Rapamycin enhances IFN-γ and IL-4 production in co-culture of gδ T and dendritic cells from mice with lipopolysaccharide-induced acute lung injury

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ABSTRACT. This study aimed to study the role of rapamycin (RAPA) in modulating the interaction between gδ T cells and dendritic cells (DCs) in a lipopolysaccharide (LPS)-induced acute lung injury mouse model. Mice were injected with LPS to establish the acute lung injury model or LPS + RAPA to assess the role of RAPA in modulating cell interactions. Mice were injected with PBS or RAPA alone as controls. gδ T cells and DCs were isolated from all mice and assessed by flow cytometry and fluorescence microscopy. The isolated gδ T cells and DCs were cultured independently or co-cultured to study their interactions. Enzyme-linked immunosorbent assay was performed to assess the expression of the cytokines, namely, interferon (IFN)-γ, interleukin (IL)-4, tumor necrosis factor (TNF)-α and IL-12 in the individually cultured or co-cultured gδ T cells and DCs, and reverse transcription-polymerase chain reaction (RT-PCR) was employed to investigate the levels of relevant mRNAs. Our study found that co-culture of gδ T cells and DCs from mice treated with LPS + RAPA have reduced expression of IFN-γ and IL-4 (but not TNF-α and IL-12) compared to mice treated with LPS only. These results were confirmed by RT-PCR, where the levels of IFN-γ and IL-4 mRNA were also reduced. This study may provide useful information in understanding the interaction between gδ T cells and DCs in the LPS-induced lung injury model in mice.

Key words: gδ T cell; Dendritic cell; LPS-induced lung injury model; ELISA; RT-PCR

INTRODUCTION

gδ T cells have attracted considerable attention in immunotherapy in recent years because of their prominent function in the rehabilitation of different diseases (Kobayashi et al., 2007; Gertner-Dardenne et al., 2009; Eberl et al., 2014; Latha et al., 2014). gδ T cells are a minor population of T cells that express a distinct T cell receptor (TCR) composed of gδ chains instead of aβ chains. Distinct from aβ T cells, gδ T do not require major histocompatibility complex (MHC) presentation of peptide epitopes but retain the ability to recognize ligands that are generated during disease (Mombaerts et al., 1993; Brandes et al., 2005; Chen et al., 2008). Therefore, these cells are an important link between innate and adaptive immunity (Morita et al., 2001; Cao and He, 2005; Holtmeier and Kabelitz, 2005; Zhao et al., 2006; Beetz et al., 2008). For example, gδ T cells in the skin epidermis can recognize an antigen expressed by damaged or stressed keratinocytes, playing an indispensable role in tissue homeostasis and repair through secretion of distinct growth factors (Ebert et al., 2006). Dendritic cells (DCs) are the most important antigen-presenting cells of the mammalian immune system, which bridges the innate and the adaptive immune systems (Holtmeier and Kabelitz, 2005). DCs and gδ T cells have been reported to interact with each other either via TCRs or regulatory secreted cytokines (Born and O'Brien, 2009; Davey et al., 2014). For example, in *Streptococcus pneumoniae*-induced inflammation, gδ T cells can resolve inflammation by regulating DCs and macrophages (Davey et al., 2014). gδ T cells have also been reported to recognize lipid A by the presentation of the glycoproteins CD1b or CD1c on DCs (Cui et al., 2009). Despite these studies, the interaction between gδ T cells and DCs in lipopolysaccharide (LPS)-induced acute lung damage has not been examined.

Rapamycin (RAPA) is a pharmacological drug developed from filamentous bacteria (Dumont and Su, 1996). It can suppress the immune functions of cells, including T cells and DCs, by binding to the intracellular membrane-bound mammalian target of rapamycin (mTOR) complex (Dumont and Su, 1996). It is also capable of inhibiting cytokine production by DCs and reducing the expression of MHC class II and co-stimulator molecules on DCs (Fischer et al., 2011). *In vivo* studies have shown that RAPA increases transplant acceptance by generating $CD4^{+}CD25^{+}FoxP3^{+}$ regulatory T cells and effector T cells (Turnquist et al., 2007). RAPA has also been reported to diminish the production of interferon (IFN)-γ in antigen presenting cells (Jin et al., 2010).

