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Venlafaxine inhibits the development and differentiation of dendritic cells through the regulation of p-glycoprotein

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ABSTRACT

Dendritic cells (DC) are professional antigen-presenting cells that have the ability to detect infectious materials; antigens to T lymphocytes, and serve as a bridge between innate and adaptive immunities. DC express the ATP-binding cassette transporters P-glycoprotein (P-gp). P-gp is a 170-kDa transmembrane protein encoded by the *mdr-1* gene, a member of highly conserved superfamily of ATP-binding cassette transport proteins. Functionally, P-gp transporters have been described to be required for efficient DC and T cell migration. We report for the first time, at the best of our knowledge, P-gp is also required for DC development and differentiation in mouse bone marrow-derived DC. In this study, we found that an *mdr-1* gene and P-gp protein level was increased during DC development and LPS-induced maturation. Moreover, the activity of P-gp was increased LPS-induced DC maturation. Next, we have attempted to determine whether the modulation of P-gp regulates surface molecules expression and cytokine production in DC. Specifically, down-regulation of P-gp by Venlafaxine (VLX) inhibits the differentiation of DC and cytokine production, such as IL-1, IL-10, and IL-12 during DC maturation. Moreover, the P-gp-decreased DC by VLX was displayed impaired induction of T cell polarizations, proliferation, and cytokine production, including IFN-γ, IL-4, and IL-2. Taken together, these findings also broaden current perspective concerning our understanding of the immunopharmacological functions of VLX and the development of therapeutic adjuvants for the treatment of DC-related acute and chronic diseases.

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1. Introduction

Dendritic cells (DC) are professional antigen-presenting cells (APC) that represent a bridge between innate and adaptive immunity [1, 2]. DC have the ability to detect tumor cells, and sample and present antigens to T lymphocytes, while at the same time preventing immune responses against self-tissues [3, 4]. DC are derived from host bone marrow, developing first into immature DC and upon activation, progress to mature DC. Mature DC present antigen in conjunction with co-stimulatory molecules such as B7-families and major histocompatibility complex (MHC) class molecules, and produce a range of cytokines, leading to the initiation of a specific immune response [5]. A critical property of DC is their ability to respond to environmental stimuli, which determine their final maturation or differentiation status [3, 6]. For example, responding to tumor antigens, pathogens binding to Toll-like receptors [7], heat shock proteins, innate cytokines [8], and inflammation cytokines, can lead to DC maturation and subsequent T cell immune responses [9]. Moreover, DC have been recognized as powerful APC, playing a key role in antitumor host responses [10, 11], and inducing primary T-cell responses *in vitro* and *in vivo*, which has generated widespread interest in DC-based immunotherapy against several types of cancer [12, 13].

P-glycoprotein (P-gp) is a 170-kDa trans-membrane protein member of a highly conserved superfamily of ATP-binding cassette transport proteins, and is encoded by the *mdr-1* gene [14]. In addition to its ability to efflux toxins, P-gp also plays a role in the resistance to pathogens as it is expressed on various tissues, including peripheral blood lymphocytes and DC [15, 16]. Further, because of its ability to efflux toxins [17], overexpression of P-gp contributes to chemotherapeutic drug resistance [18], and thus remains a critical barrier to successful chemotherapy in cancer patients treated with drugs such as verapamil (VRP), cyclosporine A (CsA) [19], and venlafaxine (VLX) [20]. VRP and CsA, a typical inhibitor of P-gp activity, particularly are used in numerous studies for P-gp function [21, 22]. Recently, P-gp has been reported to be expressed in

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Table 1
siRNA of P-gp inhibits the expression of costimulatory molecules (CD80, CD86 and CD40), MHC class I, and II on LPS-stimulated CD11c+ DC.

% of positive cell[a] (MFI)[b]				
Surface Ag	Medium	siRNA	LPS	siRNA + LPS
CD80	74 ± 2 (256 ± 21)	71 ± 1 (241 ± 15)*	86 ± 3 (950 ± 36) ##	74 ± 2 (70 ± 28)**
CD86	75 ± 14 (435 ± 22)	78 ± 2 (421 ± 47)	80 ± 1 (1205 ± 47) ##	76 ± 3 (7232 ± 86)**
MHC I	74 ± 2 (87 ± 5)	73 ± 1 (86 ± 1)	81 ± 3 (254 ± 23) ##	75 ± 1 (196 ± 23)**
MHC II	80 ± 1 (230 ± 12)	79 ± 3 (211 ± 23)*	87 ± 2 (470 ± 12) ##	85 ± 1 (341 ± 21) **

*, ** The statistical significance between samples with and without siRNA of P-gp is indicated (##$P<0.01$ *vs* unstimulated DC (medium); **$P<0.01$ *vs* LPS-stimulated DC).
[a] BM-derived DC were cultured in the absence or presence of siRNA of P-gp following the LPS (200 ng/ml) stimulation for 24 h. The expression of surface molecules was analyzed by FACSCalibur. Two-color flow cytometry was used to determine the level of Ag expression on CD11c+ DC.
[b] MFI, Mean fluorescence intensity. The results are from one of the three experiments performed.

monocyte-derived and interstitial DC during migration toward lymph nodes via afferent lymphatic vessels. In the human skin explants system, it was described that antibodies or drugs that antagonize P-pg, such as VRP, block this migration [16].

Little is known about the role of P-gp in DC maturation, differentiation, and during T cell interaction and the subsequent immune response. The functions of P-gp in immune responses are still unclear. Therefore, in this study, we sought to examine the effects of P-gp down-regulation on the development, maturation, and differentiation of DC and how this affects T cell proliferation and polarization. Taken together, these data identify a novel physiological function for P-gp in DC.

2. Materials and methods

2.1. Animals

Male 8–12-week-old C57BL/6 (H-2K^b and I-A^b) and BALB/c (H-2K^d and I-A^d) mice were purchased from the Korean Institute of Chemistry Technology (Daejeon, Korea). They were housed in a specific pathogen-free environment within our animal facility for at least 1 week before use. All mouse work was approved by the IACUC and was performed in our IACUC approved facility.

2.2. Reagents and Abs

Recombinant mouse (rm)GM-CSF and rmIL-4 were purchased from R&D Systems (Minneapolis, MN USA). Dextran-FITC (molecular mass, 40,000), and LPS (from *Escherichia coli* 055:B5) were obtained from Sigma-Aldrich. An endotoxin filter (END-X) and an endotoxin removal resin (END-X B15) were acquired from Associates of Cape Cod. Cytokine ELISA kits for murine IL-12 p70, IL-4, IL-6, IL-1α, and IFN-γ were purchased from BD Pharmingen (Rockville, MD, USA). FITC- or PE-conjugated mAbs used to detect the expression of CD11c (HL3), CD80 (16-10A1), CD86 (GL1), IA^b β-chain (AF-120.1), H2K^b (AF6-88.5), and CD4 (L3T4), or the intracellular expression of IL-12 p40/p70 (C15.6), and IL-10 (JESS-16E3) by flow cytometry, as well as isotype-matched control mAbs, biotinylated anti-CD11c (N418) mAb, were purchased from BD Pharmingen.

