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Upregulation of programmed cell death 1 by interferon gamma and its biological functions in human monocytes

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ABSTRACT

Programmed cell death 1 (PD-1) is a co-inhibitory checkpoint receptor expressed in various immune cells, especially in activated T cells. Engagement of PD-1 with its ligand leads to the exhausted T cells and impaired antitumor immunity. To date, PD-1 expression and its roles have been widely reported in T cells but not well defined in innate immune cells including monocytes. In this study, expression of PD-1 was investigated in human monocytes. Here we observed that among cytokines tested, IFN-γ significantly upregulated the PD-1 expression in both THP-1 cell line and human primary monocytes in a dose- and time-dependent manner. This effect was reduced by PI3K inhibitor, suggesting that the involvement of PI3K/AKT pathway. Furthermore, enrichment of active histone mark H3K4me3 in the *Pdcd1* promotor was also observed in IFN-γ-induced THP-1, indicating that epigenetic regulation also plays a role in IFN-γ-induced PD-1 expression. To investigate the biological functions of PD-1, *Pdcd1* was deleted in THP-1 cell line by CRISPR/Cas9 system and the phagocytic ability was investigated. The results showed that the PD-1 deficiency in THP-1 cell line resulted in significantly poor phagocytic potency against carboxylated-modified latex beads. Moreover, the PD-1 deficiency or blocking PD-1/PD-L1 interaction by immune checkpoint inhibitor resulted in an impaired induction of IL-4-induced CD163 expression in THP-1 cell line. Taken together, these results highlighted the importance of PD-1 expression in some of key monocyte functions.

1. Introduction

Monocytes perform diverse functions in infections and cancer. Monocytes in circulation give rise to precursors of macrophages and dendritic cells in peripheral tissues while directly respond to infection. In responding to infection, monocytes are stimulated by external threats such as pathogen-associated molecular patterns (PAMPs) and internal cues including cytokines that dictates their biological functions [1]. In tumor, monocytes display both pro-tumoral and anti-tumoral functions by directly killing of cancer cells and phagocytosis of tumor-derived materials serving as antigen presenting cells [2,3]. Because of the important roles monocytes play in initiating immune response, recent focuses have been on the regulatory mechanisms governing their effector functions.

Programmed cell death 1 (PD-1, also known as PDCD1 and CD279), an immune checkpoint receptor, is a type I transmembrane protein from the CD28 family expressed in wide range of cell types including T cells, B cells, dendritic cells, monocytes, and macrophages [4,5]. Signaling induced by PD-1 and its ligand, PD-L1/2, has been extensively studied in activated T cells and it is well known to serve as co-inhibitory signaling accompanying with TCR signal. PD-1 ligation with its ligand, PD-L1 or PD-L2, results in impaired of T cell functions such as decreased cytokine production, proliferation, and transcription of pro-survival factors [5,6]. Thus, PD-1 expression in T cells helps to prevent an excessive immune-mediated tissue damages, protect self-tissue from autoimmunity and maintain immune tolerance [7]. In the context of immune response to cancer, PD-1 expression on T cells dampens the effector functions [8].

PD-1 expression is also found and reported to play important roles in tumor associated macrophages (TAMs). Both mouse and human TAMs

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express high level of PD-1 along with stage of disease in colon cancer. Moreover, the presence of PD-1 in TAMs is negatively correlates with phagocytosis against tumor cells [9]. In consistent with the report of Ruan et al. that PD-1^{hi} TAMs tend to polarize as a tumor promoting M2 which can significantly suppress their phagocytic potency in T cell lymphoma patients [10]. The regulatory mechanisms of PD-1 expression in TAMs were found to be partially mediated by the MyD88/IL1 receptor via NF-κB p65 [11].

PD-1 expression is regulated by diverse mechanisms via several factors. Epigenetics, including DNA methylation and histone modification, also play roles on PD-1 expression in immune cells [12]. To date, molecular mechanisms underlying PD-1 expression and its roles have been widely studied in the context of T cells but not well defined in monocytes. Therefore, in this study we investigated cytokine(s) that induce PD-1 expression and the underlying signaling pathways including the changes in epigenetic modification of its promoter. Furthermore, the effect of PD-1 deficiency was studied in the context of phagocytosis and M1/M2 polarization.

2. Materials and methods

2.1. Cell culture

THP-1, a human monocytic cells line (reference no. JCRB0112, Japanese Collection of Research Bioresources Cell Bank, Japan), and human primary monocytes were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) HEPES, 1% (v/v) sodium pyruvate (all from Hyclone, UK), 50 mM 2-mercaptoethanol and gentamycin (Gibco Laboratories, USA). Cells were incubated at 37 °C with humidified 5% (v/v) CO_2. Primary human monocytes were purified by positive selection using CD14 beads MojoSort™ (BioLegend, USA). Handling of human samples were carried out in accordance with guidelines/regulations issued and approved by Institutional Review Board of Thai Red Cross Society (IRB approval No. 683/62).

2.2. Reagents and chemicals

Recombinant human TGF-β, IFN-γ, IL-4, GM-CSF and M-CSF were purchased from BioLegend. Phorbol 12-Myristate 13-Acetate (PMA) (Millipore, USA) and PI3K inhibitor, LY294002 (Cell Signaling Technology, USA) were dissolved in dimethyl sulfoxide (DMSO) and kept at −80 °C until use. FuGene®HD transfection reagent (Roche, USA) was used for plasmid transfection and puromycin (Thermo Fisher Scientific, USA) was used to select transduced cells. Nivolumab (Opdivo®) (Bristol-Myers Squibb, USA) was used to block PD-1/PD-L1 interaction *in vitro*.

