than three dLNs. Arrows indicate the population of transferred DCs. (B) The values on bars indicate the mean percent ± SEM of WT or PD-1 KO DCs among the total transferred DCs in each dLN. Data are representative of two independent experiments (n=3/group).

33.3±0.81% to 21.9±2.18%). As expected, the apoptosis of PD-1 KO DCs was unaffected by antibody treatment (Fig. 1D). This result suggests that PD-1 mediates apoptosis of PD-1-expressing DCs via interaction with its ligands.

To investigate the in vivo role of PD-1 in DC survival, LPS was administrated systemically, which is a well-established model to study the maturation and apoptosis of DCs in vivo [21, 22]. As noted, WT and PD-1 KO DCs showed a highly activated phenotype after LPS injection, including B7-1, B7-2, and B7-H1, with no significant differences between them (**Fig. 3A**). As DC activation by TLR ligation is reported to induce PD-1 expression in vitro [23], we also examined the expression pattern of PD-1 on DCs after LPS administration. Enhanced PD-1 expression on WT DCs but not on PD-1 KO DCs was accompanied by LPS-mediated activation of these cells (Fig. 3B). As shown in Fig. 3C, the percentage of PD-1-expressing DCs in spleens of WT mice reached its peak (~12% of splenic DCs) at 20 h after LPS injection and then decreased gradually to a basal level at 48 h. Interestingly, the number of apoptotic DCs assessed by Annexin-V binding was highest at 32 h after LPS injection, which is 12 h after the peak expression of PD-1. This result suggests that induction of PD-1 expression might be a prerequisite for the apoptosis of DCs. Interestingly, there were significantly higher fractions of DCs in PD-1 KO mice (0.92±0.09%) compared with WT DCs (0.49±0.05%) after LPS administration, whereas naive, untreated WT and PD-1 KO mice showed similar DC populations in spleens. Moreover, the average number of DCs from LPS-injected PD-1 KO mice was significantly higher than WT controls (Fig. 3D). We confirmed further that a higher number of splenic DCs in PD-1 KO mice injected with LPS were concomitant with a lower level of DC apoptosis, as assessed by Annexin-V binding (Fig. 3E). Taken together, our results suggest that inducible PD-1 on DCs plays an important role in regulating apoptosis of activated DCs.

Correlation of PD-1 deficiency with up-regulation of MAPK1 pathway and CD40–CD40L interactions

To investigate signaling molecules involved in PD-1-mediated apoptosis of activated DCs, we analyzed the mRNA level of apoptosis-related genes in WT and PD-1 KO DCs. Among the genes analyzed, Mapk1 (ERK2) and Cd40lg (CD40L, CD154) mRNA levels, which are well-known pathways involved in DC survival, were decreased significantly in WT DCs of mice injected with LPS compared with naive WT controls, whereas they were up-regulated (Mapk1) or not changed (Cd40lg) in PD-1 KO DCs after LPS injection (**Fig. 4A**). As a result, relative fold changes of Mapk1 and Cd40lg mRNA in PD-1 KO DCs were >10 and 140 times higher, respectively, than those in WT DCs after LPS administration (Fig. 4B). Consistently, the percentages of CD40, CD40L, ERK2, and p-ERK1/2 expression were significantly higher in PD-1 KO DCs than those of WT DCs [56.1±4.0% vs. 34.5±3.2%; 25.3±1.0% vs. 19.2±2.3%; 95.4±3.1% vs. 61.5±3.5%; and 60.3±3.7% vs. 45.4±2.5%, respectively (Fig. 4C)]. This result suggests that down-regulation of the MAPK1 signaling pathway and CD40–CD40L interaction might be involved in PD-1-mediated DC apoptosis.

