The detailed analysis of the changes of murine dendritic cells (DCs) induced by thymic peptide pidotimod(PTD)

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Abbreviations: PTD, pidotimod; ACP, acid phosphatase; DCs, dendritic cells; LPS, lipopolysaccharides; MTS, 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl)tetrazolium; SEM, scanning electronic microscopy; TEM: transmitted electron microscopy; DAB, 3,3'-diaminobenzidine; PBS/2% FACS, 98%PBS+2% fetal calf serum; G mean, Geometric mean.

The aim of present research is to analyze the detailed changes of dendritic cells (DCs) induced by pidotimod(PTD). These impacts on DCs of both bone marrow derived DCs and established DC2.4 cell line were assessed with use of conventional scanning electron microscopy (SEM), flow cytometry (FCM), transmission electron microscopy (TEM), cytochemistry assay FITC-dextran, bio-assay and enzyme linked immunosorbent assay (ELISA). We demonstrated the ability of PTD to induce DC phenotypic and functional maturation as evidenced by higher expression of key surface molecules such as MHC II, CD80 and CD86. The functional tests proved the downregulation of ACP inside the DCs, occurred when phagocytosis of DCs decreased, with simultaneously antigen presentation increased toward maturation. Finally, PTD also stimulated production of more cytokine IL-12 and less TNF- α . Therefore it is concluded that PTD can markedly exert positive induction to murine DCs.

Introduction

PTD is a synthetic thymic dipeptide with immunological activity on both innate and adaptive immune systems.^{1,2} In vivo studies, both from animal and human data, have documented PTD with a good activity on innate and adaptive immune responses and improved immune suppression.³⁻⁵ These activities have been applied in clinical treatment for patients with various kinds of infections, in improving the immune defense during infections, situation of immune dysfunction and other immune handicapped cases, such as cancers.⁶ Other studies demonstrated that PTD displayed an immunopotentiating activity on T cells, macrophages and granulocytes.⁷

Dendritic cells (DCs), originally identified by Steinman (1972),⁸ represent the pacemakers of the immune response. DCs are potent antigen presenting cells (APCs) that possess the ability to stimulate naïve T cells.⁹ Therefore they are crucial for the induction of T cell responses that result in cell-mediated immunity. Our previous work also provided evidence that the more matured the DCs was, the stronger the antigen presentation of DCs would be.^{10,11} Recently substantial number of articles showed great importance of DCs in both cancer therapy and

vaccine preparations.^{12,13} For instance, The DCs loaded with mRNA of a tumor antigen elicit higher cytotoxicity of T cell.⁹ However, there is so far, no report on either the concrete mechanisms or maturation changes of DCs post treatment with PTD, except a primitive study.^{14,15} The detailed changes both inside and outside DCs after treatment with PTD remains unclear. So we conducted the study to investigate and analyze the changes of murine DCs induced by PTD, in order to gain insight and a deeper understanding of the mechanisms through which PTD would be working on DCs.

Results

Determination of the DCs' growth curve by method of MTS. PTD affects the growth rate of DCs2.4 Cells. Under the influence of a range of concentrations of PTD, the DCs2.4 cells proliferated into both differential colonies, and various degrees of maturity. The most optimal concentration of PTD to boost cell proliferation is 800 μ g/ml as shown in **Figure 2**. Under 800 μ g/ml the number in the PTD group yielded 0.827 ± 0.035 , while the number in the RPMI-1640 group yielded 0.501 ± 0.085 ; and the number in the LPS group yielded 0.974 ± 0.052 .

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Figure 1. The CD11c+ cell purification with MACS. After co-culture for 6 d, the purity of CD11c+ cells were examined and the percentage were over 80%. Followed by purification with MACS, the CD11c+ cells were enriched and the results of FCM showed that the purity CD11c+ cells approached 95%.

Figure 2. The curve of the DCs' proliferation after treatment with a range of concentrations of PTD for 48h determined by method of MTS.

Morphology of PTD treated BMDCs and DC2.4 cells. BMDCs after treatment with GM-CSF and IL-4 for 6 d were observed with an inverted phase contrast microscope. The cells (Fig. 3A) showed the mature DCs were large cells with oval or irregularly shaped nuclei and many small dendrites, which were more in the PTD and LPS group compared with the cells in control group.