2.3. Pseudo-lentivirus production and transduction for Pdcd1 knockout by CRISPR/Cas9

Lentiviral plasmid vectors containing sgRNA targeting the *Pdcd1* exon 2 locus for CRISPR/Cas9-mediated genome editing (GenScript, USA), lentiviral vector packaging construct containing gene encoding VSVG, obtained from Dr. Barbara A. Osborne, and psPAX2 (AddGene, USA) were co-transfected into HEK293T by using FuGene®HD transfection reagent. After 72 h, culture supernatant containing pseudo-lentivirus was harvested by filtrating through 0.45 μm filter. The filtrated culture supernatant was used to transduce to THP-1 cell line. After 48 h, the culture supernatant was removed and replaced by fresh RPMI-1640 complete medium containing 2 μg/ml puromycin. Stable clones were obtained and subjected for limiting dilution for single cell clones. *Pdcd1* deletion was confirmed by sequencing of the PCR products corresponding to the targeted locus.

2.4. Phagocytic activity assay

THP-1 was stimulated with IFN-γ (25 ng/ml) in the presence or absence of LPS (100 ng/ml) for 24 h, and the activated cells were incubated with 2.0 μm FluoSpheres™ carboxylate-modified microspheres (Themo Fisher Scientific) at 1:4 ratio for 2 h, in dark. THP-1 was later subjected to analyze for the phagocytic activity by flow cytometry (FC500, Beckman Coulter, USA).

2.5. M1 and M2 polarization

THP-1 was pretreated with PMA (10 ng/ml) for 24 h to induce differentiation from monocytes to macrophages. Cells were then rested for 24 h before stimulating with IFN-γ (10 ng/ml) and LPS (100 ng/ml) for M1 polarization, or IL-4 (100 ng/ml) for M2 polarization. Markers of M1 or M2 were examined by flow cytometry. Cell lysates were collected and subjected to Western blot for PD-1 expression.

2.6. Western blot

Total protein was extracted by using RIPA lysis buffer. The equal amount of protein samples was analyzed on 10% SDS-PAGE by mini-Protein III system (Bio-Rad, USA). The proteins were transferred to polyvinylidene fluoride membrane (GE Healthcare, USA). The membrane was probed with mouse anti-human CD279 antibody (BioRad), rabbit anti-human pAKT (Thr308) (Cell Signaling Technology), rabbit anti-human AKT (Cell Signaling Technology) and rabbit anti-human GAPDH antibody (BioRad). The signals were detected in different exposure times depending on each protein by horseradish peroxide (HRP) conjugated secondary antibodies and enhanced chemiluminescence substrate with Alliance Q9 Advanced (UVITEC, UK) or X-ray film. All original unmanipulated Western blot images were provided as the Supplementary Information.

2.7. Flow cytometry

Cells treated as described were harvested and washed. FC receptors was blocked by 10% human serum, and subsequently washed by FACS buffer before staining with mouse anti-human CD279 primary antibody and the secondary antibody, anti-mouse IgG (H+L) Alexa Fluor 488 (BioLegend), anti-human CD274-PE (BioLegend), anti-human CD86-Cyanine 7 (BioLegend) or anti-human CD163-Cyanine 5 (BioLegend), in dark. The stained cells were then subjected to analyze by flow cytometer. The acquired data were analyzed using FlowJo data analysis software (Tree Star, Inc., USA).

2.8. Quantitative real time PCR (RT-qPCR)

Total RNA was extracted by Direct-zol™ RNA Miniprep (Zymo research, USA), then converted to complementary DNA (cDNA). The RT-qPCR was carried out by using iQ™ SYBR® Green Supermix (BioRad) according to manufacturer's protocol and performed in the CFX Connected™ Real-Time PCR Detection System (BioRad). Primer sets for *Pdcd1* (FW 5′ ATCCCTTGTCCCAGCCACTC 3′ and RV 5′ CGTGGCCTATCCACTCCTCA 3′) [13] was used. *GAPDH* served as an internal control; FW 5′ ACCACAGTCCATGCCATC 3′ and RV 5′ TCCACCACCCTGTTGCTG 3' [14].

2.9. Chromatin immunoprecipitation (ChIP) and qPCR

ChIP was performed according to the manufacturer instruction by using SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads), (Cell Signaling Technology). In brief, the cells were fixed with formaldehyde. Micrococcal nuclease was then added and incubated to digest the chromatin into DNA fragments. The digested chromatin was precipitated by using Tri-Methyl-Histone H3 (Lys4; H3K4me3) antibody (Cell

Signaling Technology), and normal rabbit IgG antibody was used as a negative control. The samples were incubated overnight at 4 °C with rotation. Immunoprecipitants were then reversed the cross-links and purified by using DNA purification buffer and spin columns (ChIP, CUT&RUN) (Cell Signaling Technology). DNA fragments were used as templates for amplification by real time qPCR using the primer sets corresponding to −1194 bp to −992 bp upstream of the transcription start site of *Pdcd1*, FW 5′ CCTCACATCTCTGAGACCCG 3′ and RV 5′CCGAAGCGAGGC TAGAAACC 3′ [15]. The signal relative to input was calculated by the following formula: Percent input = 2% x $2^{(C[T]\ 2\%\ Input\ sample\ -\ C[T]\ IP\ sample)}$, where C[T] = threshold cycle of the PCR reaction.

2.10. Statistical analysis

All statistical analyses were performed using GraphPad Prism software. Statistical significance was determined using one-way ANOVA or unpaired *t*-test. P-value of less than 0.05 was considered statistical significance.

3. Results

3.1. IFN-γ upregulates PD-1 expression in THP-1 cell line and human primary monocytes

Firstly, we investigated the effect of monocyte-related key cytokines on PD-1 expression in THP-1 cell line. THP-1 was stimulated with different recombinant cytokines (TGF-β (10 ng/ml), IFN-γ (10 ng/ml), M-CSF (10 ng/ml), GM-CSF (10 ng/ml) and IL-4 (20 ng/ml)) for 48 h and the PD-1 expression was examined by Western blot. The results showed clearly that IFN-γ and TGF-β induced PD-1 upregulation to approximately 4-folds and 2.5-folds, respectively, compared with the untreated cells (Fig. 1A and B). Furthermore, it was confirmed that IFN-γ, but not TGF-β could also upregulate PD-1 expression in human primary monocytes (Fig. 1A). Because IFN-γ significantly upregulated and provided the highest PD-1 expression in THP-1 and human primary monocytes, it was chosen for further characterization of PD-1 in monocytes in the subsequent experiments.

