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Interferon-γ upregulates Δ42PD1 expression on human monocytes via the PI3K/AKT pathway

Lin Cheng[a,b], Xian Tang[b], Liumei Xu[b], Lukun Zhang[b], Huichun Shi[b], Qiaoli Peng[b], Fang Zhao[b], Yang Zhou[b], Yun He[b], Hui Wang[b], Boping Zhou[b], Zhiliang Gao[a,*], Zhiwei Chen[b,c,**]

[a] *Department of Infectious Diseases, the Third Affiliated Hospital of Sun Yat-sen University, Guangzhou, Guangdong, China*
[b] *HKU-AIDS Institute Shenzhen Research Laboratory, Shenzhen Key Laboratory of Infection and Immunity, Guangdong Key Laboratory of Emerging Infectious Diseases, Shenzhen Third People's Hospital, Shenzhen, China*
[c] *AIDS Institute, Research Center for Infection and Immunity, Department of Microbiology, Li Ka Shing Faculty of Medicine, University of Hong Kong, Hong Kong SAR, China*

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ABSTRACT

Background: We recently identified a novel alternatively spliced isoform of human programmed cell death 1 (PD-1), named Δ42PD1, which contains a 42-base-pair in-frame deletion compared with the full-length PD-1. Δ42PD1 is likely constitutively expressed on human monocytes and down-regulated in patients infected with human immunodeficiency virus type 1 (HIV-1). The mechanism underlying the regulation of Δ42PD-1 expression in monocytes remains unknown.

Methods: By flow cytometry, we investigated the effect of Interferon-gamma (INF-γ) on the expression of Δ42PD1 in primary human monocytes as well as monocytic cell lines THP-1 and U937 cells. In addition, signaling pathway inhibitors and Δ42PD1-specific blocking antibody were used to explore the pathway involved in INF-γ-induced Δ42PD1 upregulation, and to elucidate the relationship between Δ42PD1 and TNF-α or IL-6 production by INF-γ primed monocytes in response to pre-fixed *E. coli*. Furthermore, we assessed T-cell proliferation, activation and cytokine production as enriched $CD4^+$ T cells were co-cultured with THP-1 or U937 cells, with or without Δ42PD1-blocking antibody.

Results: Treatment of human peripheral blood mononuclear cells (PBMCs) with IFN-γ resulted in an approximately 4-fold increase in the expression of Δ42PD1 on monocytes. Similarly, IFN-γ upregulates Δ42PD1 expression on human monocytic cell lines THP-1 and U937, in a time- and dose-dependent manner. IFN-γ-induced Δ42PD1 upregulation was abolished by JAK inhibitors Ruxolitinib and Tasocitinib, PI3K inhibitor LY294002, and AKT inhibitor MK-2206, respectively, but not by STAT1 inhibitor and MAPK signaling pathway inhibitors. JAK, PI3K-AKT, and MAPK signaling inhibitors abolished effectively the production of TNF-α and IL-6 in INF-γ-primed monocytes in response to pre-fixed *E. coli*. In contrast, Δ42PD1-specific blocking antibody did not affect the IFN-γ-induced priming effect. Furthermore, the MFI ratio of Δ42PD1 to full-length PD-1 (PD-1 Δ/F ratio) was significantly and positively correlated with TNF-α ($P = 0.0289$, $r = 0.6038$) produced by circulating $CD14^+$ monocytes in response to pre-fixed *E. coli*. Notably, Δ42PD1 blockage significantly inhibited $CD4^+$ T-cells proliferation and cytokine production in the co-culture conditions.

Conclusions: We demonstrated that IFN-γ increases Δ42PD1 expression on human monocytes via activating the PI3K/AKT signaling pathway downstream of JAKs, and that the PD-1 Δ/F ratio is a potential biomarker to predict the functional state of monocytes. Notably, we revealed the Δ42PD1 play a role in T-cell regulation, providing a novel potential approach to manipulate adaptive immune response.

1. Introduction

The cell surface inhibitory co-stimulatory receptor PD-1 (also known as CD279) inhibits excessive T cell and B cell proliferation and differentiation (Boussiotis, 2016; Okazaki et al., 2001) through interaction with its two native ligands PD-L1 (B7-H1 or CD274) and PD-L2

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[*] Corresponding author.
[**] Corresponding author at: HKU-AIDS Institute Shenzhen Research Laboratory, Shenzhen Key Laboratory of Infection and Immunity, Guangdong Key Laboratory of Emerging Infectious Diseases, Shenzhen Third People's Hospital, Shenzhen, China.

E-mail addresses: gaozhl@mail.sysu.edu.cn (Z. Gao), zchenai@hku.hk (Z. Chen).

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(B7-H2 or CD273) (Freeman et al., 2000; Latchman et al., 2001), playing a vital role in the maintenance of peripheral tolerance (Francisco et al., 2010; Yogev et al., 2012). In contrast to the beneficial effects of the PD-1/PD-Ls pathway in maintaining peripheral tolerance, over-expression of PD-1 by tumor- or virus-specific T cells prevents T-cell expansion and function, and promotes tumorigenesis (Iwai et al., 2002) and chronic viral infections (Day et al., 2006). Therapies targeting the PD-1/PD-Ls pathway with blocking antibodies have been shown to be associated with remarkable response rates in various tumors and viral infections (Herbst et al., 2014; Grabmeier-Pfistershammer et al., 2017; Barber et al., 2006).

We previously reported a novel alternative splice variant of the human PD-1 gene, termed Δ42PD1, generated by alternative splicing where 42 base pairs of exon 2 (extracellular IgV-like domain) is spliced out (Zhou et al., 2013). The 42-basepair in-frame deletion of PD-1 results in a conformational change of the extracellular domain. Therefore, Δ42PD1 is not engaged with PD-L1 or PD-L2, and is not recognized by several monoclonal antibodies specific to PD-1. Interestingly, an extracellular domain of Δ42PD1 fusion DNA vaccine amplifies the level of antigen-specific $CD8^+$ T cell immunity in vivo (Zhou et al., 2013), demonstrating an immunoregulatory function of Δ42PD1. The extracellular domain of Δ42PD1 and cellular surface Δ42PD1 could induce the upregulation of IL-1β, IL-6, and TNF-α in human PBMCs and dendritic cells. Moreover, the expression of Δ42PD1 is up-regulated on a subset of gut-homing γδ T cells in the acute phase of HIV-1 infection, promoting innate mucosal damage (Cheung et al., 2017).

Using home-made anti-Δ42PD1 monoclonal antibodies, we found by flow cytometry that Δ42PD1 is likely expressed on monocytes constitutively (Cheng et al., 2015). Since PD-1 is upregulated during T cell activation, we hypothesized that the IFN-γ, a potent monocytes activator, (Estaquier and Ameisen, 1997; Docke et al., 1997; Zhu et al., 1993) could modulate the expression of Δ42PD1. To test this hypothesis, we investigated the effects of IFN-γ on the expression of Δ42PD1 on human peripheral blood primary monocytes and two monocytic cell lines THP-1 and U937. We show that IFN-γ upregulates the expression of Δ42PD1 on monocytes via the activation of PI3K (phosphatidylinositol 3-kinase)/AKT (protein kinase B) pathway, and that PD-1 Δ/F ratio is a potential biomarker to predict the functional state of monocytes.