2.3. Generation and culture of DC

DC were generated from murine bone marrow (BM) cells. Briefly, BM was flushed from the tibiae and femurs of C57BL/6 and depleted of red cells with ammonium chloride. The cells were plated in six-well culture plates (10^6 cells/ml; 3 ml/well) in OptiMEM (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 5×10^{-5} M 2-ME, 10 mM HEPES (pH 7.4), 20 ng/ml rmGM-CSF and rmIL-4 at 37 °C, 5% CO_2. On day 3 of the culture, floating cells were gently removed, and fresh medium was added. On day 6 of the culture, nonadherent cells and loosely adherent proliferating DC aggregates were harvested for analysis or stimulation, or, in some experiments, replated in 60-mm dishes (10^6 cells/ml; 5 ml/dish). On day 6, 80% or more of the nonadherent cells expressed CD11c. In certain experiments, to obtain highly purified populations for subsequent analyses, the DC were labeled with bead-conjugated anti-CD11c mAb (Miltenyi Biotec, Gladbach, Germany) followed by positive selection through paramagnetic columns (LS columns; Miltenyi Biotec) according to the manufacturer's instructions. The purity of the selected cell fraction was >95%.

2.4. Stimulation of DC by P-gp molulators

P-gp modulator (VRP, CsA, and VLX) was dissolved in culture media and was added to cultures of isolated DC in six-well plates (10^6 cells/ml; 2 ml/well). Media alone was used as negative control. For the analysis of apoptosis, DC were stimulated with LPS or left without any stimuli, and analyzed over time by staining of phosphatidylserine translocation with FITC-annexin V in combination with propidium iodine kit (BD Pharmingen) according to the manufacturer's instructions.

2.5. Flow cytometric analysis

On day 6, BM-DC were harvested, washed with phosphate buffered saline (PBS) and resuspended in fluorescence activated cell sorter (FACS) washing buffer (2% fetal bovine serum and 0.1% sodium azide in PBS). The cells were first blocked with 10% (v/v) normal goat serum for 15 min at 4 °C and stained with phycoerythrin (PE)-conjugated anti-H-2K^b [major histocompatibility complex (MHC) class I], anti-I-A^b

Table 2
siRNA of P-gp inhibits the production of cytokines (IL-1α and IL-12) on LPS-stimulated CD11c+ DC.

pg/ml/10^6 cells[a]				
	Medium	siRNA	LPS	siRNA + LPS
IL-1α	21 ± 2	23 ± 1	382 ± 23##	240 ± 12**
IL-12	12 ± 0.5	15 ± 2	630 ± 47##	342 ± 42**

*, ** The statistical significance between samples with and without siRNA of P-gp is indicated (##$P<0.01$ *vs* unstimulated DC (medium) **$P<0.01$ *vs* LPS-stimulated DC).
[a] BM-derived DC were cultured in the absence or presence of siRNA of P-gp following the LPS (200 ng/ml) stimulation for 24 h. Analysis of IL-1α and IL-12p70 production was measured by ELISA with magnetic bead-purified DC (1×10^6 cells) 24 h after LPS stimulation.

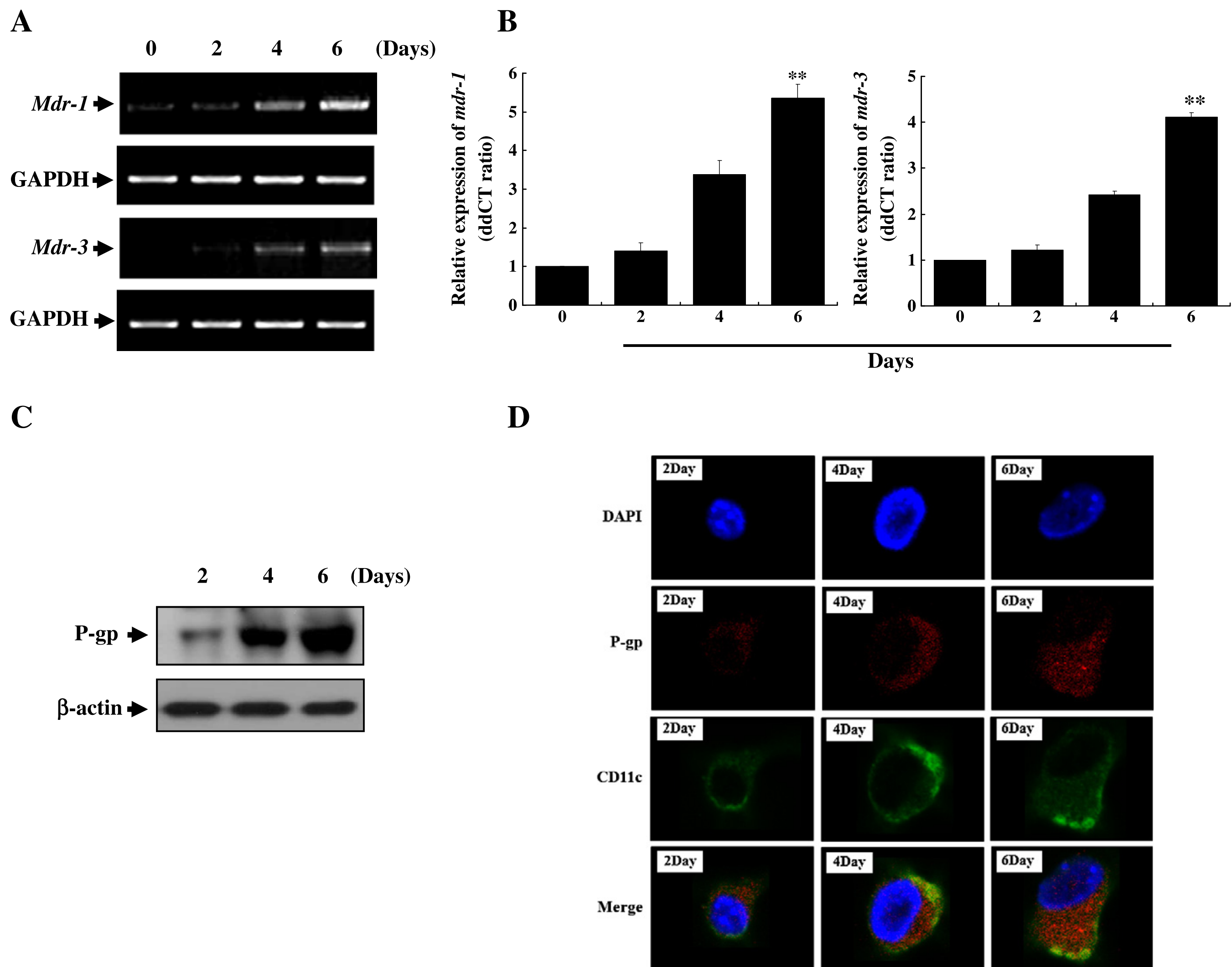


Fig. 1. Expression of *mdr-1* and *mdr-3* during DC development. (A) *mdr-1* and *mdr-3* gene expression levels during DC development for 2, 4, and 6 days. The data are representative of three experiments that gave similar results. (B) Quantitation of *mdr* mRNA levels by real-time RT-PCR. Samples were taken for RNA preparation, and quantitative real-time RT-PCR analyses were performed. A significant difference in expression between each group was detected. The histogram is from one out of three representative experiments. (C) For P-gp protein analysis, cell lysates were prepared and blotted with anti-P-gp and anti-β-actin Abs. (D) Confocal microscopy analysis of P-gp expression during DC development. The DC were stained for CD11c$^+$ (green), P-gp (red), and DAPI (blue). The data represent the means (±SD) of four separate experiments. (**$P<0.01$ *vs* control groups).