PD-1-deficient DCs elicit higher antigen-specific T cell responses than WT DCs

To investigate whether increased survival of PD-1 KO DCs leads to enhanced immune responses, WT or PD-1 KO DCs pulsed with OVA peptides were cocultured with OT-II OT-I T cells. The peptide-pulsed PD-1 KO DCs induced higher antigen-specific IFN-γ production (**Fig. 5A**) and proliferation (Fig. 5B) in OT-I and OT-II cells in vitro. Consistently, blockade of PD-1 on WT DCs up-regulated IFN-γ production in T cells (Supplemental Fig. 2). This capacity of PD-1 KO DCs to enhance T cell responses might not be attributed to the different DC subsets in PD-1 KO mice, as similar DC subsets were observed in PD-1 KO mice when compared with WT (Supplemental Fig. 3). To confirm these results further in vivo, we stimulated in vivo-transferred OT-I or OT-II cells labeled with CFSE by injecting OVA-pulsed WT or PD-1 KO DCs. At 60 h after T cell transfer, we examined proliferation of T cells in LNs (Fig. 5C) and spleens (Fig. 5D) of recipient B6 mice. Consistent with in vitro results, OVA-specific CD4 and CD8 T cells highly proliferated when stimulated by PD-1 KO DCs compared with WT DCs. These results suggest that DCs without PD-1 expression elicited antigen-specific T cell responses to a greater degree than WT DCs. Thus, PD-1 blockades might