This study examines the role of RAPA in modulating the interactions between gδ T cells and DCs, which were isolated from mice injected with PBS, LPS, RAPA or LPS +

RAPA. Enzyme-linked immunosorbent assay (ELISA) and reverse transcription-polymerase chain reaction (RT-PCR) were used to assess the expression or mRNA levels of cytokines, respectively. Our study shows that co-culture of gδ T cells and DCs from mice treated with LPS + RAPA have reduced expression of IFN-γ and interleukin (IL)-4 [but not tumor necrosis factor (TNF)-α and IL-12] compared to mice treated with LPS alone. This study may provide useful information in understanding the interaction between gδ T cells and DCs in a LPS-induced lung injury model.

MATERIAL AND METHODS

Materials

C57BL/6 mice (~20 g, 6-8 week old) were purchased from Charles River Laboratories (Beijing, China). Pelltobarbitalum natricum, LPS, and RPMI-1640 medium were obtained from Sigma (Shanghai, China). Recombinant human interleukin-2 was purchased from SF Pharmaceuticals Inc. and penicillin/streptomycin and fetal bovine serum (FBS) were obtained from Tianjin Hematosis Hospital (Tianjin, China). Anti-CD3 (FITC-labeled), anti-gδ (PE-labeled), PE-labeled rat IgG2bκ isotype, and FITC hamster IgG2κ isotype were obtained from BD Bioscience (Beijing, China). Bovine serum albumin was from Shanghai AiYan Biotech (China). RAPA was from Huabei Pharmaceutical Corp (China), and gδ T cell isolation beads and FITC-labeled CD11b beads were obtained from Miltenyi Biotech (USA). IL-4, IFN-γ, IL-12, and TNF-α ELISA kits were obtained from Beijing Jingmei Biotech (China). SYBR green was purchased from ABI Inc. (USA).

Animals

Twenty-four mice were divided randomly into 4 groups (N = 6 each): PBS, LPS, RAPA, and LPS + RAPA. LPS or PBS was injected by tracheal instillation in soluble form (10 mg/kg) and RAPA was injected intraperitoneally (4 mg/kg). All animal experiments were under the regulation of the animal protection board of Capital Medical University hospital.

Tissue collection and histological examination

Mice were sacrificed 1 day after injection and the bronchoalveolar lavage fluid was collected. Cells were collected from spleen and lung and then cultured in RPMI 1640 cell culture medium supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin. The weight of lung tissue was measured as follows: weight of tissue was measured immediately after isolation and then the tissue was dried at 70°-80°C for 72 h and weighed again. The dissected lung tissue was stained with hematoxylin and eosin and observed in paraffin section under a microscope. The thickness of the alveolar septum was calculated as: (No. of pixels in the pathological tissues) x 100 / (total No. of pixels in the image).

gδ T cell and DC isolation

T cells from mouse spleen were isolated with magnetic beads by positive selection, followed by further addition of magnetic beads for gδ T isolation. CD11b DCs were isolated from mouse lung with magnetic beads by positive selection. Both isolation procedures were performed according to the manufacturer protocols.

Cell staining

The DCs were collected by centrifugation (500 *g*, 5 min) and stained with 2.5 μL FITC hamster IgG2κ isotype control (200X dilution, 2 μg/μL), 1 μL PE rat IgG2bκ isotype control (200X dilution, 0.5 μg/μL), 2.5 μL anti-CD3 (200X dilution, 0.2 μg/μL), and 1 μL PE-labeled anti-gδ (200X dilution, 0.5 μg/μL). gδ T cells were stained with anti-CD3 (PE-labeled, 400X dilution, 0.2 μg/μL) and anti-gδ T (FITC-labeled, 400X dilution, 0.2 μg/μL), and then examined by flow cytometry.

Cell co-culture

Spleen gδ T cells and lung DCs were co-cultured. Cells were cultured on 24-well plates and were divided into 4 groups according to their treatment: PBS, LPS, RAPA, and LPS + RAPA groups. Each group contained three types of samples: gδ T cells (10^6 cells/mL), lung DCs (10^6 cells/mL), co-culture of gδ T cells, and lung DCs (10^6:10^6 cells/mL). These experiments were repeated in quadruplicate.

ELISA

Production of IL-4, IFN-γ, IL-12, and TNF-α was examined according to the manufacturer protocols.