(MHC class II), anti-CD80, and anti-CD86 with fluorescein isothiocyanate (FITC)-conjugated anti-CD11c (Pharmingen, San Diego, CA) for 30 min at 4 °C. The stained cells were analyzed using a FACSCalibur flow cytometer (BD Biosciences) (Table 1).

2.6. Cytokines assay

DC were first blocked with 10% (v/v) normal goat serum for 15 min at 4 °C and then stained with FITC-conjugated CD11c$^+$ antibody for 30 min at 4 °C. The cells stained with the appropriate isotype-matched Ig were used as negative controls. The cells were fixed and permeabilized with the Cytofix/Cytoperm kit (BD Pharmingen) according to manufacturer's instructions. Intracellular IL-12p40/p70 and IL-10 were stained with fluorescein R-phycoerythrin (PE)-conjugated antibodies (Pharmingen) in a permeation buffer. The cells were analyzed using FACSCalibur flow cytometer with the CellQuest program. Furthermore, murine IL-12p70, IL-4, IL-6, IL-1α, and IFN-γ from DC were measured using an ELISA kit (BD Pharmingen), according to manufacturer's instructions.

2.7. Mixed lymphocyte reaction

Responder T cells, which participate in allogeneic T-cell reactions, were isolated passing through mononuclear cells from spleen of BALB/c mice in a MACS column (Miltenyi Biotec). Staining with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 and anti-CD8 antibodies (BD Pharmingen) revealed that they consisted mainly of CD4$^+$ and CD8$^+$ cells (>95%). The lymphocyte population was then washed twice in PBS and labeled with CFSE, as previously described [23]. The cells were resuspended in 5 μM CFSE in phosphate-buffered saline (PBS). After being shaken for 8 min at room temperature, the cells were washed once in pure fetal bovine serum (FBS) and twice in PBS with 10% FBS. DC (1×10^4) or DC exposed to VLX (25 and 50 μM/ml) or LPS (200 ng/ml) for 24 h were cocultured with 1×10^5 allogeneic CFSE--labeled T lymphocytes in 96-well, U-bottom plates (Nunc). A negative control (CD4$^+$ and CD8$^+$ lymphocytes alone) and a positive control (CD4$^+$ and CD8$^+$ lymphocytes in 5 μg of Concanavalin A) were included for each experiment. After 4 days, the cells were harvested and washed in PBS. CFSE dilution optically gated lymphocytes were assessed by flow cytometry.

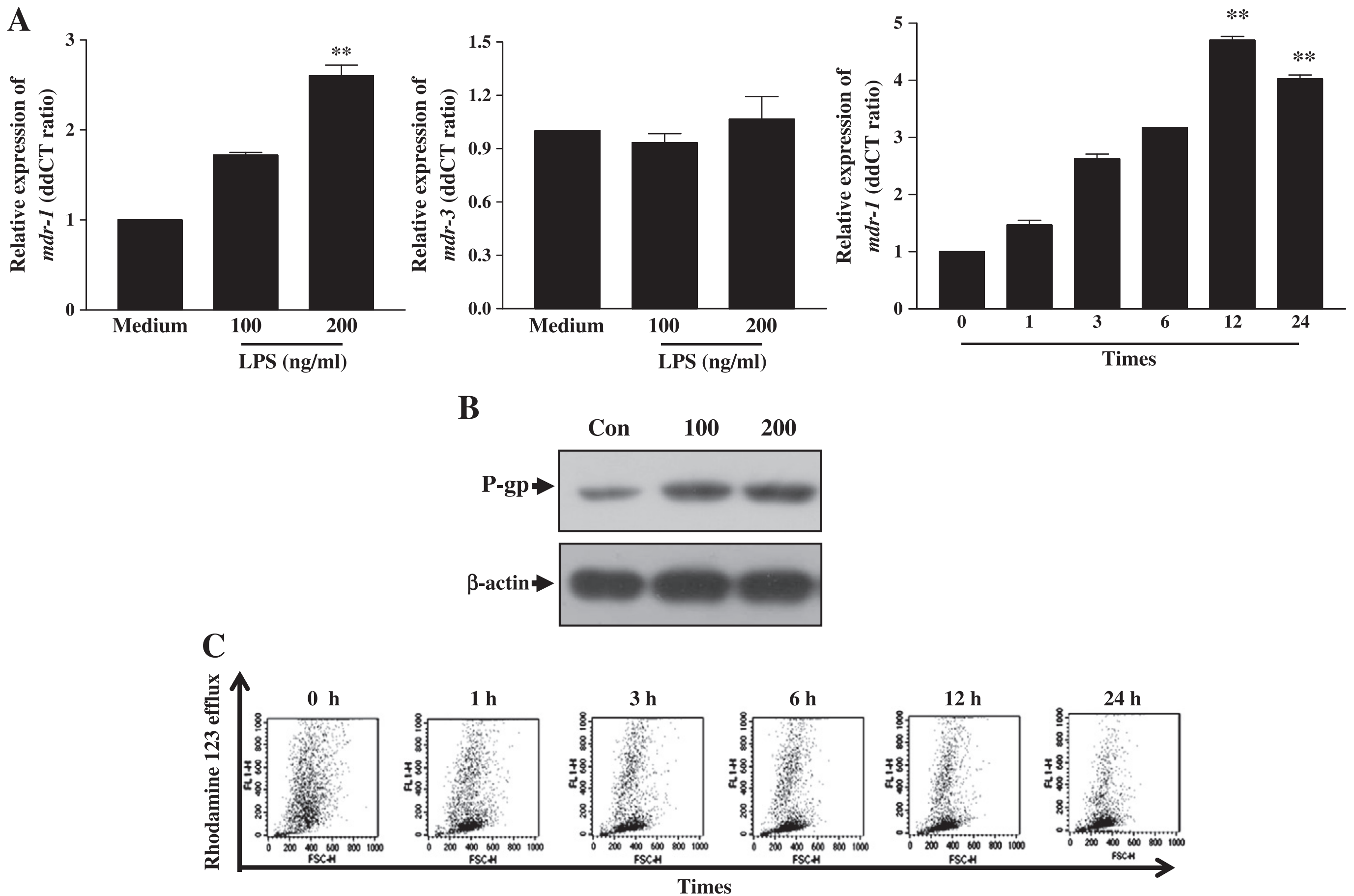


Fig. 2. DC maturation increased expression of *mdr-1* and P-gp, but not *mdr-3*. DC were generated as described in Materials and methods. On day 6, the cells were cultured under standard conditions for another 6 h and for time course experiments in the presence of 100 or 200 ng/ml LPS. Cells were then harvested and analyzed by quantitative real-time RT-PCR (A) and Western blot (B). To assess P-gp activity, DC were loaded with the fluorescent probe Rhodamine 123 for 30 min at 37 °C, washed, and incubated for a another 30 min at 37 °C to determine dye extrusion. The fluorescence was measured by flow cytometry (C). The data represent the means (±SD) of three separate experiments. (**$P<0.01$ *vs* untreated DC groups).