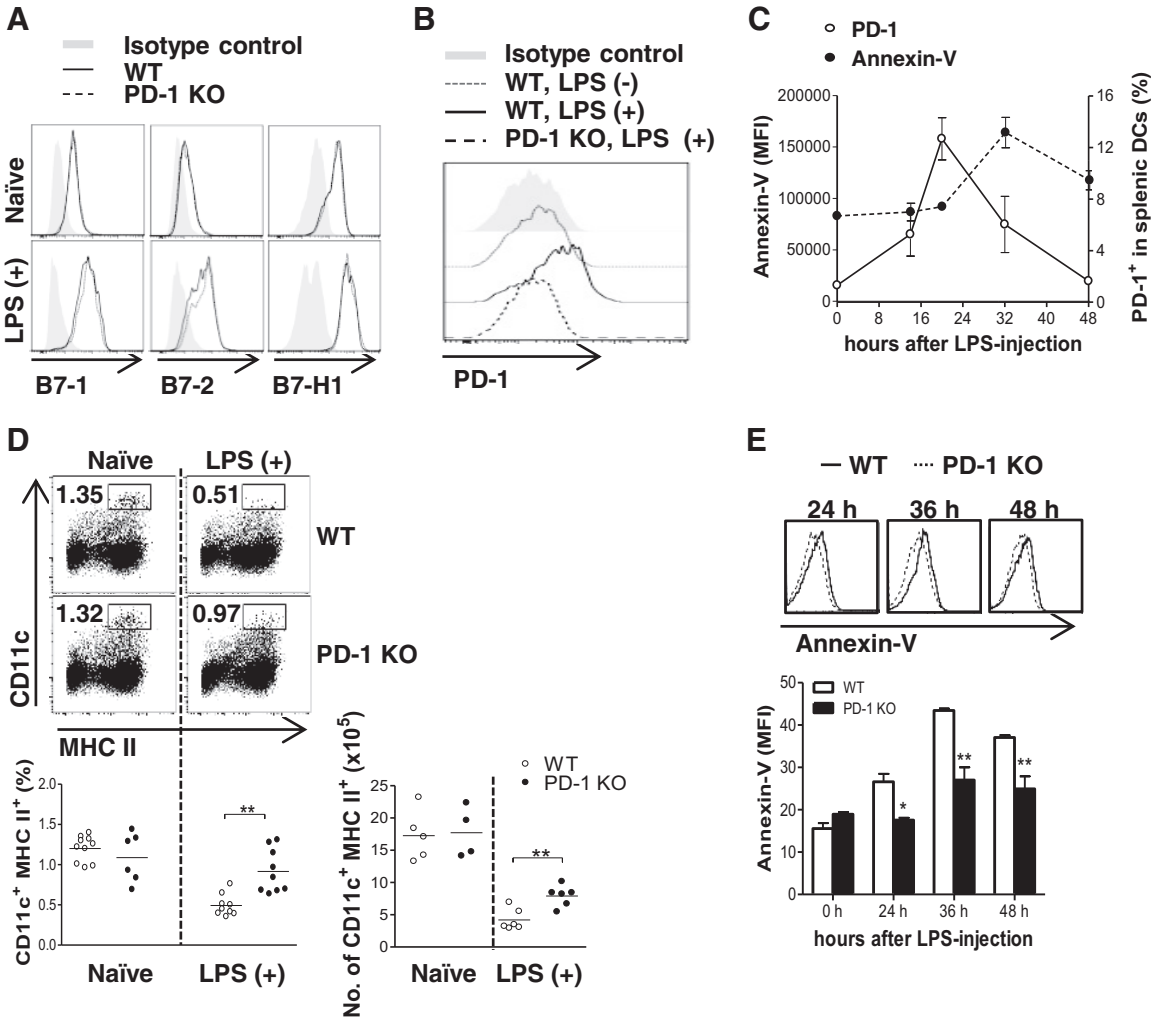

Figure 3. PD-1 deficiency reduces apoptosis of activated DCs. WT or PD-1 KO mice were i.v.-injected with 50 μg LPS (LPS-positive) or PBS as a control. At 24 h (A, B, and D) or indicated time-points (C and E) after LPS injection, DCs in spleens were analyzed by flow cytometry. Expression of (A) B7-1, B7-2, and B7-H1 and (B) PD-1 on the CD11c$^+$ MHC II$^+$ population in spleen was examined. (C) PD-1$^+$ fractions or Annexin-V binding [mean fluorescence intensity (MFI)] in WT DCs in spleens were analyzed at the indicated time-points after LPS injection. The values on dots indicate mean ± SEM (n=3/each time-point). (D) DC population in the spleens of WT or PD-1 KO mice after LPS treatment was analyzed by flow cytometry. Absolute numbers of DCs in the spleens were shown in the dot graphs (lower panel), and each dot represents a single mouse. (E) Binding of Annexin-V was examined in WT or PD-1 KO DCs at indicated time-points after LPS injection. The values on bars indicate mean fluorescence intensity ± SEM of three or four mice. Data are pooled from two independent experiments. *$P < 0.05$; **$P < 0.01$ versus WT controls, by Student's t-test.

be needed to enhance antigen-specific T cell responses, including cytokine production and proliferation, in the DC-based vaccination.

DISCUSSION

The roles of DCs in the adaptive immune responses start with antigen uptake and presentation to T cells and end by apoptosis afterward. As DCs are the initiators and regulators of immune responses, their lifecycle should be tightly controlled. Indeed, activated DCs, which stimulated T cells in lymphoid organs, undergo apoptosis rapidly [24–27]. Although the immunoinhibitory roles of PD-1 expressed on T cells have been studied extensively over the last decade, PD-1 expression and its functions in DCs have not been well addressed yet. Here, we provide the first evidence that PD-1 induces apoptosis of matured DCs.

Apoptosis of DCs after antigen presentation is an important mechanism to regulate immune responses. During antigen presentation to T cells, DCs receive survival or apoptotic signals from the interacting T cells. Ligation of Fas (CD95), expressed on maturated DCs with its Fas ligand (CD95L) on activated T cells, results in DC apoptosis via up-regulation of cellular FLICE-like inhibitory protein, long isoform [28], and MHC II induces apoptosis of DCs by inhibiting PKCδ during interaction with T cells [24]. MHC II is also responsible for DC apo-