2. Materials and methods

2.1. Cell isolation and culture

Human PBMCs were isolated by density gradient centrifugation using Ficoll-paque premium (GE Healthcare, WI, USA) from whole blood of healthy donors. Blood samples were collected with the approval of the Institutional Review Board of Shenzhen Third People's Hospital. Briefly, whole blood was diluted 1:4 with sterile PBS and centrifuged at 400 × g for 30 min without a brake. The isolated PBMCs were washed twice with PBS at 200 × g for 10 min. After washing, cells were counted and resuspended for culture. $CD14^+$ monocytes and $CD4^+$ T cells were positively or negatively isolated using CD14 MicroBeads and $CD4^+$ T Cell Isolation Kit, respectively (Miltenyi Biotec, Germany), according to the manufacturer's instruction.

PBMCs, monocytes, THP-1 cells (ATCC), and U937 cells (ATCC) were maintained in RPMI-1640 medium (Gibco, UK) supplemented with 10% fetal bovine serum (Gibco, UK), containing penicillin, streptomycin and L-glutamine. Primary $CD4^+$ T cells also received 10 U/ml rhIL-2 (PeproTech, NJ, USA). Co-cultures were performed by adding 1×10^5 THP-1 or U937 cells followed by 2×10^5 of $CD4^+$ T cells per well in round-bottom 96-well plate. For the blocking assays, 5 μg/ml Δ42PD1-blocking antibody (Clone CH101, homemade) (Cheng et al., 2015) or irrelevant mouse IgG1 (LEAF Purified IgG1, clone MODC21, BioLegend, CA, USA) was added to the culture. All above-mentioned cells were maintained in a 37 °C humidified incubator in an atmosphere of 5% CO2 - 95% air.

2.2. Reagents and chemicals

Recombinant human IL-2 and IFN-γ were purchased from PeproTech (NJ, USA). (3R,4R)-4-Methyl-3-(methyl-7H-pyrrolo[2,3-d]pyrimidin-4-ylamino)-β-oxo-1-piperidinepro panenitrile citrate (Tasocitinib) and (*R*)-3-(4-(7H-Pyrrolo[2,3-d]pyrimidin-4-yl)-1H-pyrazol-1-yl)-3-cyclopentylpr opanenitrile phosphate (Ruxolitinib) were purchased from Gene Operation (USA). (2R,3S,4S,5R)-2-(6-amino-2-fluoro-9H-purin-9-yl)-5-(hydroxymethyl)-tetrahydrofuran-3,4-diol (Fludarabine), 2-hydroxy-4-(2-(tosyloxy)acetamido)benzoic acid (S3I-201), 4-[[(4-cyclohexylphenyl) methyl][2-[methyl[(2,3,4,5,6-pentafluorophenyl)sulfonyl]amino] acetyl]amino]-benzoic acid (SH-4-54), 2-morpholino-8-phenyl-4H-chromen-4-one (LY294002), 8-(4-(1-aminocyclobutyl)phenyl)-9-phenyl-[1,2,4]triazolo[3,4-f][1,6]naphthyridin-3(2 H)-one (MK-2206), 2,3-bis (amino(2-aminophenylthio)methylene)succinonitrile, ethanol (U0126), 2H-Dibenzo[cd,g]indazol-6-one (SP600125) and 4-(4-(4-fluorophenyl)-2-(4-(methylsulfinyl)phenyl)-1H-imidazol-5-yl) pyridine (SB203580) were from Selleckchem (TX, USA).

2.3. Flow cytometry and antibodies

For surface staining, cells were resuspended and incubated with pre-cooled FACS buffer (PBS with 2% FBS and 0.1% NaN_3), containing fluorescein-conjugated antibodies for 30 min at 4 °C, followed by extensive washing.

For intracellular cytokine staining, cells were firstly stained with surface markers. After washing, cells were fixed and permeabilized using Fixation/Permeabilization Solution Kit (BD Biosciences, CA, USA), and then stained with cytokine-specific antibodies using the above-mentioned method. Data were acquired with FACSCalibur Flow Cytometer (Becton Dickinson, NJ, USA), and analyzed using FlowJo software (Tree Star V10, OR, USA).

Mouse anti-Human PD-1 (Clone EH12.1), PE-Mouse IgG1 (Clone MOPC-21), PerCP-Cy5.5 Mouse Anti-Human CD25 (Clone M-A251), FITC Mouse Anti-Human CD69 (Clone FN50) were purchased from BD Biosciences (CA, USA); PE/Cy7 anti-human CD14 Antibody (Clone 63D3), PE anti-human CD119 (IFN-γ R α chain) Antibody (Clone GIR-208), Alexa Fluor 488 anti-human IFN-γ Antibody (Clone 4S.B3), APC anti-human TNF-α Antibody (Clone Mab11), and Alexa Fluor 647-Mouse IgG1 (Clone MOPC-21) were purchased from BioLegend (CA, USA); Alexa Fluor 647-Mouse anti-Human Δ42PD1 (Clone CH101) was homemade (Cheng et al., 2015).

2.4. Western blot

THP-1 or U937 cells (1×10^6 cells/well) in 24-well plate were either untreated or treated with Janus kinase inhibitors (Ruxolitinib or Tasocitinib, 10 μM) or solvent of inhibitors (Dimethyl Sulphoxide, DMSO) for 1 h, followed by stimulation with recombinant human IFN-γ (100 ng/ml) for 10 min. Then cells were harvested and lysed with RIPA Lysis and Extraction Buffer containing protease inhibitor cocktail and Phosphatase Inhibitor Cocktail (Pierce, Thermo Fisher Scientific, MA, USA). Western blot analysis was performed as described previously (Cheng et al., 2015) and the bands were visualized using ECL Western Blotting Substrate (Pierce) by enhanced chemiluminescence. Specific antibodies used in immunoblot analysis including anti-β-Actin (Clone 13E5) and anti-p-STAT1 (Tyr701, Clone 58D6) purchased from Cell Signaling Technology (MA, USA), and anti-rabbit IgG-HRP purchased from Promega (WI, USA).

2.5. Cell apoptosis assay

Apoptosis and necrosis in THP-1 and U937 cells treated with various inhibitors were determined using the FITC Annexin V/PI Apoptosis Detection Kit (Cat.No. 640914, BioLegend, CA, USA) according to the manufacturer's protocol. Briefly, 2×10^5 cells were seeded in 48-well

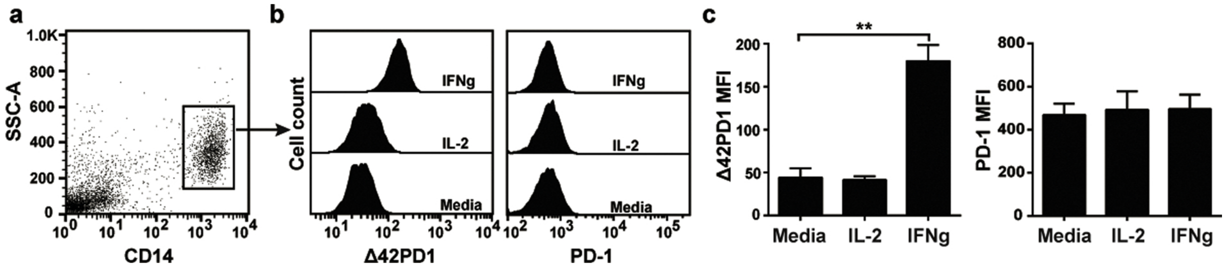

Fig. 1. Flowcytometric analysis of Δ42PD1 expression on human primary monocytes upon IFN-γ treatment.
(a) Gating strategies of monocyte subsets in human PBMCs according to CD14 expression. **(b–c)** Representative flow cytometric histogram **(b)** and bar charts **(c)** showing the expression of PD-1 and Δ42PD1 on $CD14^+$ monocytes among PBMCs from three healthy donors, following stimulation without (Media) or with indicated cytokines (50 ng/ml) for 48 h. MFI, mean fluorescence intensity. $^{**}P < 0.01$; paired *t*-test.

culture plates and treated with a series of inhibitors (0 μM, 0.01 μM, 0.1 μM, 1 μM, and 10 μM, respectively) for 48 h. Then, cells were harvested and stained with Annexin V and Propidium Iodide for 15 min at room temperature in the dark and subsequently analyzed via flow cytometry.