3.2. Dose and time-dependent upregulations of PD-1 by IFN-γ in THP-1 cell line

To determine the dose-dependent effect of IFN-γ on PD-1 expression in THP-1 cell line, THP-1 was stimulated with various concentrations of IFN-γ (1, 10 and 25 ng/ml) for 48 h, and the expression of PD-1 was examined by Western blot and Flow cytometry. As shown in Fig. 1C and D, IFN-γ induced PD-1 expression in a dose dependent manner when IFN-γ at 25 ng/ml yielded the highest level. *Pdcd1* mRNA expression level was also investigated upon treatment of THP-1 at 24 h. It was found that *Pdcd1* mRNA were also upregulated by IFN-γ in a dose-dependent manner (Fig. 1E).

To determine whether there was a time dependency to changes in PD-1 and *Pdcd1* mRNA expression upon IFN-γ treatment, THP-1 was stimulated with IFN-γ (25 ng/ml) for various incubation times and *Pdcd1* mRNA was examined at 0, 6, 12 and 24 h by RT-qPCR while the

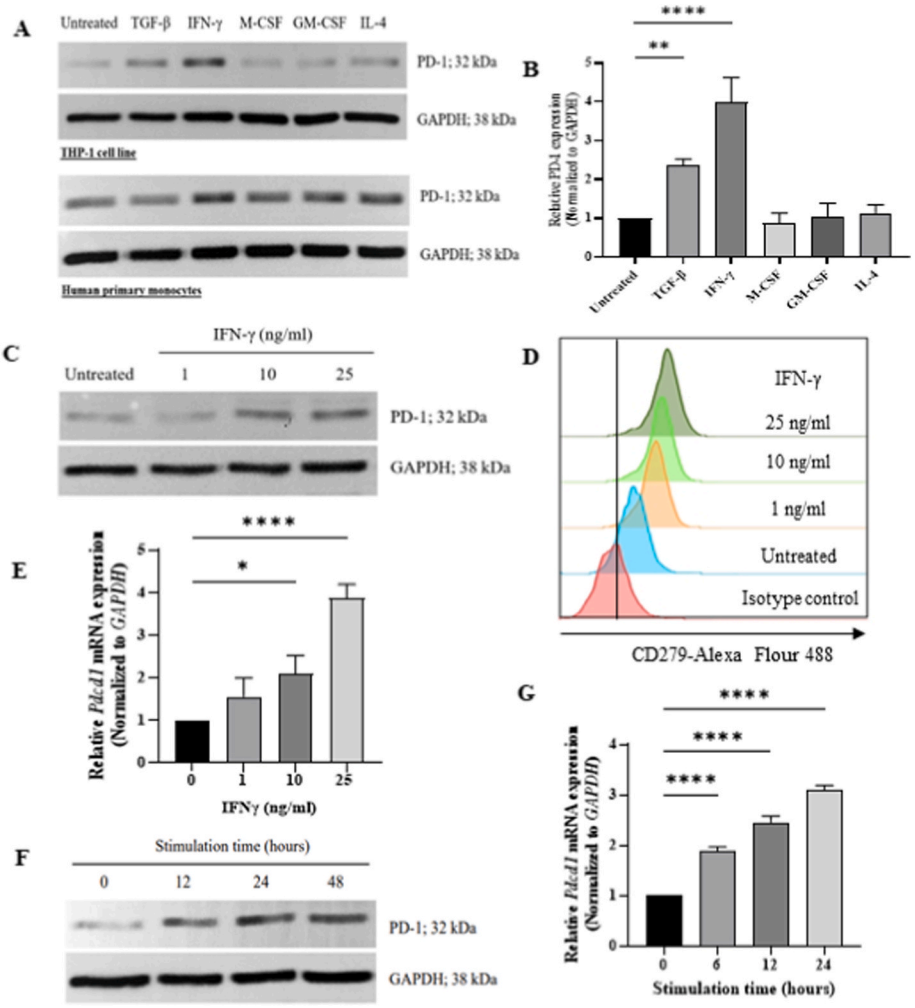

Fig. 1. IFN-γ upregulates PD-1 expression in THP-1 cell line in a dose- and time-dependent manner.
(A) Representative Western blots of PD-1 expression upon cytokine stimulation in THP-1 cell line (upper) and $CD14^{+}$ human primary monocytes (lower). (B) Relative band intensity of PD-1 in THP-1 by various cytokines, normalized by GAPDH. (C–E) Dose-dependent effect of IFN-γ on PD-1 expression in THP-1 was observed by Western blot (C) or Flow cytometry (D) and RT-qPCR (E). (F–G) THP-1 was stimulated with IFN-γ (25 ng/ml) as indicated times and PD-1 and *Pdcd1* mRNA expression were detected by Western blot (F) or RT-qPCR (G). Each bar represents a mean ± SD of triplicate independent experiments. *, ** and **** indicate statistically significant differences at *p*-value < 0.05, < 0.01 and <0.0001, respectively.

PD-1 expression was examined at 0, 12, 24 and 48 h by Western blots. Our results showed that PD-1 level was readily increased at 12 h and reached the highest level at 24–48 h (Fig. 1F). Correlating with the protein level, the *Pdcd1* mRNA level was increased by 6 h treatment and continued to increase up to 24 h (Fig. 1G).

