Programmed death-1 receptor negatively regulates LPS-mediated IL-12 production and differentiation of murine macrophage RAW264.7 cells

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\textsuperscript{1} Introduction

Programmed cell death-1 (PD-1) is a type I transmembrane protein consisting of a single IgV-like extracellular domain, a transmembrane region and a cytoplasmic tail containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) [1,2]. Based on structural homologies, PD-1 has been classified as a member of the CD28 family [3]. The PD-1 receptor is widely expressed on various immune cells, including double-negative T cells in the thymus, activated peripheral T and B cells, NKT cells and macrophages [4–7]. Engagement of T cell PD-1 by its ligands, B7-H1 (PD-L1) and B7-DC (PD-L2), inhibits CD28-mediated T cell activation, leading to apoptosis, inhibition of proliferation and cytokine production [8–10]. In B cells, PD-1 engagement also inhibits B cell receptor-mediated activation, resulting in suppression of B cell expansion and antibody synthesis [11]. In vivo study using PD-1-deficient mice revealed that PD-1 is a negative regulator of immune responses, playing a critical role in maintaining both central and peripheral tolerance [4,12]. Previously, we reported that PD-1 is expressed on various myeloid lineage cells including macrophage, dendritic cells (DC), and myeloma cell lines, and that its expression on macrophages is upregulated in response to IFN-\(\alpha\) [7]. Recent studies have shown that PD-1 receptor is expressed at low levels on nonspecific inflammatory cells and increased by various bacterial stimulus such as lipoteichoic acid, Poly-I:C, LPS and peptidoglycan [13,14], suggesting a novel immunomodulatory function of the PD-1 receptor on innate immune cells. Recently, Yao et al. [14] suggested that PD-1 expressed on dendritic cells negatively regulates DC functions as evidenced by the finding that PD-1-deficient mice have superior capacity in innate protection of mice against Listeria infection and elevated production of IL-12 and TNF-\(\alpha\). However, there are few reports describing the roles of PD-1 in differentiation of macrophages into dendritic cells and in signaling pathway associated with IL-12 production.

In vitro studies using RAW264.7, a macrophage cell line, suggested that lipopolysaccharide (LPS) induces the differentiation of RAW264.7 cells into DC-like cells, as demonstrated by the acquisition of a distinct dendritic morphology as well as mature DC surface markers such as upregulation of MHC class I and II, and co-stimulatory molecules such as CD80 and CD86 [15,16]. Furthermore, LPS-treated RAW264.7 cells enhance allostimulatory...
capacity, a functional characteristics of mature DC compared to immature DC or macrophages. In the present study, we demonstrated that PD-1 engagement with its ligand B7-H1 suppresses IL-12 production in LPS-stimulated RAW264.7 cells through a reduction of Janus N-terminal-linked kinase (JNK) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling by the recruitment of SHP-2 to PD-1 cytoplasmic tail. In addition, PD-1 engagement inhibits the differentiation of RAW264.7 cells into DC-like cells as revealed by a reduced allostimulatory activity and a downregulation of MHC and co-stimulatory molecules. These results indicate that macrophage PD-1 negatively regulates functional maturation of LPS-stimulated RAW264.7 cells.

2. Materials and methods

2.1. Reagents and antibodies

Lipopolysaccharides from Salmonella Enterica serotype Minnesota (L9763) were obtained from Sigma–Aldrich (St. Louis, MO), Recombinant mouse B7-H1/Fc chimera (1019-B7) was purchased from R&D Systems (Minneapolis, MN). Human IgG for control experiments was purchased from Sigma–Aldrich (St. Louis, MO). LY294002, PD98059, SP600125, and SB203580 were purchased from Calbiochem (San Diego, CA). Anti-mouse PD-1 (clone J43) and FcR blocker (anti-mouse CD16/CD32 2.4G2) were purchased from eBioscience (San Diego, CA, USA). Mouse monoclonal antibodies were C18.2 and C17.8 (eBioscience, San Diego, CA, USA), anti-mouse CD16/CD32 2.4G2, anti-mouse CD80 (B7-1; clone 16-10A1), anti-mouse CD86 (B7-2; clone GL1), anti-mouse CD11c (clone N418) and PE-conjugated anti-mouse H-2K\(^{\beta}\) (clone SF1-1.1) were obtained from BioLegend (San Diego, CA), and FITC-conjugated anti-mouse I-A\(^{d}\) (clone AMS-32.1) was also purchased from BD Bioscience (Franklin Lakes, NJ, USA). The cut off for each marker was based on a relevant isotype control antibody.