2.8. Quantitative real-time PCR

mdr-1 and *mdr-3* PCR primers used were as follows: forward 5′-ACATTCTTGGCTGACTTGCG-3′ and 5′-TATAGTTTTGGCTGGGCGTGG-3, reverse 5′-AAAACACCGTCCTGAAGGCT-3′ and 5′-ATTTCTCCAGCACCAGGGAC-3′, respectively. Quantitative amounts of each gene were standardized against the GAPDH housekeeping gene. Real-time PCR was performed using a BioRad MiniOpticon System (BioRad Laboratories Ltd, Missouri, USA.) with SYBR green fluorophore. Reactions were performed in a total volume of 20 μl-including 10 μl 2× SYBR Green PCR master Mix (Applied Biosystems, Carlsbad, CA, USA), 1 μl of each primer at 10 μM concentration and 1 μM of the previously reverse-transcribed cDNA template. The protocols used were as follow: denaturation (95 °C for 10 min), amplification was repeated 40 times (95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and acquisition temperature for 15 s). For each sample, ddCt (crossing point) values were calculated as the Ct of the target gene minus Dt of the GAPDH gene. Gene expression was derived according to the equation $2^{-\mathrm{ddCT}}$, and changes in gene expression were expressed as relative to basal.

2.9. siRNA studies

DC were transfected with 100 nM siRNA specific to P-gp (Santa Cruz, CA, USA) and a negative control siRNA according to the manufacturer's protocol (Santa Cruz). After 24 h incubation, the DC were rinsed with PBS and used for further analysis as described above (Table 2).

2.10. Cytoplasmic extracts and Western blot

The cells (DC) were exposed to VRP, CsA, and VLX for 24 h of incubation at 37 °C, cells were washed twice with cold PBS and lased with modified RIPA buffer (1.0% NP-40, 1.0% sodium deoxycholate, 150 nM NaCl, 10 mM Tris–HCl [pH 7.5], 5.0 mM sodium pyrophosphate, 1.0 mM $NaVO_4$, 5.0 mM NaF, 10 mM/ml leupeptin, and 0.1 mM phenylmethylsulfonyl fluoride) for 15 min at 4 °C. Lysates were cleared by centrifuging at 14,000 g for 20 min at 4 °C. The protein content of cell lysates was determined using the Micro BCA assay kit (Pierce, Rockford, IL). Equivalent amounts of proteins were separated by 10% SDS-PAGE and analyzed by Western blotting using a P-glycoprotein (Abcam, Cambridge, MA, USA) and anti-β-actin (Santa Cruz) mAb for 3 h, as described by the manufacturer of the antibodies. Following the wash for three times with TBST, membranes were incubated with secondary HRP-conjugated anti-mouse IgG for 1 h. After washing, the blots were developed using the ECL system (GE healthcare life sciences, NJ, USA), by following manufacturer's instructions.

2.11. Statistics

All results were expressed as the means ± SD of the indicated number of experiments. Statistical significance was estimated using a Student's *t*-test for unpaired observations, and the differences were compared with regard to statistical significance by one-way ANOVA, followed by Bonferroni's *post hoc* test. The categorical data from the

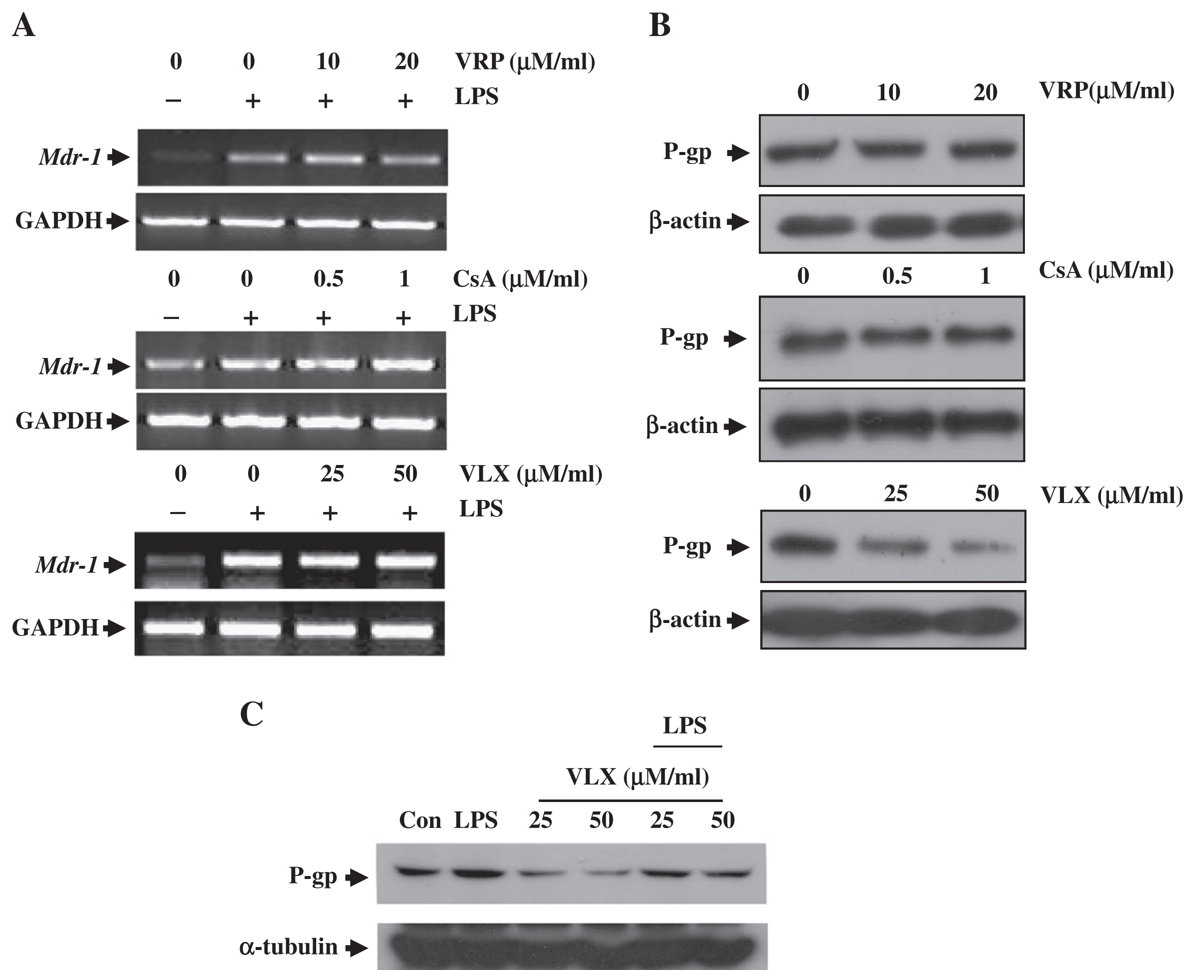


Fig. 3. VLX, but not VRP or CsA, suppresses P-gp levels during dendritic cells maturation. DC were treated VRP, CsA, or VLX at the noted concentrations, in the absence or presence of LPS (200 ng/ml). (A) Total RNA was extracted and quantitative PCR was done using sequence-specific primers to *mdr-1* gene and GAPDH. GAPDH was included as an internal control. Western blot analysis showed the protein expression of P-gp in DC treated with VRP, CsA, and VLX. The cells were treated with VRP, CsA, or VLX at the indicated doses for 24 h (B), or with 200 ng/ml LPS (C), and then collected for western blot analysis (20 μg/lane). The data are representative of three experiments that gave similar results.