3.3. PI3K/AKT pathway is involved in PD-1 upregulation in IFN-γ-treated THP-1 cell line

To investigate the signaling pathway(s) downstream of IFN-γ responsible for inducing PD-1 upregulation in THP-1, THP-1 was pretreated with phosphoinositide 3-kinases (PI3K) inhibitor, LY294002 (50 μM), for 60 min to inhibit the activity of PI3K before stimulation with IFN-γ (25 ng/ml). Phosphorylated-AKT (pAKT-T308), a downstream substrate of PI3K, and PD-1 were examined by Western blot. The results showed that the level of pAKT was increased after IFN-γ treatment but clearly reduced in the presence of LY294002 inhibitor (Fig. 2A and B). More importantly, the LY294002 treatment complexly abrogated the upregulation of PD-1 (Fig. 2A and C) and *Pdcd1* mRNA (Fig. 2D) in IFN-γ-treated THP-1. The cell viability of THP-1 was minimally reduced to 80% by LY294002 at the dose used in this experiment (50 μM) (Fig. S1). These results strongly indicated that IFN-γ induces upregulation of PD-1 mainly through the PI3K/AKT pathway in THP-1 cell line.

3.4. Active histone mark H3K4me3 is increased on Pdcd1 promoter in IFN-γ-treated THP-1 cell line

To investigate whether the expression of PD-1 in IFN-γ-treated THP-1 is associated with epigenetic changes, ChIP was performed using THP-1 stimulated with IFN-γ (25 ng/ml) for 48 h. ChIP-qPCR assay was performed by using anti-H3K4me3 antibody and the chromatin fragments were quantitated by qPCR. The results showed that IFN-γ treatment is accompanied by increasing H3K4me3 enrichment in the promoter −1194 bp to −992 bp upstream of the transcription starting site of *Pdcd1* encoding region (Fig. 2E). This result indicated that IFN-γ treatment is accompanied by histone modification that is conducive to PD-1 expression.

3.5. CRISPR/Cas9-mediated PD-1 deletion in THP-1 cell line

In order to probe the biological functions of PD-1 in THP-1 cell line, CRISPR/Cas 9 technique was used to generate PD-1 KO THP-1. Drug selection of clones that were transduced by lentiviral particles with sgRNA targeting the exon 2 of *Pdcd1* resulted in multiple clones. After screening for clones with severely reduced PD-1 expression, one of the PD-1 KO clones showed significantly reduced PD-1 level, compared with the parental and mock THP-1 cell line as observed by Western blot (Fig. 3A), flow cytometry (Fig. 3B) and RT-qPCR (Fig. 3C). To preliminarily characterize the obtained PD-1 KO THP-1 cell line, the doubling time was measured and compared with that of the parental line or mock