DCs2.4 cells morphology from secured SEM photos were compared before and after treatment with PTD. The results demonstrated that the minimal cellular adhesion in RPMI1640 group and cellular adhesion in LPS group resulted in the extensive formation of dendrites, processes, as well as ruffling, while that in testing group was intermediary between the two groups. This evidence proved that DCs after treatment with PTD exhibited more matured shape with more protrusions and more cascading folds than untreated DCs did (Fig. 3B).

TEM for DCs2.4 cells' intracellular study. Usually immature DC is stronger in phagocytosis and contains more phagosomes. When DC becomes matured the numbers of phagosomes inside the DC reduce with increased antigen presentation.

The photos from TEM were compared with control. The results demonstrated that there was greater reduction for

phagosomes inside the mature DC than those inside the immature DC as shown in Figure 4.

Acid phosphatase (ACP) activity detection. The activity of ACP represents the DC's ability to digest antigens and its number reflect the maturity of DC via an inverse relationship.

The ACP activity of the DCs was measured by the method illustrated above and yielded the following activity number: 46.012 ± 1.301 U/gprot in the LPS group and 55.760 ± 2.390 U/gprot in the PTD group ($p < 0.01$) vs. 67.832 ± 3.213 U/gprot in the RPMI 1640 group as shown in Figure 5. This is consistent with the number of phagosomes inside the DC. That meant after the DCs were treated with 800 $\mu\text{g/ml}$ PTD for 48 h, immature DCs became matured with a marked decrease in ACP activity indicating a gradual termination of phagocytosis or antigen ingestion, and accordingly, an increased antigen presentation. This also led to the more production of IL-12, which would work as an intensified signal to initiate CD4+T cells response.

Confirmation by FCM of the DCs' phagocytic effect. Immature DC is stronger in phagocytosis and contains more phagosomes. When phagocytosis inside DCs2.4 cells took place DCs2.4 cells phagocyte FITC labeled Dextran with higher G means. When DCs2.4 cells were cultured in 800 $\mu\text{g/ml}$ PTD for 48 h, they matured with decreased G means, as reflected in Figure 6. Concretely, The number in the PTD group yielded 85.968 ± 1.342 , 98.393 ± 3.034 in the RPMI-1640 group and 82.730 ± 2.424 in the LPS. This demonstrated DCs maturation after treatment with PTD.

Immunohistochemical staining of the DCs2.4 cells after treatment with PTD. After staining with DAB kit, and restaining with hematoxylin, the DC under light microscope displayed phagocytosing. The photos showed phagocytosing horseradish peroxidase was in process, and showed more phagocytosis in the immature DCs2.4 cells than that in the mature DCs 2.4 cells as shown in Figure 7. This is consistent with the ACP activity and also with phagocytic effect by DC above.

FCM analysis. *Analysis by FCM of the BMDCs' key surface molecules.* The BMDCs were cultured and induced in complete RPMI 1640 medium supplemented with a given dose of GM-CSF plus IL-4 for 6 d. The results of FCM analysis showed that the percentage of MHC II expression on PTD-treated BMDCs were 2-fold more than those on control. Simultaneously with the increased percentages of CD86 and CD40 on the PTD-treated BMDCs were detected out.

The data shown in Figure 8A indicated the distribution of the expression of the key surface markers. Namely, MHC-II yielded $52.43 \pm 1.742\%$ in the PTD group ($p < 0.01$) vs. $10.52 \pm 1.691\%$ in the RPMI1640 group, and $53.65 \pm 0.824\%$ in the LPS group. Similarly, CD86 yielded $43.81 \pm 2.075\%$ in the PTD group ($p < 0.01$) vs. $20.96 \pm 1.357\%$ in the RPMI1640 group, and $43.26 \pm 1.966\%$ in the LPS group. And CD40 yielded $44.23 \pm 2.008\%$ in the PTD group ($p < 0.01$) vs. $14.77 \pm 0.957\%$ in the RPMI1640 group and $53.80 \pm 0.364\%$ in the LPS group. These results also reflected maturation of DCs after treatment.

Analysis by FCM of the DC2.4 cells' key surface molecules. After cell culture for 48 h, the cells were harvested for FCM analysis.