2.2. Cell line

RAW264.7 cells were obtained from American Type Culture collection (ATCC, Manassas, VA, USA) and maintained at 37 °C in a 5% CO\(_2\) humidified incubator in RPMI 1640 culture medium containing 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, and 100 µg/ml streptomycin.

2.3. RT-PCR

Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed with Superscript II (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Two micrograms total RNA was reverse transcribed with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA), and 0.1 µg of the reverse transcribed cDNA was amplified by PCR. For PCR amplification, the PCR primer sequences are listed in supplementary Table 1. PCR reactions at 94 °C for 30 s, at 58 °C for 30 s, and at 72 °C for 1 min were carried out for 21–30 cycles according to target transcripts. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), twenty-five cycles of amplification at 94 °C for 30 s, at 58 °C for 30 s, and at 72 °C for 1 min were performed. PCR products were separated on a 1.2% agarose gel and stained with ethidium bromide.

2.4. Real-time PCR

For real-time PCR, total RNA was digested with RNase-free DNase (Promega, Madison, WI) to remove a contaminating genomic DNA. The first-strand cDNA was synthesized using the Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, CA). Real-time PCR was performed using a SYBR Supermix kit and an iCycler system (Bio-Rad, San Diego, CA), according to the manufacturer's instructions.

2.5. Intracellular staining, flow cytometric analysis and ELISA

For intracellular IL-12 staining, we used a BD Cytofix/Cytoperm kit (BD Pharmingen, San Diego, CA, USA) following the manufacturer's instructions and subsequently stained with biotin conjugated anti-mouse IL-12 (clone C17.8; eBioscience, San Diego, CA, USA) or isotype control antibody. Anti-mouse IL-12 was detected with fluorescein isothiocyanate (FITC)-conjugated streptavidin (BioLegend, San Diego, CA, USA). For the expression of PD-1 or other cell surface markers, cells were collected from cultures and washed with FACS buffer (PBS containing 1% FCS and 0.05% NaN\(_3\)). And then the cells were first incubated with FcR blocker (anti-mouse CD16/CD32 2.4G2; eBioscience, San Diego, CA, USA) to block nonspecific antibody binding and stained with Phycoerythrin (PE)-conjugated anti-mouse programmed death (PD)-1 (clone J43; eBioscience, San Diego, CA, USA) or with matched pairs of antibodies. Analysis was performed using FACSsort and CELLQUESTPRO software (BD Bioscience, San Diego, CA, USA). The quantities of IL-12 p70 in the culture supernatants were determined by a sandwich enzyme-linked immunosorbent assay (ELISA), using monoclonal antibodies specific for IL-12 p70. The monoclonal antibodies for coating the plates and the biotinylated second monoclonal antibodies were C18.2 and C17.8 (eBioscience, San Diego, CA, USA), respectively. Standard curves were generated using recombinant IL-12, with a lower limit of detection of 15 pg/ml.