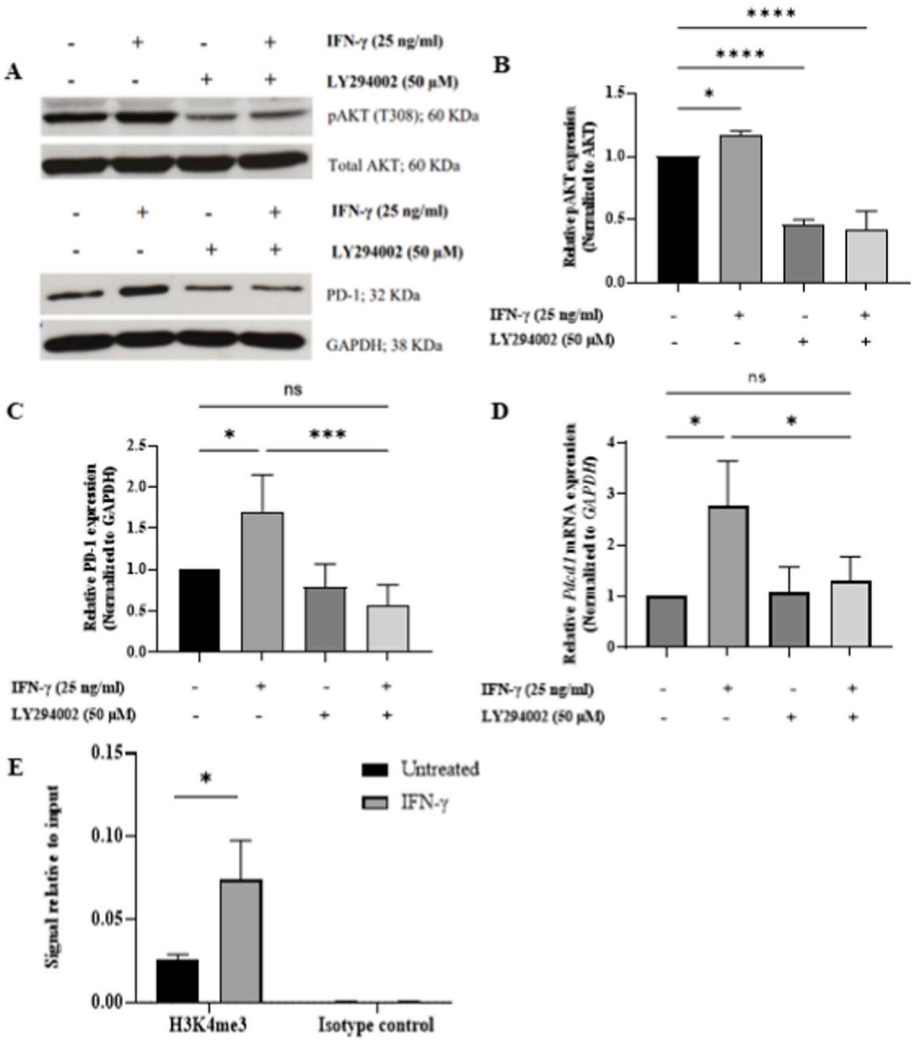

Fig. 2. IFN-γ-induced PD-1 upregulation is mediated via PI3K/AKT pathway and accompanied by histone trimethylation on H3K4 in THP-1 cell line.
(A–D) THP-1 was pretreated with LY294002 (50 μM) for 1 h followed by IFN-γ stimulation. The levels of AKT, phosphorylated AKT at threonine 308 (pAKT T308) and PD-1 were detected by Western blot after 2- and 48-hours stimulation, respectively. Representative Western blot (A) and the relative band density of pAKT normalized by total AKT (B) and the band density of PD-1 normalized to GAPDH (C) were shown. THP-1 was treated as described above and the level of *Pdcd1* mRNA was investigated by RT-qPCR (D). (E) Enrichment of H3K4me3-associated DNA fragment performed by ChIP assay in IFN-γ-treated THP-1 was quantitated by qPCR. Each bar represents a mean ± SD of triplicate independent experiments. *, *** and **** indicate statistically significant differences at p-value < 0.05, < 0.005 and <0.0001, respectively, and ns: not significance.

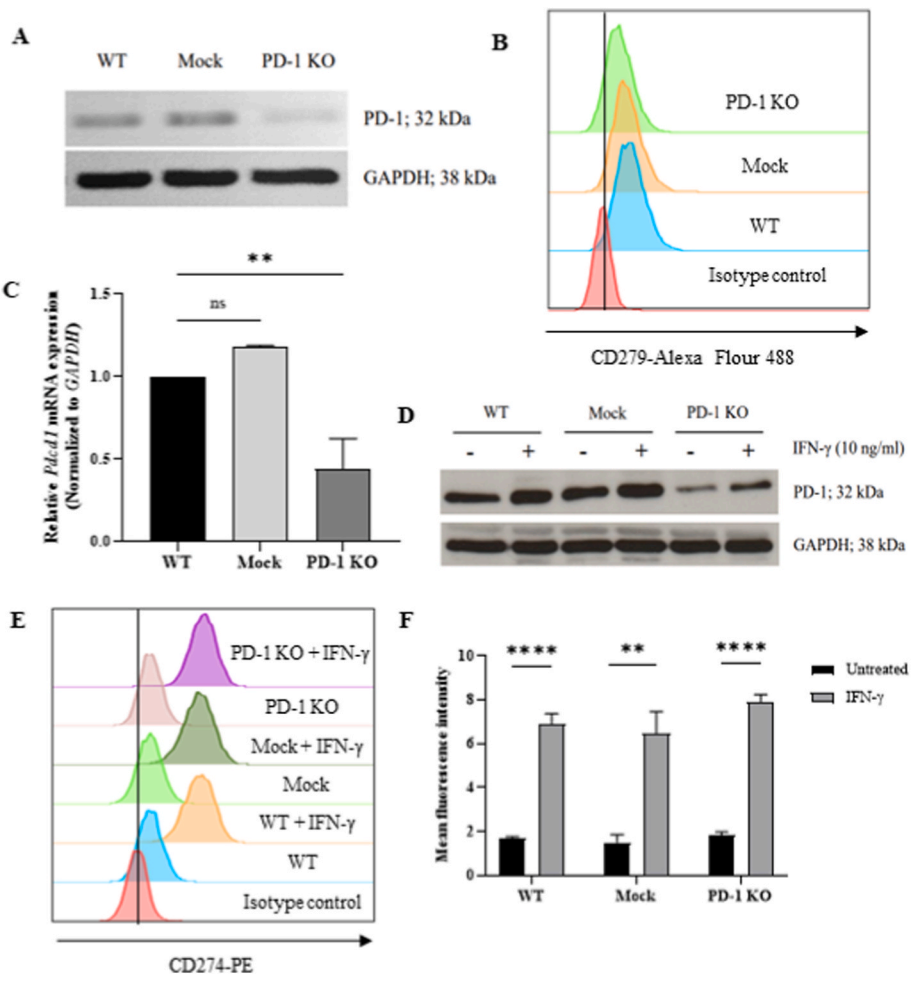

Fig. 3. Deletion of *Pdcd1* in THP-1 cell line.
(A–C) CRISPR/Cas9-mediated PD-1 knockout (KO) in THP-1 cell line was performed. PD-1 and *Pdcd1* mRNA expression in PD-1 KO THP-1 were observed by (A) Western blot (B) Flow cytometry and (C) RT-qPCR. (D) The PD-1 expression after IFN-γ treatment for 48 h in PD-1 KO THP-1 was also observed by Western blot. (E–F) PD-L1 (CD274) expression after IFN-γ treatment for 48 h in PD-1 KO THP-1 was observed by Flow cytometry (E) and calculated as mean fluorescence intensity (MFI) of CD274 expression (F). Each bar represents a mean ± SD of triplicate independent experiments. ** and **** indicate statistically significant difference at p-value < 0.0005 and <0.0001, respectively, and ns: not significance.

transduced line. As shown in Fig. S2, PD-1 deletion exhibited shorter doubling time than that of the parental or mock THP-1 cell line.

In this PD-1 deficient clone of THP-1 cell line, IFN-γ treatment only minimally induced PD-1 expression (Fig. 3D). Together with the nucleotide sequencing, the results implied that the deletion of PD-1 was successful (data not shown). Moreover, expression of PD-L1 was also examined after IFN-γ treatment by Flow cytometry. The results showed that the PD-L1 expression in THP-1 was upregulated after IFN-γ stimulation but the PD-L1 expression in IFN-γ-treated PD-1 KO THP-1 was not significantly different from the parental cell line (Fig. 3E and F) suggesting that PD-1 deficiency did not interfere with PD-L1 expression in THP-1 cell line.