fertility test were subjected to statistical analysis via Chi-square test. A *P* of <0.01 was considered significant.

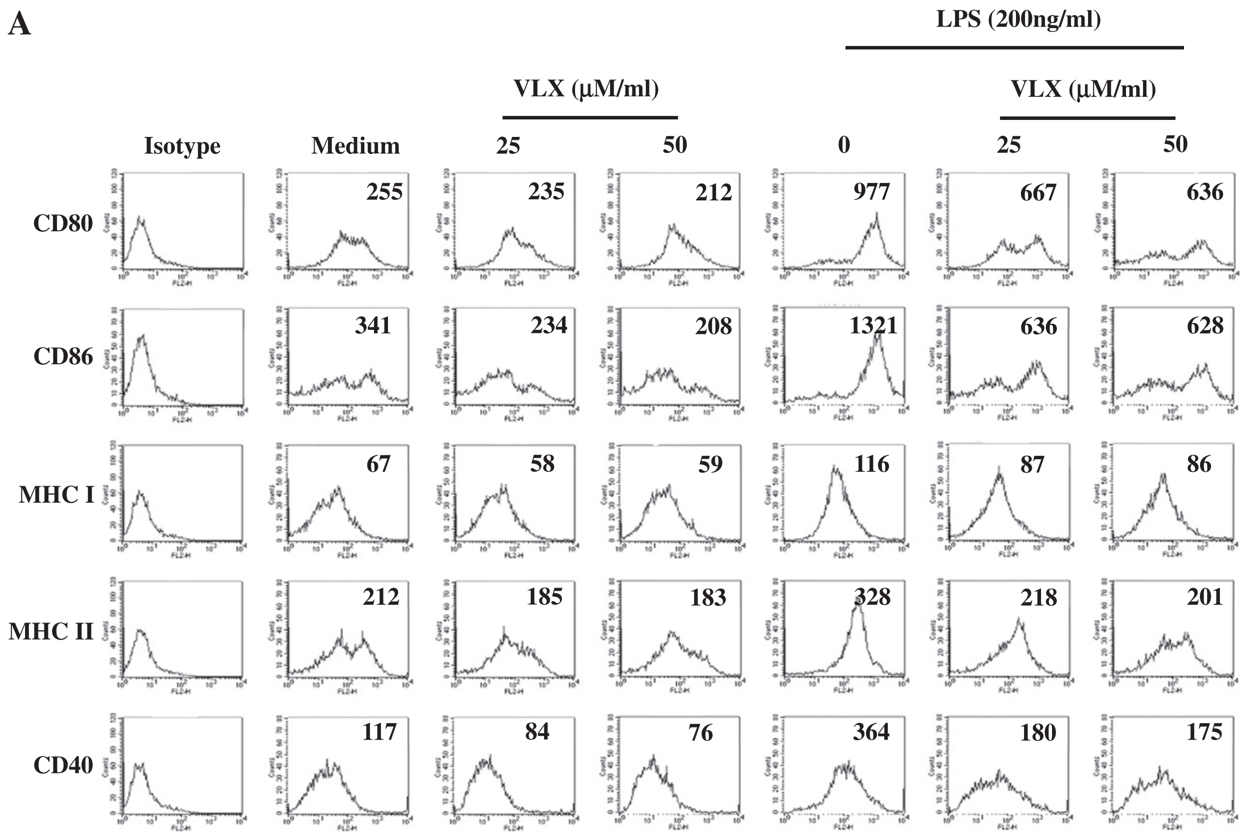
3. Results

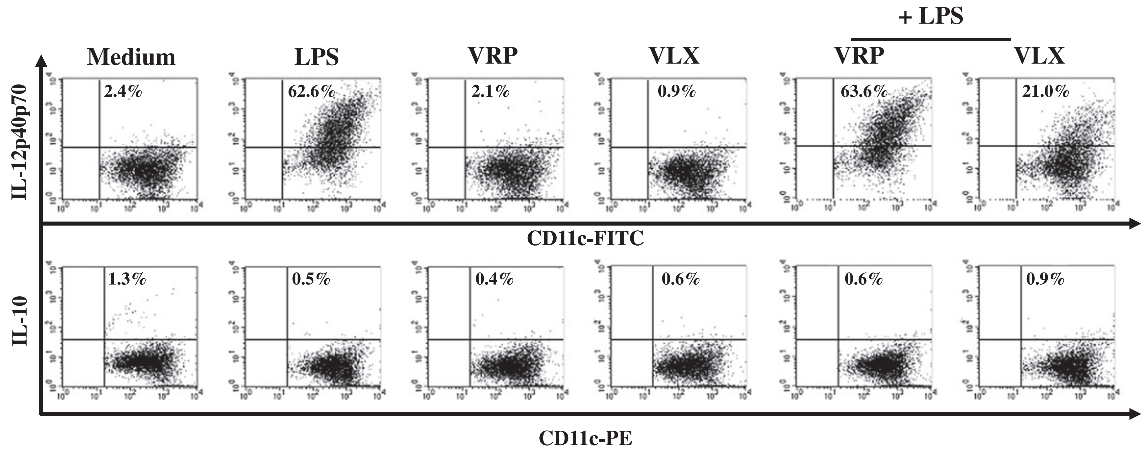
3.1. Up-regulation of mdr-1, mdr-3 and P-gp during DC development

Monocytes, T cells, NK-cells, and skin dendritic cells are known to express the *mdr-1* and *mdr-3* gene product, P-gp [18, 24]. In the initial series of experiments, we attempted to determine whether *mdr* gene expression influences DC development. Bone marrow-derived DC were cultured for 2, 4, and 6 days in OptiMEM supplemented with 20 ng/ml of GM-CSF and 20 ng/ml of IL-4. The expression levels of *mdr-1* and *mdr-3* were assessed using RT-PCR and quantitative real-time PCR, normalizing the amounts of mRNA for targeted genes to the mRNA levels of the GAPDH gene. Our quantitative real-time PCR results clearly indicate that expression of *mdr-1* and *mdr-3* was highly up-regulated during DC development (Fig. 1A and B). P-gp protein expression was evaluated during the monocyte to immature DC development by confocal microscopy and Western blot analysis. By western blot analysis, we found a pronounced up-regulation of the 170 kDa P-gp protein during differentiation from monocytes to immature DC (Fig. 1C). Cultured DC were co-stained for P-gp and the DC marker, $CD11c^{+}$. DC expressing P-gp (red) was also positive for DC marker $CD11c^{+}$ (green), suggesting that these cells are in fact increasing P-gp expression during DC development (Fig. 1D).