As reflected in increased expression of the key surface markers of MHC-II, CD86 and CD40. These molecules collaborate in triggering T cell activation.

The data shown in **Figure 8B** indicate the distribution of the expression of the key surface markers. Namely, MHCII yielded $90.71 \pm 2.031\%$ in the PTD group ($p < 0.01$) vs. $67.50 \pm 1.264\%$ in the RPMI1640 group, and $29.94 \pm 0.860\%$ in the LPS group. Similarly, CD86 yielded $89.62 \pm 1.698\%$ in the PTD group ($p < 0.01$) vs. $66.68 \pm 1.680\%$ in the RPMI1640 group, and $94.35 \pm 1.743\%$ in the LPS group. And CD40 yielded $15.41 \pm 0.836\%$ in the PTD group ($p < 0.01$) vs. $7.55 \pm 0.660\%$ in the RPMI1640 group and $16.20 \pm 0.465\%$ in the LPS group. These were corresponding to the results on BMDC.

Cytokine assay of the IL-12p70, IL-12p40 and TNF- α by ELISA. After the immature DC2.4 cells were exposed to $800 \mu\text{g/ml}$ for 96 h, the cultured DCs2.4 increased in cell numbers with simultaneous morphology maturation. In addition to the higher expression of key surface markers, the cultured DCs2.4 also produced higher levels of IL-12p70, with the following distributions: $289.000 \pm 11.303 \text{ pg/ml}$ in the PTD group, ($p < 0.01$) vs. $256.833 \pm 7.243 \text{ pg/ml}$ in the RPMI1640 group, and $310.833 \pm 4.933 \text{ pg/ml}$ in the LPS group as shown in **Figure 9A**.

Similarly, the production of IL-12p40 was with the following distributions: $388.500 \pm 19.634 \text{ pg/ml}$ in the PTD group, ($p < 0.01$) vs. $353.000 \pm 13.435 \text{ pg/ml}$ in the RPMI1640 group, and $459.500 \pm 24.003 \text{ pg/ml}$ in the LPS group as shown in **Figure 9B**.

The production of TNF- α was with the following distributions: $23.467 \pm 6.503 \text{ pg/ml}$ in the PTD group, ($p < 0.01$) vs. $62.600 \pm 7.000 \text{ pg/ml}$ in the RPMI1640 group, and $35.467 \pm 5.853 \text{ pg/ml}$ in the LPS group as shown in **Figure 9C**.

Evidently, These result shown in **Figure 8** are consistent with those observed in morphological maturation of the DCs2.4 and data from ACP test above, showing functional maturation.

Discussion

PTD, as an important immune modulator, plays an important role in both immune modulation and immune boosting based clinical treatment.¹⁶⁻¹⁹ Besides these, the influence of this enhancer is shared between innate and adaptive immune systems. In fact, PTD's function of upregulating immune system at suitable range of doses has been reported previously.²⁰ The data of their findings proved that PTD could upregulate the functions of both innate immune and the adaptive immune cells, including: significantly increasing phagocytosis of mononuclear-macrophage in mouse,⁷

improving B lymphocyte proliferation induced by LPS, enhancing the activity of monocyte by upregulating the expression of HLA-DR and other application for various cases clinically.²¹⁻²⁵

In recent years, the research data on PTD revealed that PTD alone or when combined with other drugs can inhibit tumors, resulting in marked regression of tumor cell growth and delivery systems of PTD to the body, such as water-in-oil-in-water double emulsions, an excellent delivery system of PTD in rats has been developed to optimize bioavailability.²⁶ In China, both in the past and present, this clinical application has been an alternative for patients with terminal cancer.