3.6. PD-1 deficiency results in poor phagocytic activity in THP-1 cell line

Because PD-1 expression in TAMs was reported to affect phagocytic activity of TAMs, we thus investigated whether PD-1 deficient THP-1 cell line showed any defect in phagocytosis of carboxylated-modified latex beads under different stimulation conditions. As shown in Fig. 4A, the numbers of bead-ingested parental THP-1 and mock THP-1 cell line were significantly higher than the PD-1 KO THP-1 after IFN-γ and LPS stimulation while no differences were observed in THP-1 cell line treated with IFN-γ or LPS alone. Next, we asked whether deletion of PD-1 affects inflammatory cytokine production associated with LPS stimulation. When THP-1 cell line was stimulated with LPS, relative level of mRNA of two pro-inflammatory cytokines, *IL6* and *TNFA*, were not different between control WT THP-1 and PD-1 KO THP-1 cell lines (Fig. S3). These results suggested that PD-1 in monocytes participates in regulating phagocytic activities upon stimulation with IFN-γ and LPS.

3.7. PD-1 deficiency abrogates IL-4 induced polarization in THP-1 cell line

Next, we examined the effect of PD-1 expression on the M1/M2 polarization of the PD-1 KO THP-1 cell line. Expression of the representative M1 and M2 macrophage markers, CD86 and CD163, respectively, were measured by Flow cytometry after stimulation with IFN-γ and LPS (M1) or IL-4 (M2). The results showed that stimulation of THP-1 with IFN-γ and LPS increased CD86 expression regardless of PD-1 expression (Fig. 4B). However, stimulation with IL-4 clearly induced CD163 in parental wild-type THP-1, but not in PD-1 KO THP-1 (Fig. 4C). To examine whether the inability to upregulate CD163 by IL-4 stimulation was the result of decreased IL-4R expression, the surface expression of IL-4R was investigated and compared among THP-1 cell lines used. In addition, the relative level of IL4R mRNA was also measured. As shown in Fig. S4, no statistical differences were observed in both at the protein and mRNA level, indicating that deletion of PD-1 did not affect the level of IL-4R.

We examined whether PMA-induced differentiation of THP-1 to become macrophages affected PD-1 expression. As shown in Fig. 4D, PMA treatment significantly reduced PD-1 level. After resting for 24 h, cells were treated with IFN-γ/LPS or IL-4 and the expression of PD-1 was examined. Interestingly, PD-1 remained low upon stimulation either by IFN-γ/LPS or IL-4 (Fig. 4D).

To corroborate the findings using PD-1 KO THP-1 cell line, the approach using nivolumab to interfere with PD-1/PD-L1 interaction was

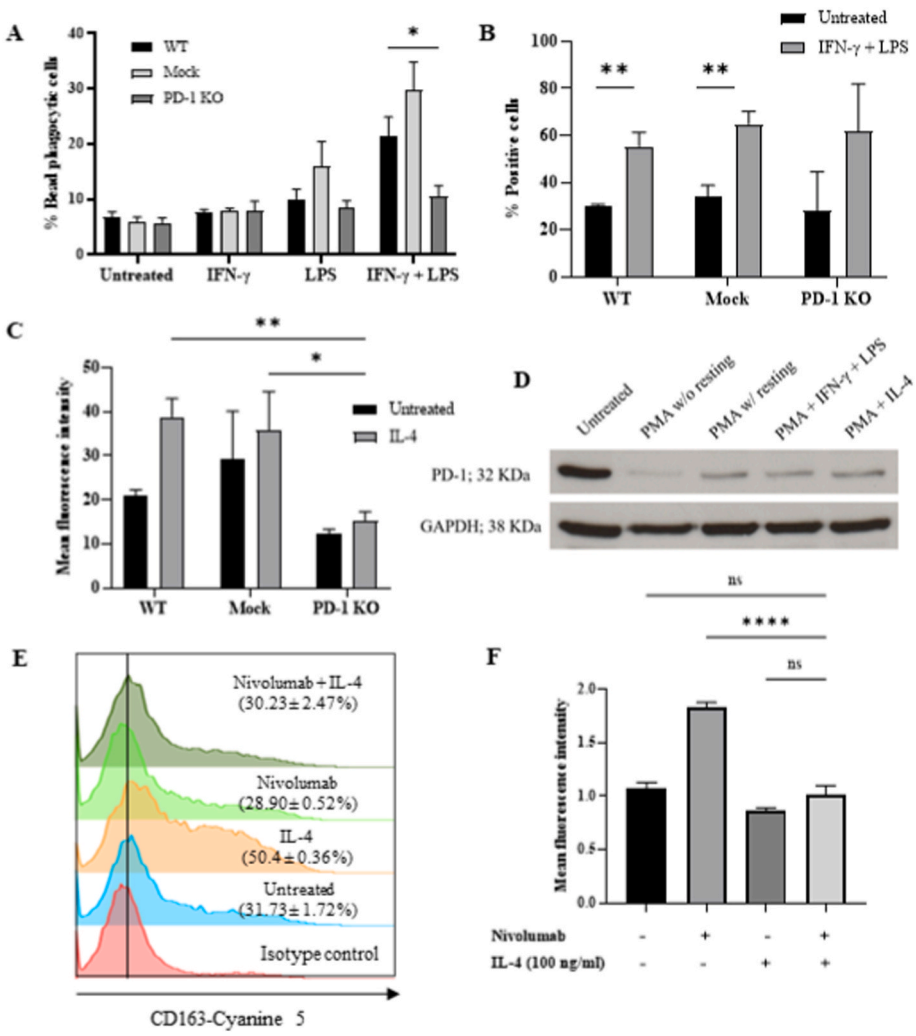

Fig. 4. Phagocytic activity and phenotypes of PD-1 KO THP-1 cell line. (A) Indicated THP-1 was stimulated with IFN-γ (25 ng/ml) or LPS (100 ng/ml) or IFN-γ (25 ng/ml) with LPS (100 ng/ml) for 24 h, and the activated cells were incubated with 2.0 μm FluoSpheres™ carboxylate-modified microspheres at 1:4 ratio for 2 h. Phagocytosis was quantitated by Flow cytometry. (B–C) Percentages of CD86 positive cells (B) and the mean fluorescent intensity (MFI) of CD163 (C) in THP-1 cell line measured by Flow cytometry. (D) Representative Western blot of PD-1 expression profile during PMA-induced THP-1 differentiation and subsequent treatment with IFN-γ/LPS or IL-4 for 24 h. (E–F) Representative Flow cytometry of CD163 expression in nivolumab-treated THP-1 cell line (E) and calculated as MFI of CD163 expression (F). Each bar represents a mean ± SD of triplicate independent experiments. *, ** and **** indicate statistically significant differences at p-value < 0.05, <0.01 and <0.0001, respectively, and ns: not significance.