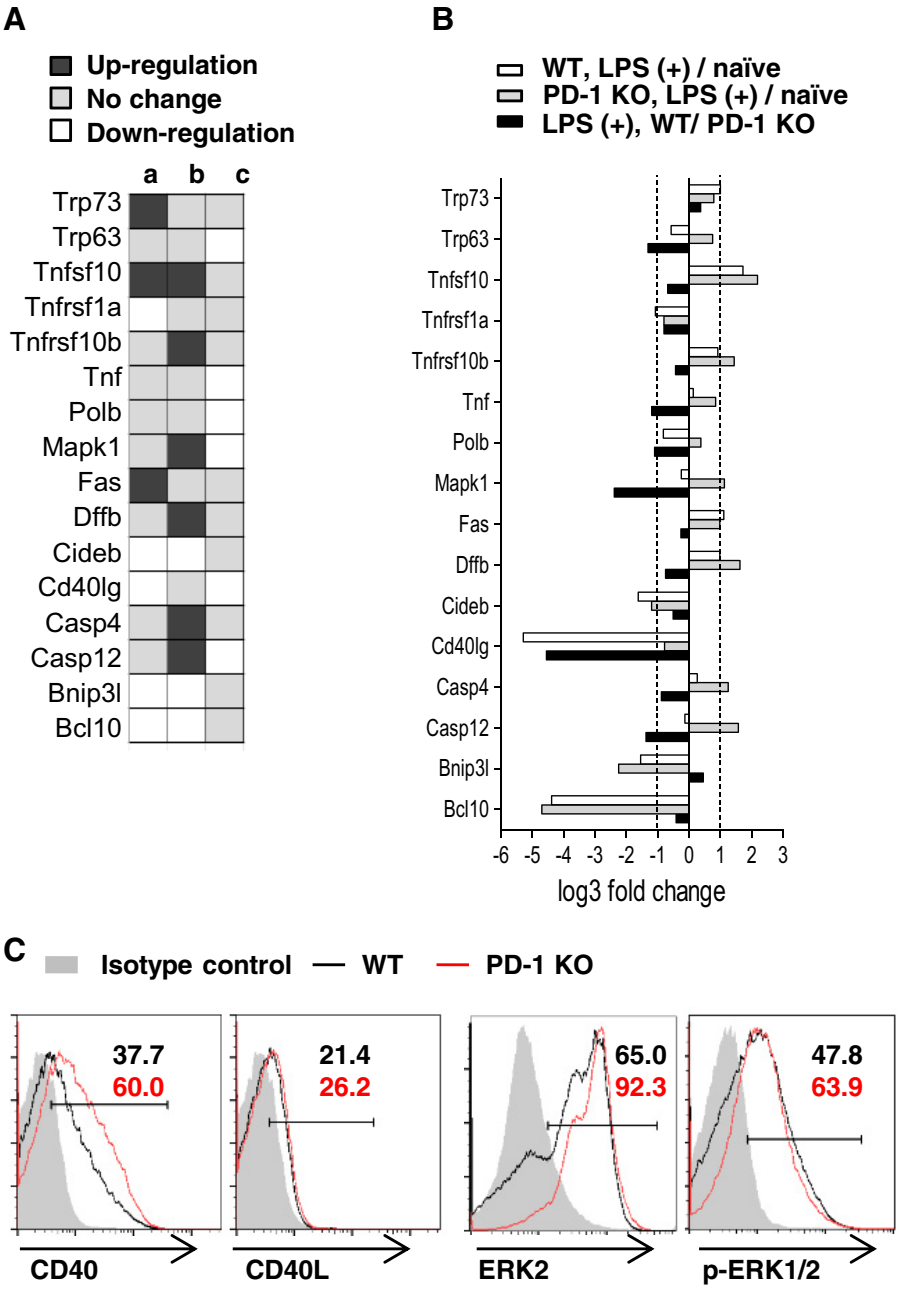

Figure 4. MAPK1 pathway and CD40–CD40L interaction are involved in PD-1-mediated apoptosis of DCs. At (A and B) 24 h or (C) 14 h after i.v. injection of PBS (naive) or LPS (LPS-positive), total RNA from CD11c$^+$ splenic DCs of WT or PD-1 KO mice was extracted. (A) Results are relative level of each gene in DCs from LPS-treated mice to the naive control (up-regulation: black; down-regulation: white), and WT versus PD-1 KO mice. (A, a) WT, LPS (+) to naive; (A, b) PD-1 KO, LPS (+) to naive; (A, c) LPS (+), WT to PD-1 KO. The genes changed more than threefold were shown. Trp, transformation related protein; Tnfsf, TNF superfamily; Polb, DNA polymerase beta; Dffb, DNA fragmentation factor subunit beta; Cideb, cell death-inducing DFFA-like effector b; Casp, caspase; Bnip, BCL2/adenovirus E1B 19 kDa protein-interacting protein 2. (B) Log_3 fold change in the relative level of the genes in DCs from LPS-treated mice to the naive control (white bar: WT; gray bar: PD-1 KO) and DCs from WT versus PD-1 KO were shown (black bar). Data are pooled from at least five mice/group and representative of two different experiments. (C) Expression of CD40, CD40L, ERK2, and p-ERK1/2 in WT DCs and PD-1 KO DCs from LPS-injected mice. Numbers in plots indicate percent of WT (black) and PD-1 KO DCs (red) expressing each molecule. Data are representative of three different experiments with similar results (n=3).

ptosis in homotypic DC–DC interactions by activating caspase-3 and -9 [29]. On the other hand, the CD40–CD40L interaction protects DCs from the apoptosis via activation of Akt1 [30]. The RANK–RANKL interaction increases the survival of DCs during DC–T cell interactions via activation of antiapoptotic signaling initiated by NF-κB and JNK pathways and Bcl-xL up-regulation [31, 32]. Our findings in this study propose an additional mechanism for DC apoptosis: that PD-1 ligation induces DC apoptosis. As two ligands of PD-1 (B7-H1 and B7-DC) are expressed on DCs and T cells [33, 34], either the DC–T cell or DC–DC interaction might contribute to PD-1-mediated DC apoptosis. It is likely that PD-1 might not be involved in the apoptosis of immature DCs as a result of the absence of PD-1 expression under steady-state. However, PD-1-mediated DC apoptosis might play a role in the DC elimination process after antigen presentation, as PD-1 expression is induced on DCs only after their activation, and DC apoptosis starts to increase after PD-1 up-regulation (Fig. 3).