2.6. Western blot and immunoprecipitation

Cells were washed with ice-cold PBS and lysed in a buffer (10 mM Tris, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 100 mM sodium pyrophosphate, 100 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 µg/ml leupeptin, 1 µg/ml aprotinin, and 1% Triton X-100). The whole cell lysates were centrifuged at 10,000 g for 20 min, and the supernatants were collected. Protein concentration of cell lysates was determined using the Protein Assay Kit (Bio-Rad). Fifty micrograms of cellular protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a polyvinylidene difluoride membrane (Amersham Bioscience, San Diego, CA, USA). After blocking with 1% Tween20 in 5% skim milk, the membrane was probed with appropriate primary antibodies, and the immune complexes were detected with ECL reagents after incubation with appropriate secondary antibodies according to the manufacturer's instruction. For immunoprecipitation, RAW264.7 cells stimulated with LPS for 12 h were washed twice with cold PBS and resuspended in 0.5 ml of PBS. The LPS-stimulated RAW264.7 cells were stimulated with rmB7-H1 or hIgG at 37 °C and pelleted at 14,000 rpm for 10 min. The whole cell lysates were centrifuged at 10,000 g for 20 min, and the supernatants were collected. Protein concentration of cell lysates was determined using the Protein Assay Kit (Bio-Rad). Fifty micrograms of cellular protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a polyvinylidene difluoride membrane (Amersham Bioscience, San Diego, CA, USA). After blocking with 1% Tween20 in 5% skim milk, the membrane was probed with appropriate primary antibodies, and the immune complexes were detected with ECL reagents after incubation with appropriate secondary antibodies according to the manufacturer's instruction. For immunoprecipitation, RAW264.7 cells stimulated with LPS for 12 h were washed twice with cold PBS and resuspended in 0.5 ml of PBS. The LPS-stimulated RAW264.7 cells were stimulated with rmB7-H1 or hIgG at 37 °C and pelleted at 14,000 rpm for 10 min at 4 °C. To detect PD-1 phosphorylation and recruitment of SHP-1 and SHP-2, whole cell lysates were incubated overnight with 5 µg/ml anti-mPD-1 (RMPI-30; eBioscience, San Diego, CA, USA). The next day samples were incubated with protein A/G-agarose (sc-6244; Santa Cruz Biotechnology) for an additional 3 h at 4 °C, then washed five times with 1% Nonidet P-40 lysis buffer and finally resuspended in SDS loading buffer. The immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose. Blots were probed with appropriate primary and secondary antibody combinations, and proteins were visualized using an ECL kit (Pierce, Rockford, CA). The following mAbs were used: anti-phosphotyrosine Ab (Cell Signaling Technology, Beverly, MA), anti-SHP-1 (sc-287), anti-SHP-2 (sc-280), anti-mouse IgG-HRP, anti-rabbit IgG-HRP Ab (Santa Cruz Biotechnology).
Cruz Biotechnology, Santa Cruz, CA) and anti-hamster IgG-HRP Ab (Southern Biotechnology Associates, Birmingham AL).

2.7. Analysis of endocytic capacity

Endocytic capacity was assessed by the uptake of FITC-dextran (FD70S; Sigma, St. Louis, MO) according to a previously reported method of Nencioni et al. [17], with slightly modifications. RAW264.7 cells were incubated with 1 μg/ml LPS, 5 μg/ml rmB7-H1, or 1 μg/ml LPS plus 5 μg/ml rmB7-H1 for 16 h. After washed with PBS, cells were incubated in a 200 μl PBS containing 5% human serum with 1 mg/ml FITC-dextran for 60 min at 37°C. The incubation was stopped with 1 ml of ice-cold PBS, and the cells were washed four times with ice-cold PBS. Cellular uptake of FITC-dextran was analyzed using a FACS flow cytometry. In addition, parallel experiments were performed at 4°C to show that the uptake of FITC-dextran by RAW264.7 cells is inhibited at low temperatures.

2.8. Allogenic mixed lymphocyte reaction

To prepare the responder cells, cells were isolated from the spleens of C57BL/6 mice. First, T cells were obtained from the splenic lymphocytes by using Dynal® Mouse T Cell Negative Isolation Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Harvested cell suspensions contained >97% CD3+ cells by flow cytometry. Raw264.7 cells which were treated with 1 μg/ml LPS, 5 μg/ml rmB7-H1, or co-treated with 1 μg/ml LPS plus 5 μg/ml rmB7-H1 for 16 h, were used as the stimulator cells. Responder T cells (1 × 10^5) and mitomycin C-treated stimulator cells (3 × 10^5) were mixed in a 3:1 ratio in a round bottomed 96-well plate. After culture for 4 or 6 days cells were pulsed with [3H]-thymidine (1 μCi/well, Amersham Pharmacia) and cultured for a further 16 h. T cell proliferation was determined by incorporation of [3H]-thymidine. The incorporation of [3H]-thymidine was measured with a β-counter (Wallac, Torrance, CA).