The work was based on our previous results^{27,28} and we optimized some methods so that better resolution was reached. From the data obtained above, we conformed that PTD's modulating effects on DCs were present and functional. In fact, PTD can markedly induce the maturation of DCs with the following evidence: (1) PTD showed bilateral regulation on DCs, which meant at both higher and lower range of concentrations PTD inhibit the DCs growth in some extent. Concretely when concentration was over $1600 \mu\text{g/ml}$ PTD inhibit growth of DCs and also same trend was found when concentration of PTD is below $200 \mu\text{g/ml}$. This happens to most of immunoregulators or modifiers because when in lower concentration of PTD the receptors on the DCs are not saturated with binded PTD and when in much higher concentration of PTD all the receptors on the DCs are stuffed with no signal transmitted into inside the DCs to initiate gene activity. In the range of $1600 \mu\text{g/ml}$ - $200 \mu\text{g/ml}$ PTD showed boosting effect to DC 2.4 cell and at the concentration of $800 \mu\text{g/ml}$ shows utmost boosting effect (**Fig. 2**); (2) the changes of morphology

Figure 3. Morphology of the BMDCs before and after treatment with PTD under a light microscope (A) ($\times 400$). The images of DC2.4 cells before and after treatment with PTD with SEM (B) ($\times 3500$).

Figure 4. TEM for changes of lysosomes inside the DCs2.4 cells before and after treatment with PTD.

of matured DC are with more protrusions as well as rougher surface (Fig. 3), which usually indicates increased expression of key surface markers or receptors; (3) reduction of phagosomes inside the DC (Fig. 4) and ACP activity (Fig. 5) are usually, parameters indicating the degree of maturation of DC, and an indicators of phagocytosis reduction (Fig. 7), with an increase in antigen

presentation, which is consistent with higher expression of MHC class II, CD86 and CD40 molecules (Fig. 8), (signals between the T cell receptor and co-receptor paths); (4) higher production of IL-12 levels (Fig. 9) which will intensify DC-CD4+T cell pathway, resulting in increased Th1 responses. Also the presence of IL-12 is necessary for CD4+T cell activation which in turn secretes other cytokines such as IL-2, IFN- γ and TNF- β , coordinating cellular immune responses. Meanwhile, IL-12 is also responsible for triggering a chain of immune responses in the immune network. Simultaneously, proinflammatory TNF- α secretion is downregulated in the ongoing process of the DCs' maturation. Usually at early stage of infection DCs secrete higher amount of TNF- α to be involved in initiating innate immunity. However, with the infection going on DCs become matured and begin to trigger adaptive immunity through mounting antigen presentation and accordingly, followed by the decreased production of TNF- α .

The morphology study by SEM, TEM for sub-cellular study and histochemistry study above, plus ACP test after treatment with 800 ug/ml PTD confirmed and reconfirmed the boosting effect to DC maturation by PTD consistently.

Additionally, it may be noted that the upregulation of IL-12 secretion not only serves DCs themselves as an autocrine and paracrine signal molecule, resulting in DCs expansion further, but also would augment the increase of CD4+T cells, and increases stability of the pathway between the DCs and CD4+T cells, which, in turn, will result in more secretion of cytokines like IL-12 by the DCs and IFN- γ by the CD4+T cells. In this way, PTD may initiate a chain of immune responses in body.²⁶

As we know, the immune system is a very complicated and diverse entity, in which numerous immune cells are involved, formulating a coordinated interaction between innate immune and adaptive systems. Moreover, there are internal interactions among the immune cells through a variety of cytokines that regulate or even police each other as part of a dynamic immune system.²⁹ And when PTD works, it can regulate DCs not only via a direct route, but also via an indirect route by triggering other immune cells such as macrophages, which can secrete high levels of IL-12, adding a synergistic effect to DCs. NK cells can also secrete IFN- γ , which can then induce the maturation of DCs and, as a result, more IL-12 is then secreted to form an immune network maintaining the balance of the body.

Now DCs have emerged as the most powerful and professional antigen presenting cells with the ability to stimulate naïve T cells and initiate T cell responses, coordinating as messengers between the innate and adaptive immunities. They can thus potentially be used in therapeutic vaccines in cancer immunotherapy and for other immune handicapped diseases.

This work could therefore contribute to the better understanding of PTD's active modulating effects on immune system, and its complicated mechanisms at subcellular or molecular level even, through which, PTD would be working. At last, this work also provides a meaningful mode of action for PTD and highlights its clinical significance as an immunotherapeutic medicine for cancer in restoring damaged immune system of cancer patients after chemotherapy.^{30,31} Furthermore, we could use PTD

Figure 5. The ACP activity of the DCs2.4 cells after treatment with 800 ug/ml PTD for 48 h.

as a potent adjuvant in vaccine formulations against human threatening diseases like AIDS.