RT-PCR

The following materials were used for RT-PCR: 12.5 μL power SYBR green master mix (10 μM), 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM), 8.5 μL double-distilled H_2O (RNase-free), and 2 μL cDNA. The extraction of RNA and reverse transcription of RNA to cDNA was performed as follows: RNA was extracted with Trizol kits (Thermo scientific, USA) and UV-Vis absorbance (ratio of 260 and 280 nm) was used to quantify the RNA, where $2.0 \geq A280/A260 \geq 1.5$ was considered a quality sample. To assess the levels of IFN-γ, IL-4, TNF-α, and IL-12 mRNA by RT-PCR, the following primers were used with the product length shown in base pairs (bp): IFN-γ (100 bp): 5'-TCAAGTGGCATAGATGTGGAAGA-3' (forward primer), 5'-GAGATAATCTGGCTCTGCAGGATT-3' (reverse primer); IL-4 (167 bp): 5'-ACCAGGAGCCATATCCAC-3' (forward primer), 5'-TTGGAAGCCCTACAGACG-3' (reverse primer); TNF-α (100 bp): 5'-GACGTGGAACTGGCAGAAGAG-3' (forward primer), 5'-GCCACAAGCAGGAATGAGAAG-3' (reverse primer); and IL-12 (226 bp): 5'-CAGGTGTCTTAGCCAGTCC-3' (forward primer), 5'-GCAGCTCCCTCTTGTTGT-3' (reverse primer). The software accompanying RT-PCR machine (Step OnePlus™) was used to analyze the data.

Statistical analysis

Data was analyzed with SPSS 13.0 (Chicago, IL, USA), with $P < 0.05$ considered as statistically significant. Independent *t*-test and ANOVA were used to analyze the data.

RESULTS

LPS-induced acute lung injury

We first established the LPS-induced acute lung injury model according to methods reported in the literature (Matute-Bello et al., 2008; Chen et al., 2010). We observed that mice injected with LPS by tracheal instillation exhibited faster breathing (tachypnea) compared to mice injected with PBS. Pink fluid bubbles were observed to flow out of the nose of the mice injected with LPS and these symptoms disappeared 72 h after the treatment. The mice used for lung tissue staining were sacrificed 24 h after injecting LPS and the thickness of the alveolar wall and wet/dry ratio of lung tissue was assessed (Figure 1A and B). Figure 1A shows the thickness of the alveolar wall in mice lung tissue in different groups. The thickness of the alveolar wall in mice treated with LPS was 64.9 ± 5.2 arbitrary units (A.U.), which is much higher than in mice treated with PBS (25.3 ± 3.7 A.U.). The wet/dry ratio of lung tissue was also measured. The mice injected with LPS had a lung wet/dry ratio 4.49 ± 0.06, which is significantly higher than in mice treated with PBS (4.28 ± 0.04) (Figure 1B). The difference in alveolar wall thickness and wet/dry ratio between LPS- and PBS-treated mice is statistically significant ($P < 0.01$).

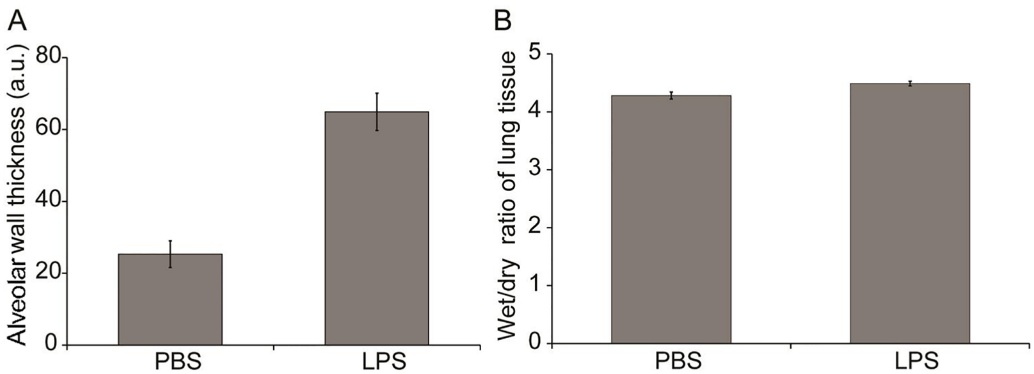

Figure 1. Establishment of LPS-induced lung damage model. Alveolar wall thickness (**A**) and wet/dry ratio of lung tissue (**B**) in mice treated with PBS or LPS (A.U. = arbitrary units). The increased alveolar wall thickness and wet/dry ratio indicates enhanced inflammation in lung tissue.