2.6. *Signaling inhibition assay*

THP-1 and U937 cells in 48-well culture plates (2×10^5 cells/well) were pre-treated with various inhibitors for 1 h, followed by stimulation or not with IFN-γ (100 ng/mL) for 48 h. Then Δ42PD1 expression was determined by flow cytometry. Percentage of inhibition of IFN-γ-induced upregulation of Δ42PD1 was calculated by the formula: 100% × ($MFI_{inhibitor}$ - MFI_{media}) / ($MFI_{INF\text{-}\gamma}$ - MFI_{media}). $MFI_{inhibitor}$, MFI_{media}, and $MFI_{INF\text{-}\gamma}$ were defined as Δ42PD1 MFI values of inhibitor pre-treated and INF-γ stimulated, DMSO pre-treated and non-stimulated, DMSO pre-treated and INF-γ stimulated, respectively.

2.7. *In vitro Stimulation with pre-fixed E. coli*

The *E. coli* strain Top10 was cultured overnight to late stationary phase at 37 °C in LB broth. Live bacteria were counted by the standard plate-counting method, and counts were expressed as CFU per milliliter. Live *E. coli* was divided in aliquots in 50% glycerol/50% FBS and stored at −80 °C until needed for functional assays. To pre-fix *E. coli* for functional assay, *E. coli* was thawed in ice-water and washed twice in PBS, fixed with 1% Paraformaldehyde for 3 min and then extensively washed and resuspended in PBS. In co-culture experiments, pre-fixed *E. coli* was added to 2×10^5 THP-1 cells, U937 cells (1 MOI) or freshly isolated human $CD14^+$ monocytes (0.5 MOI) in 48-well plates for 8 h. Then cell-culture supernatants were collected for determination of cytokine content.

2.8. *Quantification of cytokine*

THP-1 (1×10^5 cells/well) or U937 (0.5×10^5 cells/well) were seeded in 48-well plate and pre-stimulated or not with INF-γ (100 ng/mL) plus 10 μM/1 μM signaling inhibitors or 10 μg/mL mouse IgG / Δ42PD1 blocking antibody for 48 h, followed by adding pre-fixed *E. coli* (1 MOI) and culturing for further 8 h. Then cell-culture supernatants were collected for measurement of TNF-α and IL-6 by Human TNF-alpha DuoSet ELISA and Human IL-6 DuoSet ELISA (R&D Systems, Bio-Techne, MN, USA), respectively, according to the manufacturer's protocol.

2.9. *Statistics*

All quantitative data were presented as mean ± SEM of at least three independent experiments unless indicated in the figure legend. We applied paired two-tailed Student's *t*-test to calculate *P* values of paired samples, and nonparametric Mann-Whitney *U*-test for comparison of two groups. A p value < 0.05 was considered statistically significant. Statistical analysis was carried out using Prism 6 (GraphPad Software, CA, USA).

3. Results

3.1. *INF-γ upregulates Δ42PD1 expression on primary human monocytes*

Activation of T cells induces a parallel increase in the transcription of PD-1 alternative splice variants (Nielsen et al., 2005). PD-1 expression on monocytes is upregulated after activation (Said et al., 2010). We, therefore, speculate that the expression of Δ42PD1 on monocyte (Cheng et al., 2015) will be regulated by cytokines released by activated T cells. To test this hypothesis, we stimulated freshly isolated human PBMCs with IL-2 or INF-γ, and examined the expression of PD-1 and Δ42PD1 on $CD14^+$ monocytes by flow cytometry (Fig. 1a). INF-γ is known as a potent activating mediator of monocyte behavior (Dallagi et al., 2015; Espinoza-Delgado et al., 1995). Indeed, the Δ42PD1 level was significantly increased (~4-fold increase, $P =$ 0.0034) by INF-γ but not by IL-2 (Fig. 1**b** and **c**). In contrast, INF-γ did not significantly alter PD-1 expression (Fig. 1**b** and **c**). IL-2 did not affect the expression of PD-1 or Δ42PD1 significantly. These results indicated that upon INF-γ stimulation, human primary monocytes upregulate selectively the expression of Δ42PD1 but not of PD-1.

3.2. *INF-γ upregulates Δ42PD1 expression on human monocytic cell lines*

To confirm our findings, we further tested the responsiveness of human monocytic cell lines THP-1 and U937 cells to IFN-γ. We firstly determined the expression of CD119, the alpha chain of IFN-γ receptor, on primary monocytes and monocytic cell lines by flow cytometry. Primary $CD14^+$ monocytes constitutively express CD119 (Fig. 2a), consistent with a previous report (Delneste et al., 2003). Likewise, THP-1 and U937 monocytic cells also express the IFN-γ receptor constitutively (Fig. 2b). We then examined IFN-γ-triggered signaling in THP-1 and U937 monocytic cells by immunoblotting. The phosphorylation of STAT1, signal transducer and activator of transcription, occurred upon IFN-γ stimulation in both human monocytic cell lines. This process, however, was suppressed by pre-treatment with Janus kinase inhibitors Ruxolitinib and Tasocitinib (Fig. 2c). Collectively, these data suggested that monocytic THP-1 and U937 cell lines could be a useful model to study the effect of INF-γ on the expression of Δ42PD1.

We subsequently determine if Δ42PD1 is expressed and upregulated in response to IFN-γ on THP-1 and U937 cell lines. Expression of Δ42PD1 and PD-1 on THP-1 and U937 cells with or without stimulation with IFN-γ or IL-2 was analyzed by flow cytometry. Inconsistency with primary human $CD14^+$ monocytes, THP-1 and U937 cells expressed Δ42PD1 (Fig. 3a). The expression was increased by 3-fold and 6-fold (P $=$ 0.0001 and P $=$ 0.0021) for THP-1 and U937 cells, respectively, upon IFN-γ stimulation (Fig. 3**b** and **c**). IL-2, consistent with the above results, did not alter Δ42PD1 level on THP-1 and U937 cells. Of note,