3.2. Increased mdr-1 gene expression during DC maturation, but not mdr-3

We found that *mdr-1* and *mdr-3* genes were essential for DC development, and next examined the individual role of these genes in DC maturation. To determine if expression of *mdr-1 and mdr-3* genes is modulated during DC maturation we examined the expression of these genes in LPS-induced DC maturation. As shown in Fig. 2, the expression of *mdr-1*, but not *mdr-3*, is up-regulated by DC maturation in a dose- and time-dependent manner. These data indicate that *mdr-1* gene expression is required for DC development as well as DC maturation. This was further confirmed on the protein level by western blot analysis (Fig. 2B), as the level of P-gp expression increased during DC maturation. Next, the efflux activity of the transmembrane P-gp was studied using rhodamine dye as a substrate. As shown in Fig. 2C, DC maturation also corresponded with an increase P-gp efflux activity, indicating that P-gp activity was increased during DC maturation.





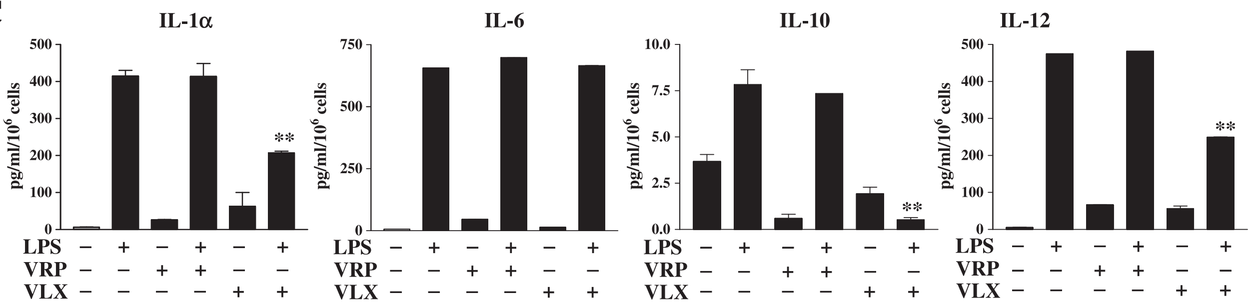


Fig. 4. VLX treatment suppresses the expression of co-stimulatory molecules and impairs cytokine production in LPS-induced DC maturation. DC were harvested and analyzed using two-color flow cytometry. The cells were gated on $CD11c^+$. VLX was added to the DC for 24 h at concentrations of 25 and 50 μM, and stimulation was induced with 200 ng/ml LPS. Surface molecule expression was then analyzed (A). The histogram shown is from one out of four representative experiments. The numbers indicate mean fluorescence indensity (MFI) (B) DC were stimulated with VRP (20 μM, such as P-gp blocker), or VLX (50 μM, such as P-gp suppressor) for 24 h, with or without LPS. This figure represents the analysis of IL-12p40p70 and IL-10 expression in $CD11c^+$DC by intracellular cytokine staining after 24 h of LPS stimulation. The numbers indicate the percentages of $CD11c^+$ cells expressing IL-12p40p70 or IL-10. (C) Analysis of IL-1α, IL-6, IL-10, and IL-12p70 production was measured by ELISA with magnetic bead-purified DC (1×10^6 cells) 24 h after LPS stimulation (200 ng/ml). The data represent the means (±SD) of four separate experiments. (**P<0.01 *vs* control groups).