Materials and methods

Reagents. PTD (> 99% purity) was a gift from Sunstone pharmaceutical company, Tangshan, China. The effect of a range of concentrations of PTD from 200 µg/ml to 1600 µg/ml on the proliferation of DCs in vitro was tested, and the optimal concentration was found to be 800 µg/ml. Based on these studies, the optimal concentration was used in current study. IL-4 and GM-CSF were obtained from PeproTech Inc. The mAbs used in this study include FITC-conjugated anti-CD40, PE-anti-MHC-II and PE-anti-CD86, which were purchased from eBioscience and BD PharMingen. The ELISA assay kits for IL-12 and TNF-α were purchased from eBioscience. Lipopolysaccharide (LPS) was a product of Sigma-Aldrich. Other chemicals frequently used in our laboratory were all from Sigma-Aldrich or BD PharMingen.

Preparation of bone marrow-derived dendritic cells (BMDCs). The preparation of BMDCs was adjusted from a previously described method.^{27,32} Briefly, bone marrow cells from the femurs and tibias of female C57BL/6 mice (4–6 weeks old from pathogen free animal house, China Medical University) were first depleted of red cells with lysis buffer. Approximately 106 cells were then placed in 24-well plates in 1 ml of RPMI 1640 supplemented with 10% fetal bovine serum, recombinant murine granulocyte macrophage colony stimulating factor (GM-CSF) (10 ng/ml), interleukin (IL-4) (10 ng/ml), 2 mM L-glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin. Cells were incubated for 4 h. Plates were then gently swirled and the medium containing non-adherent cells was removed and replaced with fresh medium as described above. Supplemented medium was replaced every three days and on day 6 of culture, all the cells expressing CD11c in the different wells were isolated respectively using MACS (Miltenyi Biotec) according to the manufacturer's instruction and seeded into new wells with fresh medium. Finally, the CD11c-positive cells were treated with or without PTD at a dose of 800 µg/ml another 48 h in order to get mature BMDCs. Purity of the sorted cells were confirmed by fluorescence-activated cell sorting (FCM)

Figure 6. Confirmation by FCM of the DCs2.4 cells' phagocytic effect. Results represent the mean ± SD of three independent samples.

analysis (Fig. 1). Finally the separated BMDCs were used for the phenotypic studies.

DC2.4 cells culture. The murine DCs 2.4 cell line was an immature one established through the transfection of GM-CSF, myc and raf genes into the C57BL/6 mouse.²⁸ It was kindly donated by Dr. Feili GONG from Hubei Medical University, China, and was passaged several times in our laboratory.

The DCs2.4 cells were grown in RPMI1640 supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 1.2% sodium bicarbonate. All media contained antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin). Unless otherwise indicated, the cells were grown in a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Cells were plated and counted 24 h later to determine the seeding efficiency before experimentation.

MTS assay for effect on DCs2.4 cells proliferation by a range of concentrations of PTD. DC cells were digested into cell suspension and the concentration of the DCs was adjusted to 1 × 10⁵ / ml. DCs2.4 cells were added to 96 well microplates, 100 µl/well, and triplicate per well.

PTD was dissolved in RPMI1640 medium, filtrated, and diluted at range of concentrations from 200 µg/ml to 1600 µg/ml. LPS (10 ng/ml) was used as positive control in this study. The absorbance of each well was measured at 570 nm (A570), using a dichromatic microplate reader after treatment with PTD for 48 h by method of MTS assay for cell proliferation.

Scanning electron microscopy (SEM) for morphology of the DCs2.4 cells. The cultured DCs2.4 cells, after treatment with 800ug/ml pidotimod for 48 h, were collected and checked for morphological study by SEM (JEOL, JSM-7300). Cell preparation steps include glutaraldehyde fixation, cleaning, osmium tetroxide post-fixation, cleaning, replacement, critical point drying, and spraying gold. Finally, the prepared samples were analyzed by SEM.

Transmitted electron microscopy (TEM) for intracellular phagosomes inside the DCs2.4 cells. The cultured DCs2.4 cells, after treatment with 800 ug/ml PTD for 48 h, were collected and checked for intracellular phagosomes by TEM (JEOL, JSM-7300). Cell preparation steps include cultured cell digestion by trypsin, centrifugation at 1000 RPMI for 10 min, glutaraldehyde fixation, slicing and finally, the prepared samples were analyzed by TEM.