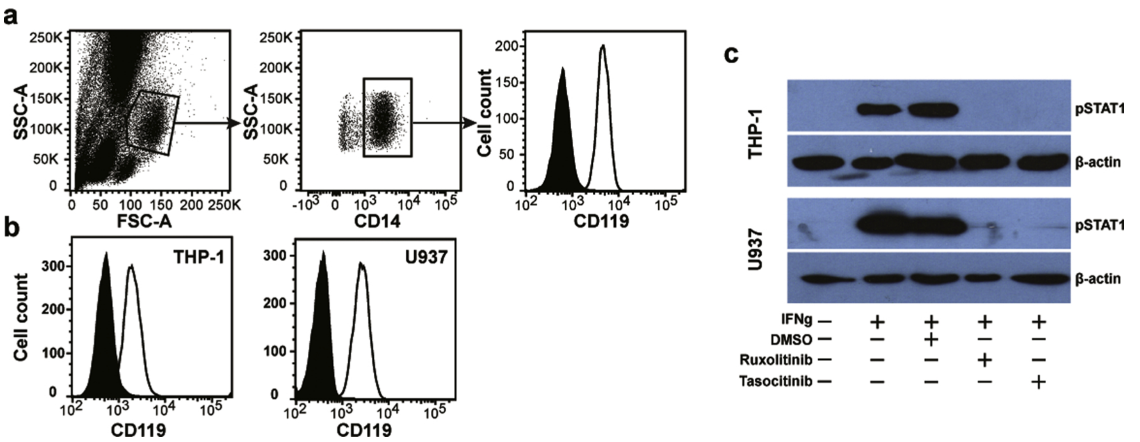

Fig. 2. The potential responsive capacity of THP-1 and U937 cells to IFN-γ.
(a–b) Representative flow cytometric histogram of CD119 expression on human CD14$^+$ monocytes among peripheral white blood cells from healthy donors (n = 3) **(a)**, and on monocytic cell lines THP-1 and U937 **(b)**. **(c)** THP-1 and U937 cells were pre-treatment with or without JAK inhibitors (Ruxolitinib or Tasocitinib), then stimulated with or without INF-γ for 10 min, followed by analysis of the pSTAT1 and β-actin by Western blot.

we did not detect PD-1 on the surface of THP-1 and U937 cells (Fig. 3a).

To further explore the effect of IFN-γ on Δ42PD1 expression, we stimulated THP-1 and U937 cells with various concentrations of IFN-γ for 48 h, and then analyzed the expression of Δ42PD1 and PD-1. In both THP-1 and U937 cells, IFN-γ induced a dose-dependent increase in Δ42PD1 expression with the detectable upregulation of Δ42PD1 at the low concentration of 1 ng/ml (~1.2-fold increase on THP-1, ~2.1-fold increase on U937), and the maximal effect was observed at the high concentration of 1000 ng/ml (~2.8-fold increase on THP-1, ~5.9-fold increase on U937). In contrast, the PD-1 expression was not affected on U937 cells by IFN-γ of all concentrations tested, and was moderately elevated (~1.3-fold) on THP-1 cells by high concentrations of IFN-γ (Fig. 3d). We also examined the expression of Δ42PD1 and PD-1 on THP-1 and U937 cells upon IFN-γ treatment at different time-points. Δ42PD1 on THP-1 was rapidly enhanced from 6 h to 48 h, then increased slowly up to 96 h. Δ42PD1 level on U937 cells was increased from 3 h to 24 h (~7.5-fold increase) upon IFN-γ stimulation. Consistently, the expression of PD-1 on U937 cells was not affected at all-time points and began to increase slightly on THP-1 cells after 48 h of IFN-γ stimulation (Fig. 3e).

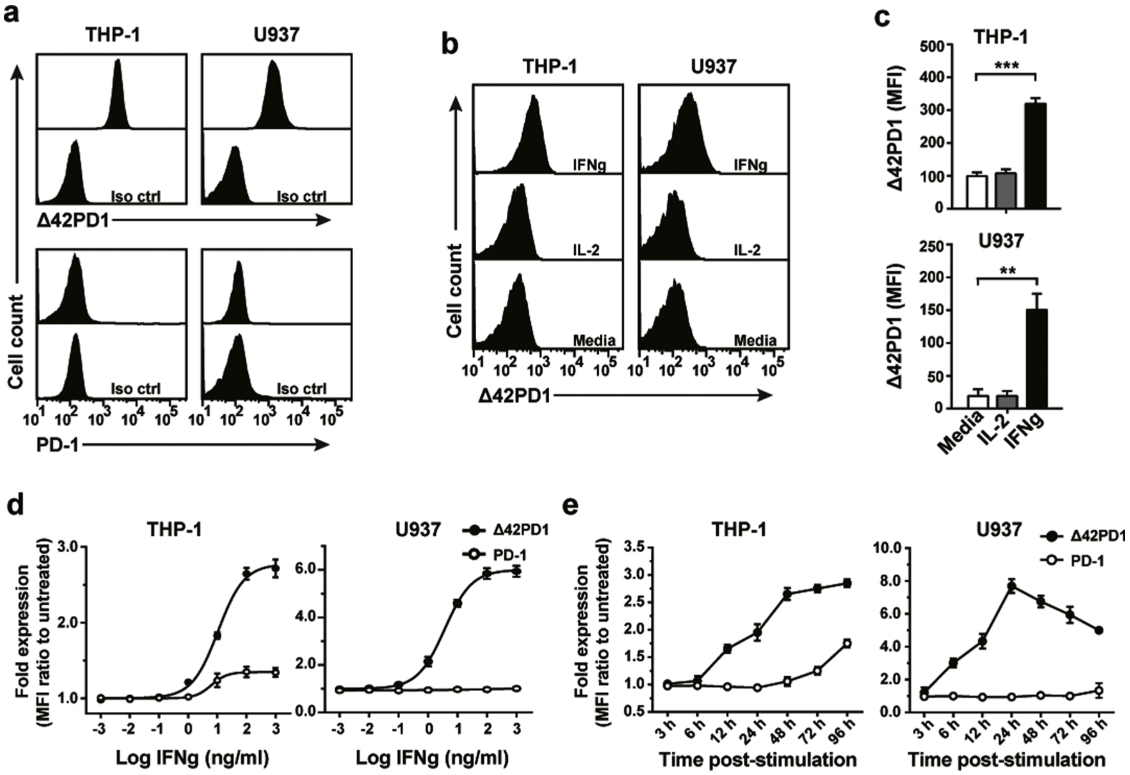

Fig. 3. Characterization of Δ42PD1 expression on THP-1 and U937.
(a) Representative flowcytometric histogram showing the expression of Δ42PD1 and PD-1 on THP-1 and U937 cells (one of three experiments is shown). **(b–c)** Representative flowcytometric histogram **(b)** and bar charts **(c)** showing Δ42PD1 expression on THP-1 and U937 cells treated without (Media) or with indicated cytokines (100 ng/ml) for 48 h. **(d–e)** Fold expression of Δ42PD1 and PD-1 on THP-1 and U937 cells following treated with indicated concentration of INF-γ for 48 h **(d)**, or with 100 ng/ml human INF-γ for indicated time-points **(e)**, determined by flow cytometry. **(c–e)** Data were presented as mean ± SEM from 3 independent experiments. **$P < 0.01$, ***$P < 0.001$; paired *t*-test.