2.9. Statistical analysis

Data were expressed as mean ± standard deviation (S.D.) of the indicated number of experiments. The statistical significance of data was estimated using a Student’s t-test for unpaired observations and the level of significance (*) was determined at P < 0.05.

Fig. 1. Expression of mouse PD-1 on RAW264.7 cells. (A) RAW264.7 cells were stimulated with LTA (2 μg/ml), poly-I:C (2 μg/ml) or LPS (1 μg/ml) for 16 h. Total RNA was isolated and analyzed for mouse PD-1 mRNA expression by RT-PCR. (B) RAW264.7 cells were stimulated with 1 μg/ml LPS for 16 h, stained with PE-conjugated anti-mouse PD-1 antibody, and analyzed by flow cytometry. Data are presented as mean fluorescence intensity (MFI).

Fig. 2. Expression of immune-related genes in RAW264.7 cells in response to LPS. RAW264.7 cells were pretreated with the indicated concentrations of recombinant mouse B7-H1.Fc (rmB7-H1) or hIgG for 2 h and stimulated with 1 μg/ml LPS for 16 h. RT-PCR was performed to analyze the expression of cytokine genes (IFN-γ, IL-10, IL-12, TNF-α, IL-1β and IL-6) (A) and inflammatory mediators (iNOS, Cox2, M-CSF, IP-10 and IRF1) (B).
3. Results

3.1. PD-1 engagement inhibits the expression of immune-related genes in LPS-stimulated RAW264.7 cells

To assess the effect of PD-1 signaling on immune-regulated gene expression in macrophages, we first examined PD-1 expression on RAW264.7 macrophage cell line using RT-PCR and flow cytometry. RT-PCR analysis showed that PD-1 transcripts were detectable in unstimulated RAW264.7 cells, and upregulated in response to Toll-like receptor (TLR) ligands such as poly-I:C and LPS, but not lipoteichoic acid (LTA) (Fig. 1A). Specifically, PD-1 mRNA level is about three times higher in LPS-stimulated RAW264.7 cells than that of unstimulated cells. Flow cytometric analysis also revealed the upregulation of PD-1 in LPS-stimulated RAW264.7 cells (Fig. 1B). Next, we examined the expression of genes involved in immune regulation in RAW264.7 cells treated with recombinant mouse B7-H1.Fc (rmB7-H1) in the presence of LPS. As shown in Fig. 2, PD-1 engagement with rmB7-H1 inhibited the expression of cytokine genes including IFN-γ, IL-10 and IL-12, but not TNF-α, IL-1β, and IL-6 (Fig. 2A). PD-1 engagement also decreased inflammatory mediators such as iNOS and Cox2, but not M-CSF, IP-10, and IRF-1 in LPS-stimulated RAW264.7 (Fig. 2B). Since IL-12 is a key cytokine in the development of Th1 cell-mediated immune responses, we focused on the regulation of IL-12 gene expression in RAW264.7 cells treated with rmB7-H1. IL-12 gene expression was...
increased about 17-fold in LPS-stimulated RAW264.7 cells compared to the unstimulated cells, whereas PD-1 engagement with rmB7-H1 in LPS-stimulated RAW264.7 cells greatly suppressed the IL-12 gene expression in dose-dependent manner (Fig. 3A and B). Consistent with this result, flow cytometric analysis also showed a reduced expression of intracellular IL-12 in rmB7-H1-treated LPS-stimulated RAW264.7 cells compared to the control hIgG-treated cells (Fig. 3C). When IL-12 protein amount in cell cultures was estimated by ELISA, the IL-12 secretion greatly decreased in the supernatant of rmB7-H1-treated LPS-stimulated RAW264.7 cells compared to hIgG-treated LPS-stimulated RAW264.7 cells (Fig. 3D). Furthermore, we obtained similar results in M-CSF-induced bone marrow-derived CD11b+ macrophages (Supplementary Figs. S1A and B) and in DC2.4, a mouse DC cell line (Supplementary Fig. S2) as revealed by RT-PCR and flow cytometry. In order to confirm whether the inhibition of IL-12 expression is mediated by PD-1 specific signaling, we further investigated IL-12 production in LPS-stimulated RAW264.7 cells using antagonistic anti-PD-1 mAb (J43) which is reported to block B7-H1.Fc binding to PD-1 [18]. As expected, blockade of PD-1 engagement with anti-PD-1 mAb restored IL-12 gene expression as revealed by RT-PCR (Fig. 3E). These results indicate that PD-1 delivers a negative signal into LPS-stimulated RAW264.7 cells, leading to suppression of IL-12 production, some inflammatory cytokines and mediators.