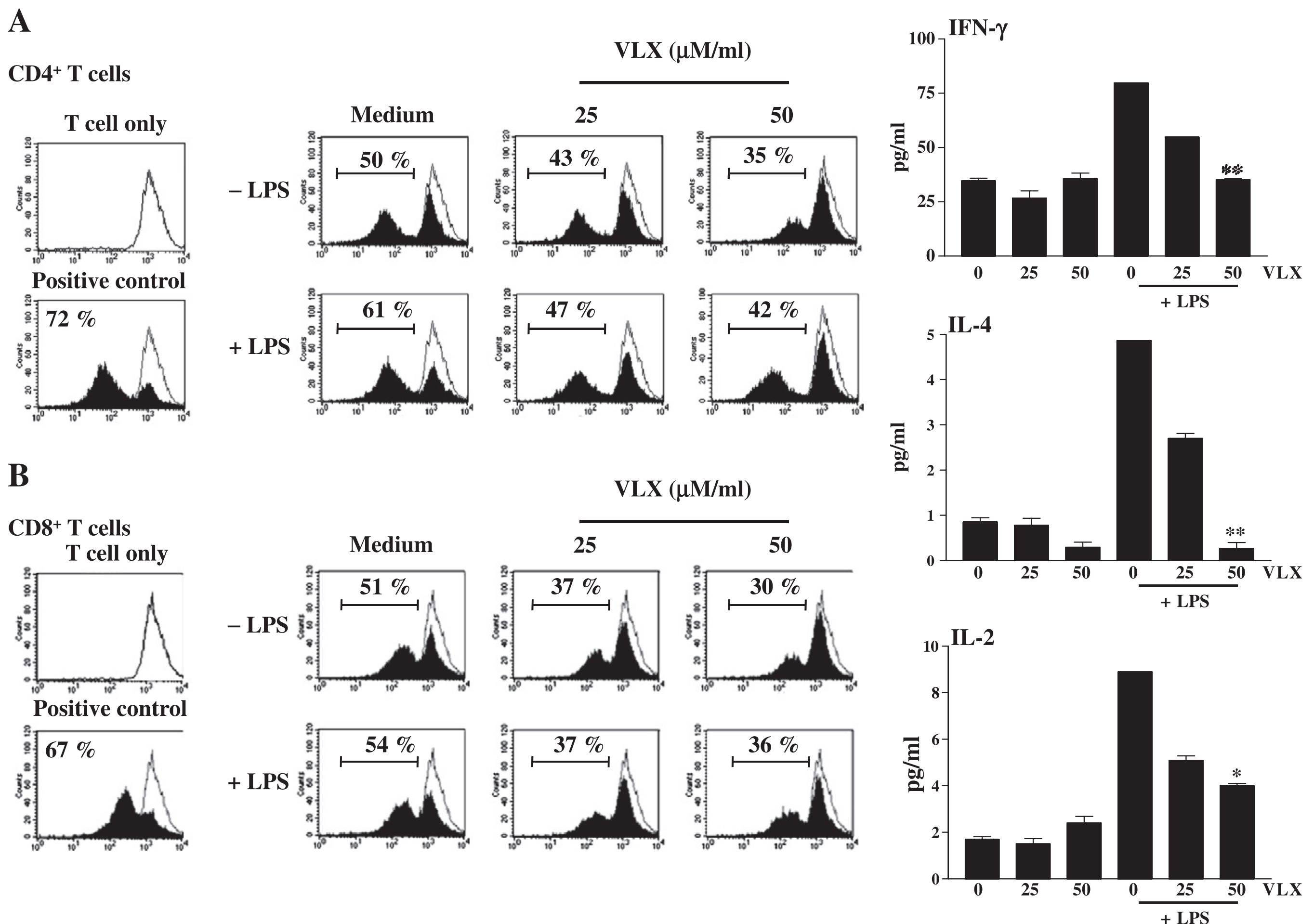


Fig. 5. P-gp depleted-DC impairs proliferation of allogeneic T cells, and inhibits Th1/2 polarization. The DC were incubated for 24 h in medium alone, positive control (Concanavalin A), in either 25 or 50 μM VLX, 200 ng/ml LPS, or in VLX coupled with LPS. The treated DC were harvested and thoroughly washed to remove the VLX and LPS. A mixed lymphocyte reaction was allowed to proceed for 3 days, as described in the Materials and methods section of this article. (A) T cell proliferation was analyzed by flow cytometry and is presented as a percentage of dividing cells. (B) Supernatants were examined for cytokine release after 72 h. IL-2, IL-4, and IFN-γ in culture supernatants were measured by ELISA. Data are expressed as pg/ml/10^6 cells ± SD of triplicate cultures. Medium represents the chemically untreated control group. (**$P<0.01$ *vs* control groups).

3.3. VLX suppresses P-gp levels during DC maturation, but not mdr-1 gene expression

To verify the function of P-gp in DC, a Western blot analysis was used to detect changes in P-gp expression following treatment with specific inhibitors, including VRP, CsA, and VLX. Generally, these specific inhibitors of P-gp have been reported to block drug efflux and function of ATP-binding cassette protein subfamily B (ABCB) transport, but not down-regulate the protein expression [25–27]. In preliminary experiments, DC were treated with VRP, CsA, and VLX, and we observed no marked differences in the percentage of dead cells, as evidenced by CD11c$^+$ cell and annexin-V/propidium iodide (PI) staining (data not shown). As shown in Fig. 3A and B, we found that VRP and CsA did not affect expression of P-gp or *mdr-1* expression, but VLX treatment significantly decreased the expression level of P-gp. Moreover, VLX treatment suppressed P-gp expression in LPS-induced DC maturation (Fig. 3C).

3.4. P-gp suppressor inhibits DC maturation and activation through IL-12 production

The observed changes in expression of P-gp during DC maturation suggest that VLX exposure may lead to a profound regulation of the phenotypic and functional maturation of DC. We then investigated the effects of VLX on DC maturation. As shown in Fig. 4A, VLX treatment significantly attenuated surface expression of CD80, CD86, MHC classes I and II on the CD11c$^+$ cells in a dose-dependent manner in comparison to the control group. Exposure to VLX in the presence of LPS resulted in impaired expression of MHC classes I and II. Interestingly, a significant down-regulation of the costimulatory molecules, CD80 and CD86, was also observed under these conditions. It is known that inducing DC activation will result in the release of cytokines, including IL-1α, IL-6, IL-10, and IL-12, and this can be modulate DC-mediated T cell immune responses [28, 29]. IL-12 production was identified previously as a specific marker of DC activity, a critical marker for DC maturation, and can be used as a select Th1-dominant adjuvant [30]. We next assess the ability of DC to generate pro-inflammatory cytokines. To do this, we analyzed the production of cytokines following treatment with each P-gp blocker (such as VRP) and suppressor (such as VLX) from both immature and mature DC. As shown in Fig. 4B and C, DC stimulated with VLX expressed small amounts of IL-1α, IL-10, and IL-12, in comparison to unstimulated DC. Interestingly, the P-gp blocker VRP had no effect on cytokine levels during DC maturation. These data indirectly indicate that P-gp is necessary for DC maturation and activation.

3.5. P-gp of DC is required for DC-mediated T cells polarization and proliferation

Changes on surface molecule expression and cytokine production from P-gp modulated-DC show that the down-regulation of P-gp