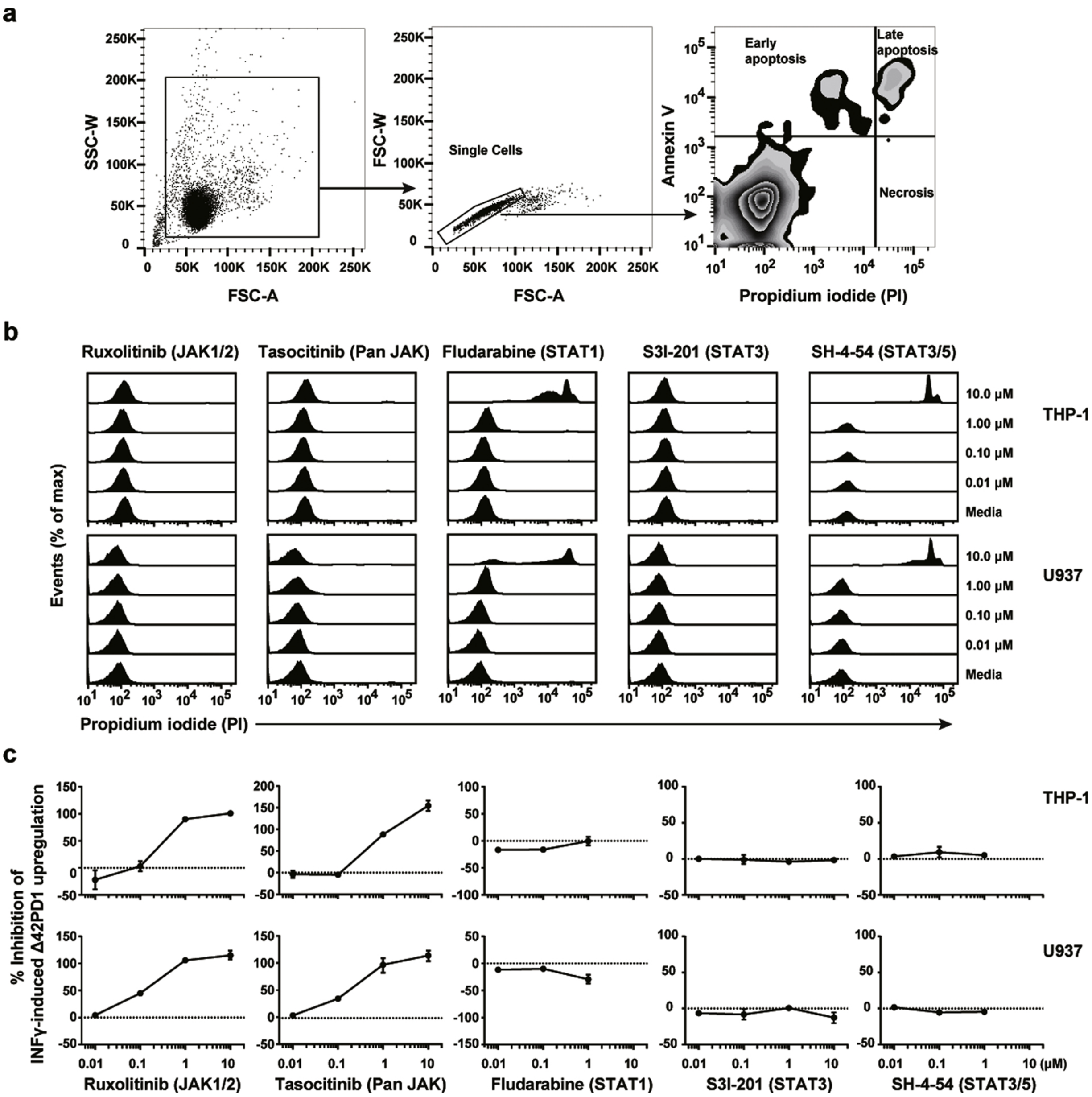

Fig. 4. Effect of JAK/STAT signaling inhibitors on Δ42PD1 expression.
(a) Flow chart of flow cytometric analysis of apoptotic and necrotic cells. **(b)** Analysis of apoptosis in THP-1 and U937 cells following treatment with JAK/STAT inhibitors (Ruxolitinib is a JAK1 and JAK2 inhibitor, Tasocitinib inhibits all four JAK isoforms, Fludarabine is a STAT1 inhibitor, S3I-201 is a STAT3 inhibitor, and SH-4-54 is a STAT3 and STAT5 inhibitor) for 48 h with indicated concentrations. **(c)** THP-1 and U937 cells were pre-treated with indicated JAK/STAT inhibitors and doses for 1 h, followed by stimulation or not with IFN-γ (100 ng/mL) for 48 h. Then Δ42PD1 expression was determined by flow cytometry. And the percentage of inhibition of IFN-γ-induced upregulation of Δ42PD1 was calculated. Data were presented as mean ± SEM from three independent experiments.

3.3. PI3K/AKT signaling pathway contributes to IFN-γ-induced upregulation of Δ42PD1

IFN-γ interacts with a membrane surface receptor, which is composed of two subunits, and associated with JAK1 (Janus activated kinase 1) and JAK2. Classically, activation of JAKs by IFN-γ results in phosphorylation and homodimerization of STAT1 that translocates to the nucleus and initiates gene transcription (Platanias, 2005). We, therefore, investigated if JAK/STAT1 signaling inhibitors could reverse IFN-γ-induced Δ42PD1 upregulation. We first conducted an apoptosis assay using flow cytometry to assess the cytotoxicity of selected inhibitors (including JAK1/2 inhibitor Ruxolitinib, pan-JAK inhibitor Tasocitinib, STAT1-specific inhibitor Fludarabine, STAT3/5 inhibitors S3I-201 and SH-4-54) to THP-1 and U937 cells. No cytotoxicity was detected for all inhibitors when the dosage was less than 1 μM. As a control, most cells underwent apoptosis after exposure to 10 μM Fludarabine or SH-4-54 (Fig. 4**a** and **b**). As expected, IFN-γ-induced Δ42PD1 increase was not inhibited by STAT3/5 inhibitors, and was completely inhibited by JAK1/2 or pan-JAK inhibitors, in a dose-dependent manner, with almost complete inhibition achieved at the dosage of 1 μM for both inhibitors. Since Fludarabine (a STAT1-specific inhibitor) did not block IFN-γ-induced Δ42PD1 upregulation (Fig. 4c), our results suggested STAT1-independent signaling pathway(s) is involved in Δ42PD1 increase upon INF-γ stimulation.

Besides the STAT1 signaling pathways, activation of JAK1/2 tyrosine kinase by IFN-γ seems to regulate several other downstream signaling cascades, such as PI3K (Blahoianu et al., 2014) and mitogen-activated protein kinase pathways (Matsuzawa et al., 2012;