Fig. 4. Effect of PD-1 engagement on signaling pathway responsible for IL-12 gene expression. (A) RAW264.7 cells were pretreated with the indicated inhibitors for 30 min and stimulated with 1 μg/ml LPS for an additional 16 h in the presence or absence of various signaling inhibitors. Total RNA was prepared and analyzed by RT-PCR for mouse IL-12 or GAPDH. NT (no treatment); LY (PI3K inhibitor); PD (ERK inhibitor); SP (JNK inhibitor); SB (p38 inhibitor). (B) RAW264.7 cells were pretreated with indicted concentrations of rmB7-H1 for 2 h and stimulated with 1 μg/ml LPS for 15 min. Whole cell lysates were subjected to western blotting with the indicated antibodies. (C) RAW264.7 cells were cultured with 1 μg/ml LPS for 24 h and stimulated with 5 μg/ml rmB7-H1 for indicated time. Cells were lysed in lysis buffer, and supernatants were collected by centrifugation. The cell lysates were immunoprecipitated overnight with 5 μg/ml of anti-mPD-1 (RPMI-30), and protein A/G-agarose was used for precipitation. The suspended pellets were subjected to western blotting with anti-PD-1 (J43), anti-phosphotyrosine, (D) anti-SHP-2 (sc-280), and (E) anti-SHP-1 (sc-287) antibodies.
3.2. JNK pathway is responsible for the regulation of PD-1-mediated suppression of IL-12 production

Having demonstrated that PD-1 engagement with rmB7-H1 inhibits IL-12 gene expression in LPS-stimulated RAW264.7 cells, we next examined the early signaling pathways responsible for the PD-1-mediated suppression of IL-12. It has been reported that LPS-induced IL-12 production is mediated by activation of c-Jun N-terminal kinase, the activation protein-1 (AP-1) and NF-kB transcription factors [19,20]. We found that SP600125 (a JNK inhibitor) and LY294002 (a PI3K/AKT inhibitor), but not PD98059 (a MEK 1/2 inhibitor) and SB203580 (a p38 inhibitor), strongly suppressed IL-12 gene transcription in LPS-stimulated RAW264.7 cells (Fig. 4A). This result suggests that JNK and PI3K/AKT signaling pathway are involved in IL-12 induction in macrophage in response to LPS. Interestingly, PD-1 engagement greatly inhibited JNK and to a lesser extent AKT phosphorylation in LPS-stimulated RAW264.7 cells (Fig. 4B). There was no significant difference in phosphorylation of other kinase such as ERK in LPS-stimulated RAW264.7 cells in the presence or absence of rmB7-H1.

Since Okazaki et al. [11] reported that PD-1 engagement by PD-L1 induces the phosphorylation of tyrosine residue from immunoreceptor tyrosine-based inhibitory motif (ITIM) in cytoplasmic domain, recruiting SHP-2 in B cells, we investigated whether PD-1 engagement could affect phosphorylation of PD-1, and SHP-1 and SHP-2 recruitment in RAW264.7 cells. When RAW264.7 cells were stimulated with rmB7-H1, PD-1 was phosphorylated at early time compared to the control hlgG-treated cells (Fig. 4C). Furthermore, PD-1 engagement with rmB7-H1 actively recruited SHP-2 to the PD-1 cytoplasmic tail (Fig. 4D) but weakly recruited SHP-1 (Fig. 4E). Taken together, PD-1 signaling may predominantly attenuate the JNK phosphorylation through the phosphorylation of tyrosine residue in PD-1 receptor and the recruitment of SHP-2 to PD-1 receptor, leading to the reduction of IL-12 production.