Acid phosphatase (ACP) activity detection. The concentration of DCs2.4 cells was adjusted to 1 × 10⁶ /ml. The ACP

Figure 7. Immunohistochemical staining of DCs2.4 cells after treatment with PTD.

activity inside the DCs after treatment with 800 $\mu\text{g/ml}$ PTD for 48 h was measured at OD 520 nm by the phenol-4-AAP (amino anti-pyrene) method in conjunction with ACP testing kit (Jiancheng Bio-engineering Institute of South).

Confirmation by FCM of the DCs2.4' phagocytic effect. The cultured DCs2.4 cells, after treatment with 800 $\mu\text{g/ml}$ PTD for 48 h, were collected and tested for phagocytic effect. 100 μl FITC-Dextran was added to the DC culture, incubated at 4°C for 2 h, subsequently at 37°C for 1 h and finally samples were checked by FCM of the DCs' phagocytic effect.

Cellular immunohistochemistry of phagocytosis by DC. The DCs2.4 cells at $1 \times 10^5/\text{ml}$, treated with 800 $\mu\text{g/ml}$ PTD were grown for 48 h. Cell preparation steps include 0.08 mg/ml horseradish peroxidase was added to the culture, followed by fixation with methanol for 4 h, staining with DAB kit, restaining with hematoxylin. Finally, the sample was observed under light microscope.

Analysis by flow cytometry (FCM). The cultured BMDCs and DCs2.4 cells served as both testing groups (after treatment with 800 $\mu\text{g/ml}$ PTD for 48 h) and control groups were respectively collected and stained with anti-CD40, anti-CD86 and anti-MHC II antibodies for 30 min for optimal staining, and then washed twice with PBS/2% FACS. The cells were harvested and immediately fixed in 4% paraformaldehyde, and subsequently were collected using FACS Calibur (Becton Dickinson). The data, obtained from the analysis of the fixed cells by FCM, were then analyzed using WinMDI2.9 (Joseph Trotter, BD Biosciences).

Cytokine assay. The DCs2.4 culture supernatant after treatment with 800 $\mu\text{g/ml}$ PTD for 96 h was collected for IL-12p70/IL-12p40 and TNF- α assaying. Per the instruction included in the ELISA kit, the concrete steps were done in a double antibody sandwich. The absorbance at 450 nm (A_{450}) was determined using a dichromatic microplate reader.

Statistical analysis. Statistical analysis was performed using the statistical program SPSS (Statistical Package for Social Sciences, Version 16.0) for Windows. All variables are presented as mean $\pm$ sd. Differences were evaluated by ANOVA for multiple groups and by the student t-test two groups using the Prism (Graph Pad Software). Tukey test used for post hoc analysis indicated significance when $p < 0.05$ by ANOVA.

Conclusion

As far as we know, this is the first ever publication to exhibit data about detailed analysis of morphology, cellular, subcellular and molecular structure of DC, which help us with understanding positive modulation by PTD to DC through mechanism explained above. PTD can markedly enhance DC maturation and function, supplying extra IL-12 and MHC class II molecules, plus other co-stimulatory molecules to the upregulation of antigen presentation and thus potentially activating CD4+T cell, resulting in a marked enhancement in the DC-CD4+T cell pathway. In other words, PTD can exert positive modulation on DCs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Figure 8. Upregulation of key surface molecules on BMDCs (A) and DCs2.4 cells (B) after treatment with PTD for 48 h. The cells were respectively collected and stained with mAbs to CD40, CD86, and MHCII. Expression of surface markers was analyzed by FCM, which was displayed respectively by the single parameter diagrams. The values shown in the profiles were the gated % and the mean fluorescence intensity indexes (MFI). Results represent the mean $\pm$ SD of three independent samples.

Figure 9. (A, B) The production of IL-12p70, IL-12p40 by the DCs 2.4 cells after treatment with PTD. The histograms above showed the IL-12p70, IL-12p40 production levels after PTD stimulation. Results represent the mean $\pm$ SD of three independent samples. (C) The production of TNF- α by the DCs 2.4 cells after treatment with PTD. Results represent the mean $\pm$ SD of three independent samples.

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