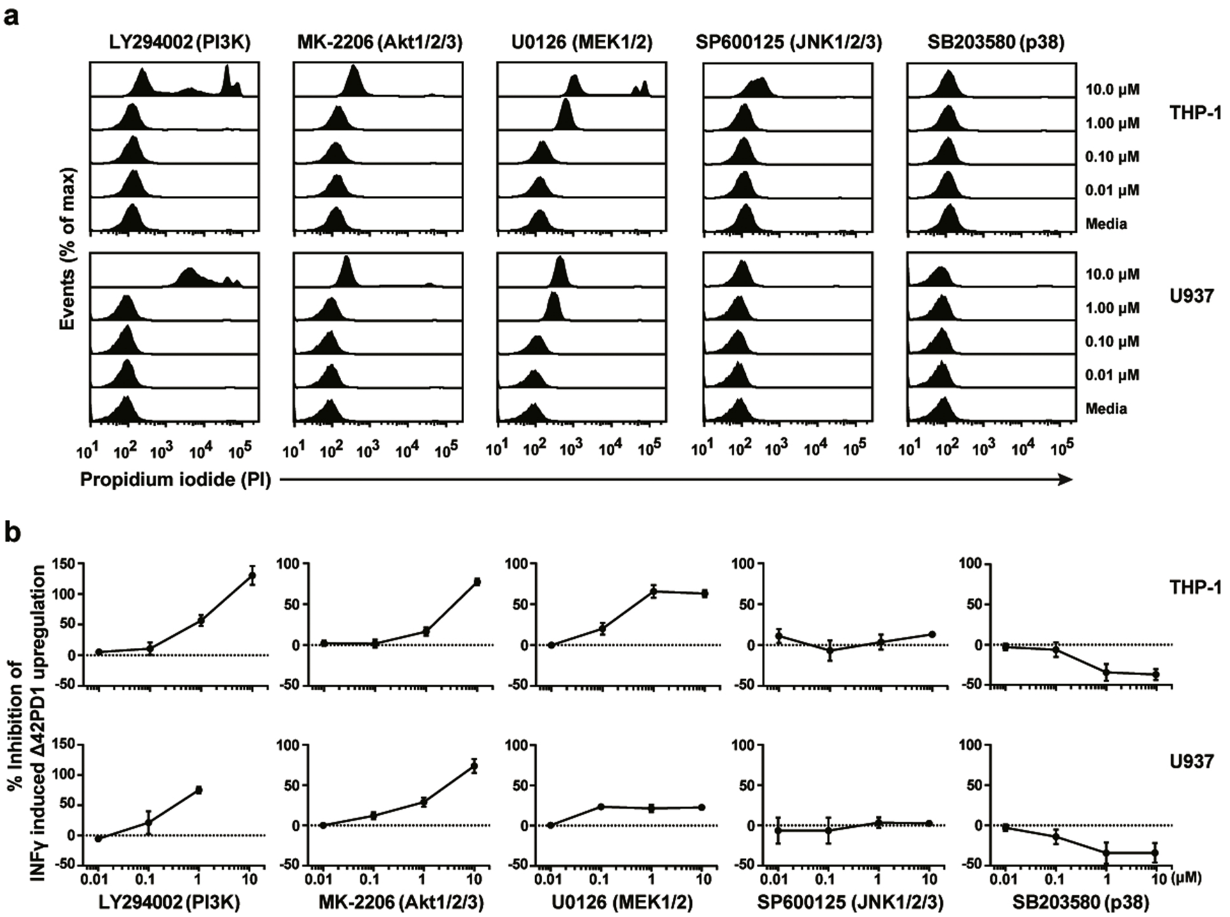

Fig. 5. Effect of non-STAT pathway inhibitors on Δ42PD1 expression.
(a) Analysis of apoptosis in THP-1 and U937 cells following treatment with indicated signaling pathway inhibitors (LY294002 is a PI3K inhibitor, MK-2206 inhibits all three AKT isoforms, U0126 is an ERK1 and ERK2 inhibitor, SP600125 is a JNK1, JNK2 and JNK3 inhibitor, and SB203580 is a P38 inhibitor) for 48 h with indicated concentrations. **(b)** THP-1 and U937 cells were pre-treated with indicated inhibitors and doses for 1 h, followed by stimulation or not with IFN-γ (100 ng/mL) for 48 h and flowcytometric analysis of Δ42PD1 expression. Data were presented as mean ± SEM from three independent experiments.

Giannopoulou et al., 2006). Accordingly, corresponding inhibitors were tested for cytotoxicity and then used to block IFN-γ-induced upregulation of Δ42PD1. PI3K inhibitor LY294002 and pan-AKT inhibitor MK-2206 blocked IFN-γ-induced Δ42PD1 expression in dose-dependent manners. In contrast, IFN-γ-induced upregulation of Δ42PD1 was not affected by JNK1/2/3 inhibitor SP600125 and P38 inhibitor SB203580. Lastly, ERK1/2 inhibitor U0126 partially blocked IFN-γ-induced Δ42PD1 expression on THP-1 cells, but did not seem to affect Δ42PD1 expression on U937 cells dose-dependently (Fig. 5).

3.4. Δ42PD1/PD-1 ratio as a potential indicator of monocyte functional state

It is well established that when monocytes are pre-treated with IFN-γ, the LPS response in these cells is augmented, producing substantially larger quantities of TNF-α (Hayes et al., 1995). Consistently, IFN-γ pretreated THP-1 cells or U937 cells produced significantly higher levels of TNF-α (~6.2-fold increase, $P = 0.0005$) and IL-6 (~37.4-fold increase, $P < 0.0001$), respectively, in response to *E. coli* (Fig. 6a). To assess whether Δ42PD1 is involved in elevated *E. coli* response, we pre-treated THP-1 and U937 cells with IFN-γ as well as various signaling inhibitors. Pan-JAK inhibitor Tasocitinib completely abolished the IFN-γ-induced priming effect as expected. PI3K-AKT signaling inhibitors, which could completely inhibit IFN-γ-induced upregulation of Δ42PD1 (Fig. 5b), partially inhibited production of TNF-α and IL-6; besides, MAPK (mitogen-activated protein kinase) signaling inhibitor SP600125 and SB203580 shown equal or better inhibitory effects of cytokine release (Fig. 6b), suggesting a non-essential role of Δ42PD1 in IFN-γ-primed effects. Furthermore, Δ42PD1-specific blocking antibody (Cheung et al., 2017; Cheng et al., 2015) did not affect TNF-α and IL-6 secretion from IFN-γ-primed human monocytic cells upon stimulation with *E. coli* (Fig. 6c), indicating lack of a cause relationship between Δ42PD1 and production of TNF-α and IL-6 from IFN-γ-primed monocytic cells.

To assess if Δ42PD1 can be used as a biomarker for evaluating the function state of human monocytes, we enriched $CD14^+$ monocytes from freshly isolated PBMCs of thirteen healthy donors. Enriched human circulating $CD14^+$ monocytes were analyzed for surface expression of PD-1 and Δ42PD1 by flow cytometry, and were stimulated with *E. coli* for determination of TNF-α production. Neither Δ42PD1 nor PD-1 exhibited any significant association with TNF-α production. However, the MFI ratio of Δ42PD1 to PD-1 (PD-1 Δ/F ratio) was significantly and positively correlated with TNF-α production ($P = 0.0289$, $r = 0.6038$, Fig. 6d).