![Fig. 5. Effect of PD-1 engagement on the expression of co-stimulatory molecules and MHC proteins in LPS-stimulated RAW264.7.](image-url) RAW264.7 cells were pretreated with 5 μg/ml rmB7-H1 for 2 h and stimulated with 1 μg/ml LPS for 16 h under the indicated conditions. Cells were stained with FITC-conjugated antibody specific for CD80, CD86, MHC class I, MHC class II, and CD11c, and analyzed by flow cytometry. Data are presented as the mean fluorescence intensity (MFI) and shown as means ± S.D. of three independent experiments.
The effect of PD-1 engagement on endocytic capacity of LPS-stimulated RAW264.7 cells. RAW264.7 cells were pretreated with the indicated concentrations of rmB7-H1 or hIgG for 2 h and further cultured for 16 h with 1 μg/ml LPS. After washed with PBS, cells were incubated in a 200 μl PBS containing 5% human serum with 1 mg/ml FITC-dextran at 37 °C for 60 min. The incubation was stopped with 1 ml of ice-cold PBS, and the cells were washed four times with ice-cold PBS. Phagocytic activity was determined by flow cytometry using FITC-conjugated dextran. Data are presented as phagocytic indices ranked by fluorescence intensity. Values are presented as means ± S.D. of three separate experiments.

3.3. PD-1 engagement downregulates the expression of co-stimulatory molecules and MHC proteins in LPS-stimulated RAW264.7 cells

RAW264.7 macrophage cell line is known to differentiate into DC-like cells when activated by LPS [15]. RAW264.7 cells activated with LPS markedly upregulated co-stimulatory molecules including CD80 and CD86, MHC class I and II proteins, and CD11c, a DC surface marker (Fig. 5). Interestingly, PD-1 engagement with rmB7-H1 greatly downregulated CD86 and MHC class I protein, to a lesser extent CD80 and MHC class II, but not CD11c in LPS-stimulated RAW264.7 cells compared to the cells treated with control hIgG. In addition, there was no significant change in cell morphology of LPS-stimulated macrophages in the presence or absence of rmB7-H1 (data not shown). These results indicate that PD-1 engagement with rmB7-H1 negatively regulates phenotypic maturation of LPS-stimulated RAW264.7 cells.

3.4. PD-1 engagement increases endocytic capacity of LPS-stimulated RAW264.7 cells

To examine the effect of PD-1 signaling on endocytic activity of LPS-stimulated RAW264.7 cells, we next investigated the ability of the cells to ingest FITC-conjugated dextran to compare the endocytic capacity. As shown in Fig. 6, experiment with FITC-dextran revealed that LPS enhanced phagocytosis of dextran in RAW264.7 cells. Furthermore, uptake of dextran is significantly increased in LPS-stimulated RAW264.7 cells treated with rmB7-H1 in dose-dependent manner compared to the control hIgG-treated cells. RAW264.7 cells treated with rmB7-H1 alone showed a similar endocytic capacity to the untreated wild type cells, indicating that PD-1 signaling does not affect a basal endocytic capacity of RAW264.7 cells. As immature DCs have a higher degree of endocytic activity than mature DCs [21,22], these results suggest that PD-1 signaling partially inhibits the LPS-mediated differentiation of macrophages into functionally mature DC-like cells.

3.5. PD-1 engagement inhibits the allostimulatory capacity of LPS-stimulated RAW264.7

It has been reported that mature DCs display a higher allostimulatory capacity than immature DCs [23]. Therefore, we further examined the effect of PD-1 signaling on the allostimulatory capability using allogeneic mixed lymphocytic reaction assays. Thymidine incorporation assay showed that allogeneic T cells were greatly expanded in the coculture with LPS-stimulated RAW264.7 cells at day 4 and to a lesser extent day 6. However, T cell proliferation was significantly decreased in LPS-stimulated RAW264.7 cells in the presence of rmB7-H1 (Fig. 7). There was no significant difference in allostimulation between the wild type RAW264.7 cells and the rmB7-H1-treated cells in the absence of LPS. Taken together, PD-1 engagement reduces the allostimulatory capacity of RAW264.7 cells stimulated with LPS, but does not affect a basal allostimulatory ability of wild type RAW264.7 cells, suggesting that PD-1 signaling actively inhibits the allostimulatory capability of activated macrophages.