PD-1 expression in T cells and DCs is regulated by the distinct pathway. Whereas PD-1 expression is induced by TCR activation in T cells [15], it is regulated through TLRs in DCs [23]. However, PD-1 downstream signaling appears to be universal in each subset of immune cells. PD-1 engagement on T cells inhibits the TCR downstream PI3K-Akt-NF-κB pathway, which results in the down-regulation of cytokine production and proliferation [14]. Similarly, several reports on the PD-1 signaling pathway in innate-immune cells have shown that PD-1 inhibits NF-κB activation, leading to the down-regulation

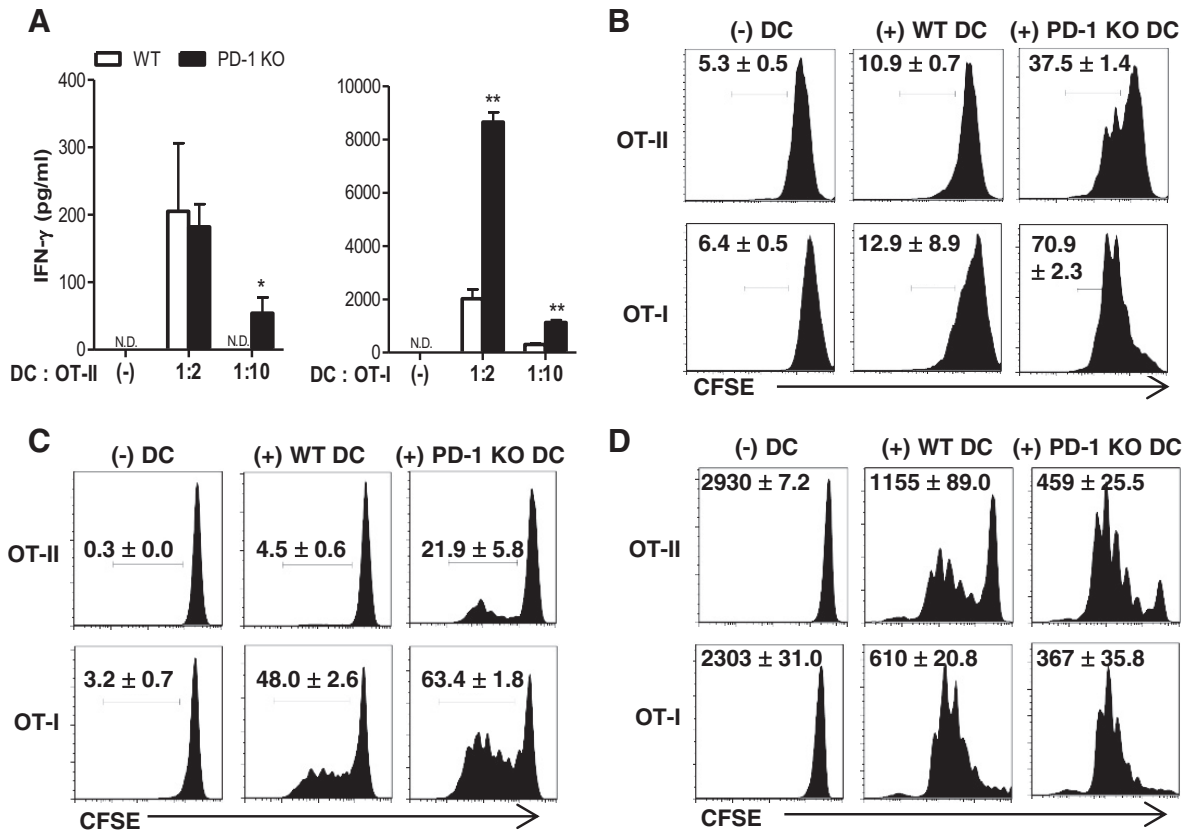

Figure 5. PD-1 KO DCs augment antigen-specific T cell responses. (A and B) Thy1.1$^+$ OT-I or Thy1.1$^+$ OT-II cells were stimulated with CD11c$^+$ WT or PD-1 KO DCs, pulsed with $OVA_{257–264}$ or $OVA_{323–339}$ peptides at an indicated DC:T ratio. (A) IFN-γ production in the culture supernatants was measured at 60 h by ELISA. The values on bars indicate mean ± SD of triplicated samples. N.D., Not detected. $*P < 0.05$; $**P < 0.001$ versus WT, by Student's *t*-test. Data are representative of two independent experiments with similar results. (B) CFSE dilution in OT-II and OT-I cells, cultured with or without DCs (DC:T=1:10) for 60 h. Numbers on histograms indicate mean percent ± SD of CFSE^{low} cells. Data are representative of two independent experiments with similar results. (C and D) OVA-pulsed DCs (0 h; 3×10^5) and 1.5×10^6 Thy1.1$^+$ OT-I or Thy1.1$^+$ OT-II cells (5 h) were transferred i.v. into Thy1.2$^+$ B6 recipients. At 60 h after T cell transfer, proliferation of Thy1.1$^+$ OT-II or Thy1.1$^+$ OT-I T cells in inguinal LNs (C) and spleens (D) was examined by flow cytometry. Numbers on histograms indicate mean ± SEM values of (C) percent CFSE^{low} cells; (D) mean fluorescence intensity of CFSE. Representative data from two independent experiments are shown (n=3–5/group).

of inflammatory cytokines, such as IL-12 and TNF-α in macrophage RAW264.7 cells and tumor-infiltrating DCs [20, 35]. We observed that expressions of ERK2, CD40, and CD40L were higher in PD-1 KO DCs than those in WT DCs after LPS injection (Fig. 4). The CD40–CD40L interaction is recognized to be activated by TLR4 ligation in innate-immune cells [36], and both TLR4 and CD40–CD40L ligation induces activation of MAPK1 signaling, which is required for DC survival [37–39]. Consistent with our data, it has been reported previously that PD-1 ligation with its ligand inhibits not only the PI3K-Akt pathway but also the MAPK1 pathway in human T cells activated with anti-CD3ε mAb [40]. Another study has also shown that blockade of PD-1 and B7-H1 interaction rescues exhausted CD8 T cells via up-regulation of the CD40–CD40L ligation pathway [41]. Taken together, these results suggest that downstream of PD-1, in T cells and DCs, is regulated by the common signaling pathways; PD-1 induced in DCs, as well as T cells, acts as a negative-feedback mechanism for the maintenance of immune homeostasis.