3.5. Δ42PD1 blockage affects T cells proliferation and cytokine production

As antigen-presenting cells, monocytes have the function of presenting antigens to cognate T cells, resulting in T-cell proliferation and activation (Jakubzick et al., 2017). It is now well documented that T cells express Toll-like receptor 4 (TLR4), and that direct activation of human T cells by TLR4 induces T cell survival and proliferation, boosts the production of proinflammatory cytokines (Reynolds and Dong, 2013). In addition, we recently reported that Δ42PD1 promotes innate mucosal damage via engagement with TLR4 during acute HIV type 1

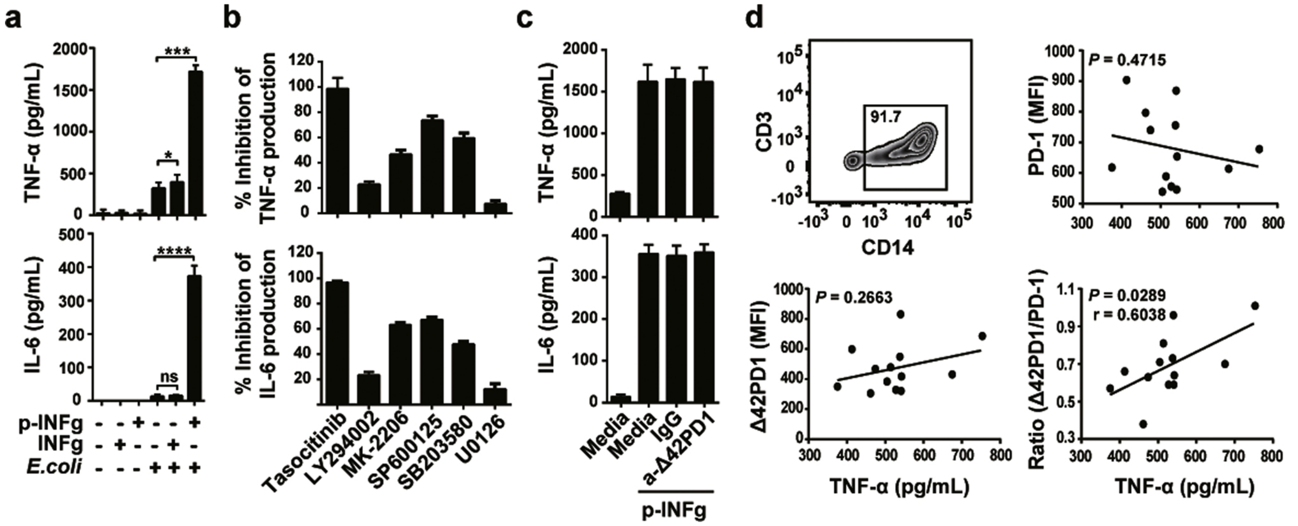

Fig. 6. The Δ42PD1/PD-1 MFI ratio is correlated with monocytes production of TNF-α.
(a) Secretion of TNF-α by THP-1 (upper) and IL-6 by U937 (lower) unprimed or primed by IFN-γ (p-IFNg) upon stimulated with pre-fixed *E. coli* (1 MOI) for 8 h, assayed in triplicate by ELISA. **(b–c)** Inhibition of production of TNF-α or IL-6 by IFN-γ-primed THP-1 (upper) and U937 (lower) by indicated signaling inhibitors **(b)** or by Δ42PD1 blocking antibody **(c)** upon stimulation with pre-fixed *E.coli* (1 MOI) for 8 h. **(d)** upper left, representative flow cytometric plot showing the percentage of enriched CD14$^+$ monocytes (one of five experiments is shown). Enriched CD14$^+$ monocytes were flow cytometrically analyzed for surface expression of PD-1 and Δ42PD1, and were stimulated with pre-fixed *E.coli* for TNF-α production. TNF-α concentration in relation to PD-1 MFI (upper right), Δ42PD1 MFI (lower left), and Δ42PD1/ PD-1 ratio (lower right). (**a–c**) Data were presented as mean ± SEM from 3 independent experiments. *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$; paired *t*-test.

infection (Cheung et al., 2017). Therefore, we hypothesized that Δ42PD1 play a role during antigen presentation, and assessed the effects of Δ42PD1 blockage on T-cell proliferation, cytokine production, and activation.

Enriched CD4$^+$ T cells were cultured alone or co-cultured with THP-1 or U937 in the presence or absence of Δ42PD1-blocking antibody, and the expression of IFN-γ, TNF-α and IL-2 were determined by flow cytometry after 24 h. As expected, co-culture conditions led to an increase of cytokine production, and this effect was significantly inhibited by Δ42PD1 blockage (Fig. 7a). Similarly, T-cell proliferation was also partially inhibited by Δ42PD1 blockage as CFSE-labeled CD4$^+$ T cells were co-cultured with THP-1 or U937 for 4 days (Fig. 7b). We furthermore determined whether Δ42PD1 blockage also affected the phenotype of CD4$^+$ T cells. We assessed the expression of two markers of activation, CD69 and CD25, after 24 h of culture in the up-mentioned model. Although a strong upregulation of CD69 and CD25 on CD4$^+$ T cells was also detected in the co-culture conditions, we did not find any significant effect of Δ42PD1 blockage on the expression of CD25 (data not shown) and CD69 (Fig. 7c).

4. Discussion

To investigate the biological function of human Δ42PD1, we previously generated monoclonal antibodies that specifically recognize Δ42PD1 by flow cytometry. Using these antibodies, we discovered the constitutive expression of Δ42PD1 on monocytes, consistent with our real-time quantitative PCR data that the relative expression of Δ42PD1 transcripts was found mainly in monocyte/macrophage but less in T- and B-cells (Zhou et al., 2013; Cheng et al., 2015). In contrast to PD-1 that is up-regulated on monocytes during chronic HIV-1 infection (Said et al., 2010), we found that the expression of Δ42PD1 on monocytes was significantly down-regulated in HIV-1 infected patients. However, the mechanism underlying the regulation of Δ42PD1 expression in human monocytes remains unknown. In this study, we found that IFN-γ remarkably upregulates Δ42PD1 on both circulating monocytes and monocytic cell lines mainly via the PI3K/AKT pathway.

Activation of T cells induces a parallel increase in the transcription of all PD-1 alternative splice variants (Nielsen et al., 2005). However, we did not observe PD-1 increase while IFN-γ triggered the upregulation of Δ42PD1 on primary human monocytes. Similarly, TNF-α, IL-1β or IL-6 induce an increase in PD-1 expression (Said et al., 2010) but fails to augment Δ42PD1 level (data not shown) on monocytes. These observations indicate a unique transcriptional regulation mechanism underlying the regulation of PD-1 and Δ42PD1 expression.

IFN-γ is produced as early as the acute phase and continually detected throughout the course of HIV-1 infection (Roff et al., 2014). However, our previous work demonstrated that the expression of Δ42PD1 on monocytes was significantly down-regulated in HIV-1 infected compared with healthy individuals. Besides IFN-γ, multiple cytokines and chemokines were also elevated in plasma of HIV-1 infected individuals, including IFN-α, IL-1β, IL-6, IL-8, IL-10, IL-15, inducible protein 10 (IP-10), tumor necrosis factor alpha (TNF-α), and monocyte chemotactic protein 1 (MCP-1) (Kedzierska et al., 2003; Barqasho et al., 2009). It is possible that one or more of these cytokines and chemokines induced by HIV-1 infection could overwhelm the effect of IFN-γ on the expression of Δ42PD1, resulting in a down-regulation phenotype of Δ42PD1 on monocytes. Future study should investigate the factor(s) that contribute to the down-regulation of Δ42PD1 on monocytes after HIV-1 infection.