4. Discussion

Our study reveals that PD-1 engagement suppresses IL-12 production through inhibition of JNK and to a lesser extent PI3K/AKT phosphorylation in LPS-stimulated macrophage cell line, RAW264.7 cells. Furthermore, this inhibition is mainly involved in the recruitment of SHP-2 to the PD-1 cytoplasmic tail. We further demonstrate that PD-1 is a negative regulator of LPS-mediated differentiation of macrophages to DC-like cells as
Fig. 7. The effect of PD-1 engagement on allostimulatory capacity of LPS-stimulated RAW264.7 cells. RAW264.7 cells were treated with 1 μg/ml LPS, 5 μg/ml rmB7-H1 or co-treated with 1 μg/ml LPS plus 5 μg/ml rmB7-H1 for 16 h, and then treated with mitomycin C (50 μg/ml) for 1 h. Responder T cells (1 × 10⁴) purified from C57BL/6 splenocytes and mitomycin C-treated RAW264.7 stimulator cells (3 × 10⁵) were mixed in a 3:1 ratio in a round Bottomed 96-well plate and cultured for the indicated time periods. Cells were pulsed with [³H] thymidine (1 μCi/well) for the last 16 h of the culture, and the incorporation of [³H] thymidine was measured with a β-counter. Data are presented as means ± S.D. of three separate experiments.

evidenced by the findings that rmB7-H1-mediated PD-1 engagement decreases the expression of co-stimulatory molecules and MHC proteins, enhances the endocytic capacity and suppresses the allostimulatory capability of LPS-stimulated RAW264.7 cells.

PD-1 is a surface glycoprotein, belonging to an immunoglobulin superfamily. In addition to T and B cells, PD-1 can be induced on splenic DC by TLR2, TLR3, and TLR4 engagements [14]. In this study, we observed that PD-1 expression was upregulated by poly-l:C (TLR3 ligand) and LPS (TLR4 ligand), but not LTA (TLR2 ligand) in RAW264.7 cells. The discrepancy of PD-1 expression in DC and macrophage cell line in response to TLR ligands may reflect the fact that PD-1 expression is regulated in cell-type specific mode rather than controlled by one universal mechanism.

PD-1 engagement leads to phosphorylation of tyrosine residue from immunoreceptor tyrosine-based switch motif (ITSM) in cytoplasmic domain, recruiting both SHP-1 and SHP-2 in T cells [24]. Very low levels of PD-1 can block CD28-mediated activation of PI3K, causing potent inhibition of the earliest stages of T cell activation and apoptosis by suppression of anti-apoptotic bcl-xl gene expression [10]. Cross-linking of PD-1 expressed on B cells inhibits B cell receptor (BCR) signaling by recruiting only SHP-2 phosphatase and reducing phosphorylation of B cell receptor proximal kinase [25], leading to inhibition of memory B cell proliferation and antibody production [26]. However, there are few reports that illustrate the roles of PD-1-expressed on innate immune cells in the production of pivotal cytokines involved in cell-mediated immunity and cellular differentiation.