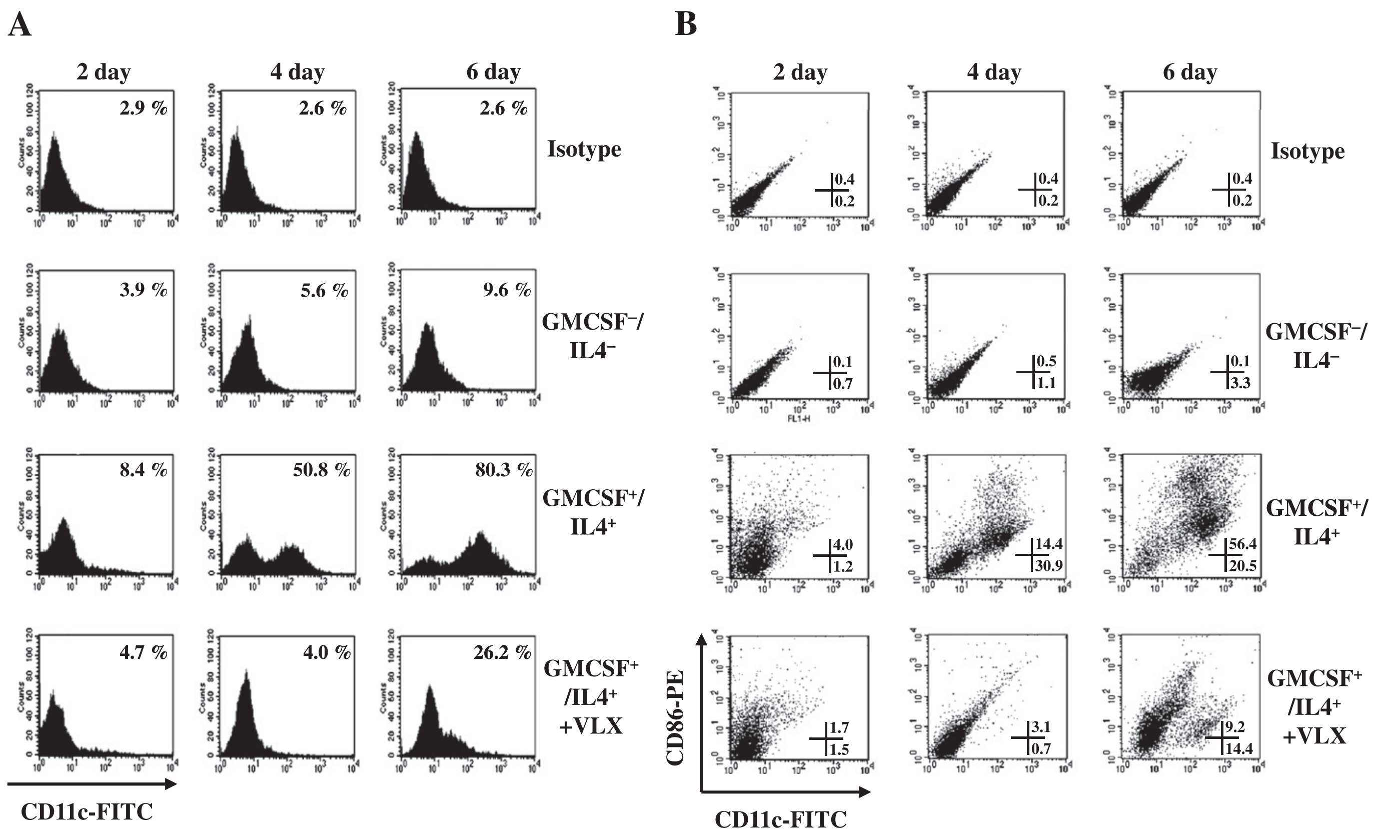


Fig. 6. The down-regulation of P-gp inhibits DC development and differentiation. As a measure of development and differentiation, we quantified the percentage of cells staining for each marker protein that was associated with the DC arbor. The DC marker proteins CD11c^{+} (A) and CD86 were already preferentially localized to the dendritic arbor on day 2, and their polarization increased to immature levels by day 6 (B). The percentage of cells expressing these proteins associated with the dendritic membrane increased significantly during DC development. DC were cultured with GM-CSF, IL-4, with or without VLX then collected and immunophenotyped on the days indicated. Cells were labeled with the designed monoclonal antibodies and analyzed by flow cytometry. The quadrants shown were set based on isotype control staining. The data are representative of three experiments that gave similar results.

leads to a profound inhibition of the phenotypic and functional changes associated with the maturation of DC. However, these results did not permit us to exclude the possibility that P-gp induces a general inhibition of the physiological functions of DC. Therefore, we sought to determine if VLX-induced decrease of P-gp on DC is associated with the reduced proliferation, differentiation, and cytokine production in T cells. More specifically, we attempted to determine whether the stimulation of DC with a P-gp suppressor alters the ability of the DC cells to activate T cells in a mixed lymphocyte reaction (MLR). As shown in Fig. 5A and B, the LPS-treated DC exhibited a far more profound proliferation than did the controls, whereas treatment with a P-gp suppressor appeared to impair to proliferation responses in the allogeneic T cells elicited by the LPS-stimulated DC. Significant inhibition of T cell proliferation occurred at concentrations of VLX as low as 25 μM in MLR stimulated assays. The concentrations of IL-2, IL-4, and IFN-γ measured in 72-h co-culture supernatants, when cell counts are approximately equal, were also reduced, suggesting a possible cytokine-dependent mechanism and inhibit DC maturation following treatment with P-gp suppressor (VLX).

3.6. P-gp suppressor blocks DC development

While the effects of P-gp suppressor treatment during DC development have not been previously investigated, our data suggest that P-gp is a very important to DC differentiation, maturation, and T cell immunity. To investigate possible roles of P-gp in DC development, a P-gp suppressor, at nontoxic does, was added to interstitial DC cultures. The addition of the P-gp suppressor VLX markedly inhibited DC development as seen by CD11c-FITC values (Fig. 6). In addition, when analyzing marker surface expression, VLX treatment had suppressive effect on CD86 expression. The DC marker proteins such as CD11c and CD86 were already preferentially localized to the DC membrane during DC development. Moreover, the percentage of cells expressing these proteins associated with the dendritic membrane increased significantly during DC activation.

4. Discussion

This is, to the best of our knowledge, the first report of the effects of P-glycoprotein (P-gp) expression levels on the phenotype, and the differentiation and development capabilities of murine bone marrow-derived DC. The aim of the work described here was to investigate whether regulation of P-pg, a protein previously shown to be involved in the migration of mature DC, altered the development of DC and DC-mediated T cell immunity [16], the functional and development capabilities have not been assessed. The physiological role of P-gp is not completely understood; however, it has been related to multi-drug resistance in cancer cells [31], T cells immunity [32], and DC migration [16]. Previous reports show that P-gp is a trans-membrane protein, a molecule of the ABC transporter superfamily, and is well-known that it mediates efflux of chemotherapeutic agents from the intracellular environment, thereby contributing to drug resistance [33, 34].

In this study, we suggest that P-gp expression alters physiological functions of DC. Down-regulation of P-gp inhibits the maturation and activation of DC, as demonstrated by low expression of costimulatory molecules on DC surfaces and the reduced cytokine levels, including IL-1α, IL-10, and IL-12 (Fig. 4). Moreover, the production of IFN-γ, IL-4, and IL-2 cytokines was significantly decreased in P-gp suppressed-DC in

a co-culture system with T cells (Fig. 5). Functionally, the down-regulation of P-gp in DC inhibited primary T cell stimulatory activity in an allogeneic MLR, and drives Th1/Th2 polarization in both $CD4^+$ and $CD8^+$ T cells.

DC are the most potent APC that amplify and initiate immune responses. In addition, DC play a crucial role in peripheral tolerance mechanisms, induce different types of immunity and tolerance, recognizing tumors, while capable of preventing immune responses against environmental antigens and self tissues [3, 33, 35–38]. Moreover, DC are among the benign cells affected by anti-cancer drugs, inducing an immune deficient state [39]. Therefore, we investigated the function role of P-pg and the influence of anti-cancer drugs on DC function.

Here, we show that the down-regulation of P-gp inhibits expression of CD80, CD86, MHC I, and MHC II on DC. Moreover, P-gp suppressor (VLX)-treated DC expressed reduced levels of IL-1α, IL-10, and IL-12 following LPS-induced maturation (Fig. 4). These results suggest that P-gp directly inhibits maturation and activation of DC. In addition, we report that DC differentiation requires *mdr-1*, but not *mdr-3*. Down-regulation of P-gp following VLX treatment during DC differentiation resulted in alteration of cell morphology and phenotype (Figs. 1 and 2). When DC were cultured in the presence of a P-gp suppressor during development, cells displayed decreased levels of $CD11c^+$. In addition to phenotypic and morphological changes, the down-regulation of P-gp in DC induced to differentiate with LPS treatment resulted in altered DC function. Specifically, these DC exhibited lowered secretion of IL-1α and IL-12p40p70, and a reduced capacity to stimulate allogeneic T cell proliferation. This reduction in IL-12 production suggests that these cells might be weaker in inducing a Th1 response. Taken together, this report shows that murine DC expresses the ABC-transporter P-gp on the plasma membrane (Fig. 1C and D), and was capable of actively extruding chemotherapeutic drugs from the cells. We further propose that P-gp activity is required for DC development and differentiation, and when combined with prior reports on its requirement in DC migration, this transporter becomes even more interesting as a potential target in DC-based immunotherapies. For example, increasing P-gp expression in DC could perhaps result in more potent APC, allowing for use as a tool combined with chemo-immunotherapy treatments.

In conclusion, down-regulation of P-gp by VLX inhibits the differentiation of DC and cytokine production, such as IL-1, IL-10, and IL-12 during DC maturation. Moreover, the P-gp-decreased DC inhibits T cell proliferation and cytokine production. In addition, these observations are important to consider when developing new P-gp modulators and the development of therapeutic adjuvants for the treatment of DC-related acute and chronic diseases.

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