IFN-γ signals mainly through the JAK1/2-STAT1 intracellular signaling pathway to achieve transcriptional activation of IFN-γ-inducible genes. In addition, JAK1/2 tyrosine kinase activation by IFN-γ activates several other downstream pathways, including ERK1/2, JNK, p38 MAPK, and PI3K/AKT signaling cascades (Platanias, 2005). We found that IFN-γ-induced changes in Δ42PD1 expression were completely blocked by the JAK inhibitors Ruxolitinib and Tasocitinib, but not by inhibitors of STAT1/3/5, indicating that IFN-γ induces the expression of Δ42PD1 in a STAT1-independent manner. PI3K/AKT but not MAPK signaling pathway inhibitors completely blocked IFN-γ-induced Δ42PD1 expression in THP-1 and U937 cells, revealing that PI3K/AKT signaling is required for IFN-γ-induced Δ42PD1 upregulation.

It is well established that IFN-γ is capable of activating human monocytes (Volk et al., 1996; Turina et al., 2006), and enhancing TNF-α release in response to LPS. Cell-surface Δ42PD1 increased significantly upon INF-γ stimulation, therefore, could be potentially used as a biomarker of monocyte functional state. Interestingly, the PD-1 Δ/F

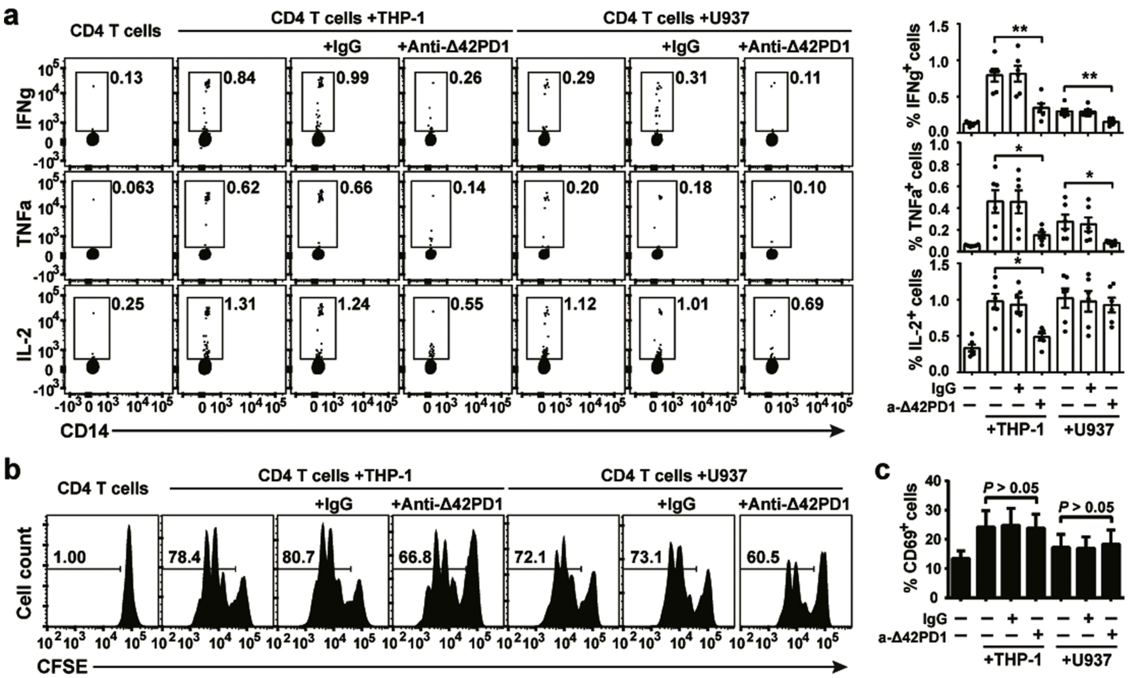

Fig. 7. Effect of Δ42PD1 blockage on proliferation and cytokine production of T cells.
(a) Flow cytometry plots showing production of IFN-γ, TNF-α and IL-2 when enriched CD4$^+$ T cells from healthy donors (n = 6) were cultured alone or co-cultured with THP-1 or U937 for 24 h, in the presence or absence of Δ42PD1-blocking antibody or isotype-matched control antibody. **(b)** The representative Flow cytometry plots showing proliferation of CFSE-labeled CD4$^+$ T cells (n = 4), as described in **a** when cells were cultured for 4 days. **(c)** CD69 expression on CD4$^+$ T cells (n = 4) measured by Flow cytometry as described in **a**. (**a, c**) Data were presented as mean ± SEM. $^*P < 0.05$, $^{**}P < 0.01$; paired *t*-test.

ratio is significantly and positively correlated with TNF-α release by primary monocytes in response to *E. coli*. Monocytes are critical components of host antimicrobial innate defense. These cells are functionally heterogeneous and highly plastic in nature, they also contribute to damage during some infections and inflammatory diseases and are closely linked to disease progression (Campbell et al., 2014; Sampath et al., 2018). Human circulating monocytes are primarily responsible for the systemic release of inflammatory cytokines, such as IL-1, IL-6 and TNF, which ultimately causes cytokine release syndrome in the setting of chimeric antigen receptor-modified T-cell (CAR-T) therapy, bispecific T-cell receptor engaging (BiTE) immunotherapy, PD-1-directed checkpoint-blockade therapy and other immunotherapies (Norelli et al., 2018; Rotz et al., 2017). Similarly, a burst in the production of inflammatory mediators known as a cytokine storm was frequently observed in many infectious diseases, such as sepsis, influenza, dengue, Ebola and HIV-associated immune reconstitution inflammatory syndrome (Campbell et al., 2014; Tisoncik et al., 2012). It is interesting to assess the value of Δ42PD1 or the PD-1 Δ/F ratio on monocytes in predicting the progress and outcome of up-mentioned situations.

T cell activation results from a complex integration of signals conjointly received by the TCR, costimulatory molecules, cytokines, and alternate surface receptors. Extensive research has demonstrated that T lymphocytes constitutively express TLR4 that mediates regulation of T lymphocyte activation, proliferation and function (Reynolds and Dong, 2013; Caramalho et al., 2003). We have previously identified that TLR4/MD2 served as an endogenous receptor for Δ42PD1 to perform its immune stimulatory function (Cheung et al., 2017). In this study, by using a blocking antibody, we describe for the first time to our knowledge that the Δ42PD1-TLR4 pathway plays a critical role in regulating CD4$^+$ T lymphocytes proliferation and cytokine production during monocyte:T cell interaction. These data highlight the complexity of the control of human T cell activation, and the discovery of the Δ42PD1-TLR4 pathway involved in T cell plasticity may provide a novel approach to manipulate adaptive immune response.

5. Conclusions

This study demonstrated that IFN-γ is a critical regulator for the expression of Δ42PD1 in human monocytes. IFN-γ upregulates significantly the expression of Δ42PD1 on primary peripheral blood monocytes and monocytic cell lines THP-1 and U937 cells, in a time- and concentration-dependent manner, through a PI3K/AKT-dependent, STAT1-independent signaling pathway. In addition, we evaluate the potential of surface expression of Δ42PD1, PD-1 and their relative ratio in predicting the functional state of monocytes. Correlation analysis indeed supported the use of the PD-1 Δ/F ratio as a potential biomarker of human monocyte functional state. Notably, we revealed the critical role of Δ42PD1-TLR4 pathway in regulating T-cell proliferation and cytokine production, providing novel insights into the regulation of human T cell activation. Further studies are needed to assess the biological function of Δ42PD1 on human monocytes as well as the mechanisms of Δ42PD1-TLR4 pathway in regulating T-cell function.

Conflict of interest statement

The authors declare no financial or commercial conflict of interest.

Acknowledgments

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