IL-12 is a Th1-type cytokine that plays a critical role in cell-mediated immune response by upregulating IFN-γ from NK and T cells and generating CTL activity [19]. The present study using specific inhibitors of signaling pathways reveals that JNK and to a lesser extent PI3K/AKT are responsible for IL-12 gene transcription in RAW264.7 cells in response to LPS. This result is in part consistent with the previous report displaying that downregulation of JNK activation by a specific inhibitor and a dominant negative mutant is associated with inhibition of IL-12 production in LPS-stimulated human promonocytic THP-1 cell line [19]. Interestingly, we found that PD-1 engagement followed by LPS stimulation of RAW264.7 cells inhibits IL-12 gene transcription by downregulation of JNK activation and to a lesser extent AKT activation through the recruitment of SHP-2 to PD-1 receptor, indicating that PD-1 signaling negatively regulates IL-12 synthesis in macrophages by diminishing JNK and AKT phosphorylation. The PD-1 engagement following LPS stimulation also diminishes IL-12 expression in RAW264.7 cells (Supplementary Fig. S3). There are several reports showing that IL-12 production is greatly augmented upon LPS stimulation in macrophages in an NF-κB- and AP-1-dependent manner [27–29]. NF-κB and AP-1 are well known as a pibovial transcription factors that regulates the expression of numerous genes during the activation of macrophages [30]. Although PD-1 engagement with B7-H1 inhibits LPS-induced JNK and PI3K/AKT activation, it did not affect the nuclear translocation of NF-κB and degradation of IκB (data not shown). Therefore, the results suggest that PD-1 signaling suppresses IL-12 gene expression by inhibiting the activation of AP-1 transcription factor via JNK phosphorylation. Further study is needed to clarify the exact molecular mechanism of PD-1-mediated downregulation of IL-12 gene transcription after LPS stimulation.

Activated macrophages with TLR ligands such as LPS acquire functional and phenotypic characteristics including upregulation of co-stimulatory molecules, MHC proteins and increase of allostimulatory capacity, a finding that can be seen in the maturation process of DC. These observations implicate that TLR signaling may be responsible for the differentiation of macrophages into DC-like cells. We observed a significant upregulation of CD80, CD86 and MHC class I and class II molecules in LPS-stimulated RAW264.7 cells compared to the unstimulated cells. Interestingly, PD-1 engagement with rmB7-H1 in LPS-stimulated RAW264.7 cells leads to a significant downregulation of CD86 and MHC class I expression, and to a less extent CD80 and MHC class II expression. Further analysis demonstrates that allostimulatory capacity of LPS-stimulated RAW264.7 cells is significantly inhibited in the presence of rmB7-H1, which can be explained by the fact that PD-1 signaling reduces IL-12 production and MHC protein expression in LPS-stimulated RAW264.7 cells. We also found that endocytic capacity of LPS-stimulated RAW264.7 cells is augmented through PD-1 signaling.

Butte et al. [31] demonstrated that B7-H1 also interacts with CD80, resulting in suppression of TNF-α, IFN-γ, and IL-2 gene expression which were induced in T cell upon CD-3 stimulation. In our experiment, however, the downregulation of IL-12 gene expression in LPS-stimulated RAW264.7 cells upon rmB7-H1 stimulation was restored after blocking PD-1 (Fig. 3E). Furthermore, stimulation of CD80 with anti-CD80 antibody does not affect the LPS-induced IL-12 expression in RAW264.7 cells (data not shown). PD-1 is known as a regulator of apoptosis because the level of PD-1 surface expression is the primary determinant of the apoptosis sensitivity of virus-specific CD8+ T cells [8]. However, when RAW264.7 cells were treated with rmB7-H1, there was no apoptosis detectable by MTT assay and FACS analysis (data not shown). These data indicate that PD-1 does not transmit pro-apoptotic signals following B7-H1 engagement in innate immune cells. From a different point of view, since B7-H1 is also expressed on activated T cells [32], PD-1/B7-H1 ligation may mitigate T cell-mediated activation of innate immune cells, resulting in the inhibition of LPS-induced IL-12 expression.

The molecular mechanism leading to the downregulation of allostimulatory activity and enhancement of endocytic ability via PD-1 signaling in LPS-stimulated RAW264.7 cells remains to be illustrated. In conclusion, PD-1 signaling negatively regulates differentiation of macrophages into DC-like cells and IL-12 production in LPS-stimulated RAW264.7 cells. Thus, the interaction of macrophage PD-1 with B7-H1 in LPS-induced inflammatory settings may serve as a negative feedback mechanism to downregulate innate immune response, and reduce a subsequent tissue damage.
Acknowledgments

This work was supported by National Research Foundation of Korea Grant funded by the Korea Government (KRF-2006-311-E00047) (S.W. Lee and I. Choi) and by the Korea Science and Engineering Foundation (KOSEF) Grant funded by the Korea government (MOST) (R13-2007-023-00000-0) (S.W. Lee).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.imlet.2009.08.011.

References


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