employed. As shown in Fig. 4E and F, blocking of PD-1/PD-L1 interaction by nivolumab significantly abrogated CD163 upregulation upon IL-4 stimulation in THP-1 cell line. These results imply that PD-1 expression and PD-1/PD-L1 interaction may play a critical role in regulating CD163 expression in IL-4-treated THP-1 cell line. Thus, manipulating PD-1/PD-L1 signaling may have an impact on immune response such as in tumor and fibrosis where M2 polarization is crucial for disease outcome.

4. Discussion

Monocytes are important components in the mononuclear phagocytic system, playing diversely essential roles during acute infection, inflammation, and tissue repair [16]. Cytokines have been widely reported to be important regulatory factors on PD-1 expression in T cells and myeloid cells through several different transcription factors and signaling pathways [12,17]. The results of cytokine screening to identify one(s) that affects PD-1 expression in human monocytic cell line, THP-1, we found that TGF-β and IFN-γ significantly upregulated the expression of PD-1. TGF-β controls innate and adaptive immunity, including promoting Treg expansion, inhibiting effector T cell and dendritic cell functions [18]. Bao et al. (2021) reported that TGF-β1 enhanced PD-1 expression in T cells and abrogated T cell cytotoxicity in hepatocellular carcinoma in both *in vitro* and *in vivo* [19]. Moreover, it could induce PD-1 expression in tumor infiltrating T cells via Smad 3 transcription factor [20]. In our study, TGF-β upregulated approximately 2.5-fold increase in PD-1 expression, however, this effect was not observed in human primary monocytes. Whether or not TGF-β promotes PD-1 expression in human monocytes in other cellular context, which might be influenced by the surrounding environment and origin of the cells, remains unresolved.

IFN-γ is produced predominantly by Th1/cytotoxic T cells and NK cells in response to inflammatory stimuli and viral infection. Furthermore, it is also produced by other cell types such as monocytes/macrophages and cancer cells [21]. IFN-γ binds to its receptors and signals through the canonical JAK/STAT1 signaling pathway to activate IFN-γ-regulating gene transcription [22]. In addition, JAK1/2 activation by IFN-γ activates several other downstream pathways, including ERK1/2, JNK, p38 MAPK, and PI3K/AKT signaling cascades [23]. In this study, we found that the expression of PD-1 was statistically significant upregulated by IFN-γ over time and in a concentration-dependent manner. Inhibition of PI3K/AKT signaling abrogated PD-1 upregulation, indicating the central role of this pathway. Activation of PI3K/AKT pathway by IFN-γ is shown to be mediated by JAK kinase, which subsequently is responsible for STAT1 phosphorylation [24]. In murine macrophages, LPS/TLR4 stimulation via NF-κB p65 is responsible for PD-1 expression [25]. Thus, we proposed that in human monocytes, IFN-γ activates PI3K/AKT pathway, leading to STAT1 activation and the translation of *Pdcd1*.

Besides the transcriptional regulation by specific transcription factors, the epigenetic regulations of *Pdcd1* by DNA methylation and histone modifications are also well documents in T cells [17]. Two upstream conserved regulatory regions, −1.1 kb and −100 bp upstream

conserved regulatory regions, CR-B and CR-C, respectively, are associated with *Pdcd1* transcription which contain specific DNA binding sites for different transcription factors. Demethylation in the CR-C or CR-B and maintaining the low levels of repressive histone marks H3K9me3 and H3K27me3 during LCMV and HIV infection resulted in enhancing *Pdcd1* transcription in T cells [26,27]. However, to date, there was no report on the epigenetic regulation on *Pdcd*1 transcription in innate immune cells, especially in monocyte/macrophages. Our ChIP-qPCR data clearly revealed that active histone mark, H3K4me3, was found to be enriched on *Pdcd1* promoter surrounding the CR-C after stimulation of THP-1 by IFN-γ, indicating that histone modification was also responsible for enhancing the *Pdcd1* transcription in IFN-γ-treated THP-1. PI3K/AKT pathway is shown to increase H3K4 trimethylation in models of breast cancer by regulating subcellular localization of histone H3K4 demethylase [28]. Currently, the enzyme(s) targeted by PI3K/AKT pathway in IFN-γ-treated monocytes are under investigation.

Several studies showed that PD-1 expression is involved in suppressing innate inflammatory responses to sepsis [29] and myeloid cell-specific PD-1 deletion induced increased T effector memory cells with improved functionality, and mediated anti-tumor protection [30]. In macrophages, recent study reported that PD-1 blockade significantly increase phagocytic activity against *Mycobacterium tuberculosis* [31]. Expression of PD-1 in TAMs was also reported to be correlated negatively with phagocytic potency against tumor cells, and blockade of PD-1/PD-L1 axis *in vivo* significantly increased macrophage phagocytosis [9]. To investigate the biological functions of PD-1 in monocytes, we successfully generated PD-1 KO THP-1 cell line by CRISPR/Cas9 approach. Deletion of PD-1 in THP-1 cell line did not affect LPS-stimulated pro-inflammatory cytokine expression but showed slight reduction in the cell doubling time (Figs. S2 and S3). However, interestingly, our study revealed the contradictory results that PD-1 deficiency in THP-1 cell line resulted in poorer phagocytic activity against fluorescent latex beads, than the control THP-1 upon IFN-γ and LPS stimulation. Yao et al. (2014) also found that PD-1 deficiency in mouse bone marrow-derived macrophages (BMDMs) decreased phagocytic activity against carboxylate-modified red fluorescent latex beads [32] which was in line with our findings.