It has been reported that various types of tumor cells express B7-H1, which gives immunosuppressive signals to tumor-infiltrating T cells expressing PD-1, thus inhibiting anti-tumor activity of T cells [42, 43]. Little is known about the role of PD-1 expressed on DCs in the tumor immunity. However, a recent report showed that tumor-associated DCs that express PD-1 inhibited T cell proliferation and activation in ovarian cancer via down-regulation of NF-κB activation, cytokine production, and costimulatory molecule expressions [35]. Our study, demonstrating PD-1-mediated DC apoptosis with its negative effects on T cell responses, further supports the role of PD-1 on DCs, which contributes the immunosuppressive tumor environments. Thus, our results provide a novel mechanism that in addition to PD-1-mediated inhibition of cytokine productions in DCs, PD-1-mediated DC apoptosis might play a role in the immune suppression in cancer.

DC manipulation has been applied to various DC-based vaccination or immunotherapies for cancers and other infectious diseases in mouse models and human clinical trials [44–47].

As DCs are key players in regulating the magnitude of T cell responses, studies on the regulation of DC apoptosis by PD-1 and other molecules might improve the efficacy of DC-based immunotherapies. In conclusion, our findings provide a novel mechanism for PD-1-mediated immune regulation that PD-1 negatively modulates the survival of DCs. In concert with PD-1-mediated inhibition of DC functions, as well as T cell effector functions, our study has important implications for our understanding of immune regulations by PD-1 in the adaptive immunity and gives new insights into identifying new targets for the design of DC-based vaccinations.

AUTHORSHIP

S.J.P. designed and performed most of the experiments and analyzed the data. H.N. contributed in vivo immunological assays. J.D. and J-C.C. contributed to the ex vivo imaging study. B-G.Y. contributed to DC transfer experiments. Y.P. and Y.C.S. contributed to experimental design, data analysis, and manuscript writing for all studies, together with S.J.P.

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DISCLOSURES

The authors declare no conflicts of interest.

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