Our results suggest the possibility that PD-1 or the downstream signaling cascade regulates phagocytosis in monocytes. PD-1/PD-L1 may directly or indirectly interfere with phagocytic activity. For the direct regulation scenario, the signaling downstream of PD-1/PD-L1 may negatively regulate kinase(s) that negatively influences phagocytosis, such as the Src kinase family member, Fgr [33]. PD-1/PD-L1 triggers phosphatase activity that may suppress the activity of such kinase(s). For the potential indirect regulatory mechanism, blocking or suppression PD-1 expression might cause compensatory upregulation of the other immune checkpoint molecules in THP-1 cell line leading to an impaired phagocytic activity. Interestingly, Huang and co-workers (2017) reported that blockade of PD-1 resulted in increasing expression of other inhibitory receptors including LAG-3 and CTLA-4, while blockade of LAG-3 upregulated the PD-1 expression [34].

Lastly, the involvement of PD-1 during pro-inflammatory (M1) or anti-inflammatory (M2) responses was investigated [35]. Ruan and co-worker revealed that PD-1 expression in macrophages significantly inhibited polarization of M1 macrophages and phagocytosis [10]. Likewise, in the context of tumor, most PD-1^{+} TAMs were reported to exhibit M2-like phenotypes, while PD-1^{-}TAMs expressed M1-like phenotypes [9]. We found that there was no significant difference in the level of CD86, induced by IFN-γ and LPS with or without PD-1 expression. However, lacking PD-1 in THP-1 cell line significantly reduced polarization capability toward M2 state after stimulation with IL-4 using CD163, one of M2 marker, which was consistent with previous reports [9,36]. Importantly, no difference on the level of IL-4R expression was found between PD-1 KO THP-1 and control THP-1, indicating that the inability to polarize to be M2 in PD-1 KO THP-1 was derived from signaling downstream of IL-4/IL-4R (Fig. S4).

In our study, PMA-treated THP-1 was used as a model for studying macrophage polarization to M1/M2. In contrast to undifferentiated THP-1, PMA treatment resulted in drastic decrease in PD-1 level and M1 (LPS/IFN-γ) or M2 (IL-4) stimulation did not rescue this down-regulation. Paradoxically, we found that CD163 upregulation as a marker of M2 polarization was severely impaired when PD-1 was deleted or PD-1/PD-L1 was blocked by nivolumab. Even when PD-1 was not upregulated, it was reported that IL-4 could induce PD-L1 upregulation in human monocytes [37,38]. Thus, we speculated that optimal M2 polarization induced by IL-4 might require PD-1/PD-L1 signaling. In parental PMA-treated THP-1, stimulation with IL-4 might trigger PD-1/PD-L1 signaling by upregulation of PD-L1, leading to enhance M2 polarization. In contrast, blocking PD-1/PD-L1 or lacking PD-1 in PMA-treated THP-1 could hamper this signaling pathways, resulting in poorer M2 polarization. Therefore, these results suggested that PD-1 expression might play a role in macrophage polarization toward M2 but was not responsible for M1 polarization.

In conclusion, this study provides the novel mechanisms underlying the PD-1 expression in IFN-γ-treated human monocytic cell line THP-1 and some biological functions of PD-1 in THP-1 cell line as depicted in Fig. 5. The biological function of PD-1 in IFN-γ-treated monocytes warrants further investigation that may be beneficial for better treatment of diseases associated with phagocytosis and wound healing.

Author contributions

KS is responsible for conceptualization, data curation, investigation, and methodology. PK assisted on methodology. TPa is responsible for conceptualization, funding acquisition, project administration and overall project supervision. TPi provided reagents and experimental design. TPa and KS were in charge of manuscript original draft and editing.

Declaration of competing interest

The authors declare that they have no known competing financial

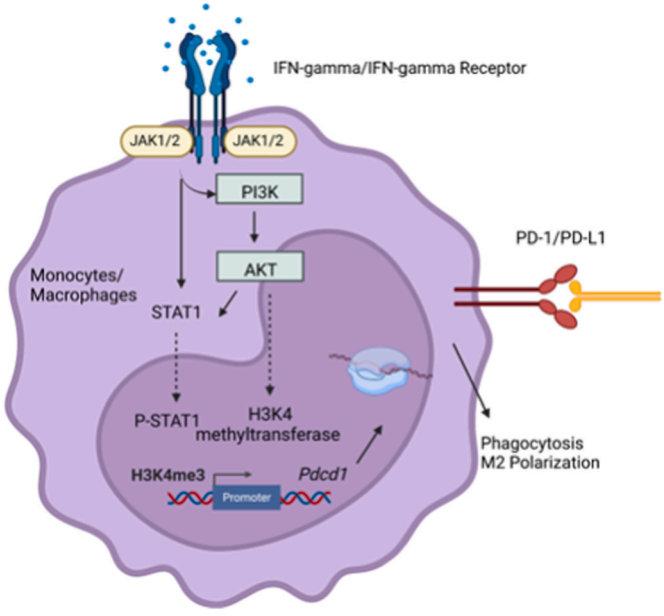

Fig. 5. A proposed model depicting the role of IFN-γ in inducing PD-1 expression in monocytes.
In human monocytes, IFN-γ triggers activation of PI3K/AKT pathway and STAT1 that is responsible for transcription of *Pdcd1* and accumulation of active histone mark, H3K4me3 by H3K4 methyltransferase. PD-1/PD-L1, in turn, regulates phagocytosis and M2 polarization. Created with BioRender.com.

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2022.101369.

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