We also stained the lung tissue by the paraffin section method and this is shown in Figure 2A-D. Compared to mice treated with PBS (Figure 2A), mice treated with LPS had fluid leaking out from the alveolus, a wider alveolar wall, and appearance of inflammatory cells. There was no significant difference between the RAPA and PBS groups (Figure 2A and C). The mice treated with LPS + RAPA had thinner alveolar walls compared to those treated with LPS only (Figure 2B and D). Figure 2E shows the lung injury score: the LPS group had an injury score of 2.5 ± 0.4, which is significantly higher than that of the PBS group (1.8 ± 0.7; $P < 0.05$). The LPS + RAPA group had a clinical score of 2.4 ± 0.8.

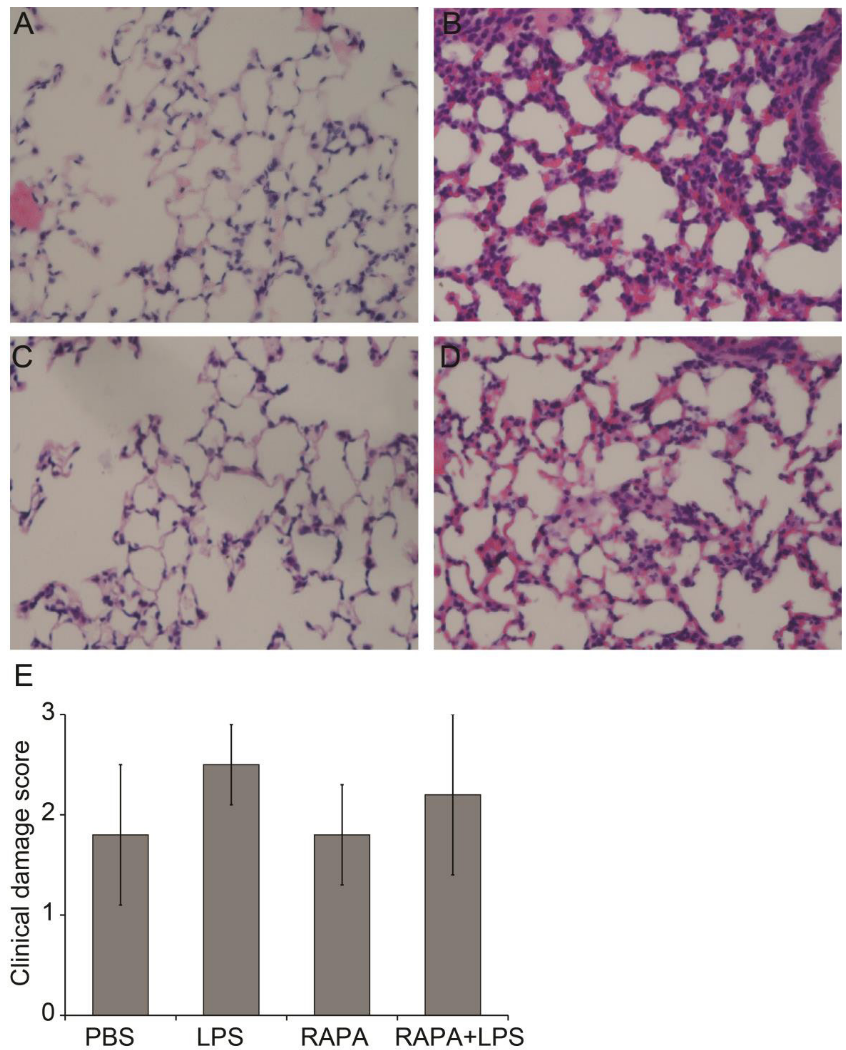

Figure 2. Histological staining of lung tissue from mice treated with PBS (**A**), LPS (**B**), RAPA (**C**), or LPS + RAPA (**D**). The lung tissues from mice treated with LPS have thicker alveolar walls and more inflammatory cells compared to treatment with PBS. Mice treated with LPS + RAPA have thinner alveolar walls and fewer inflammatory cells than treatment with LPS alone, indicating reduced damage by RAPA modulation. **E.** Clinical damage score of mice lung tissue following treatment with PBS, LPS, RAPA, or LPS + RAPA.

The total number of cells and the number of neutrophil granulocytes and macrophages in bronchoalveolar lavage fluid in mice from the different groups were determined. Our study found that the LPS-treated mice had more cells and neutrophil granulocytes than PBS- and LPS + RAPA-treated groups (Figure 3). We also counted the number of macrophages in in bronchoalveolar lavage fluid in mice from different groups but there was no significant difference.

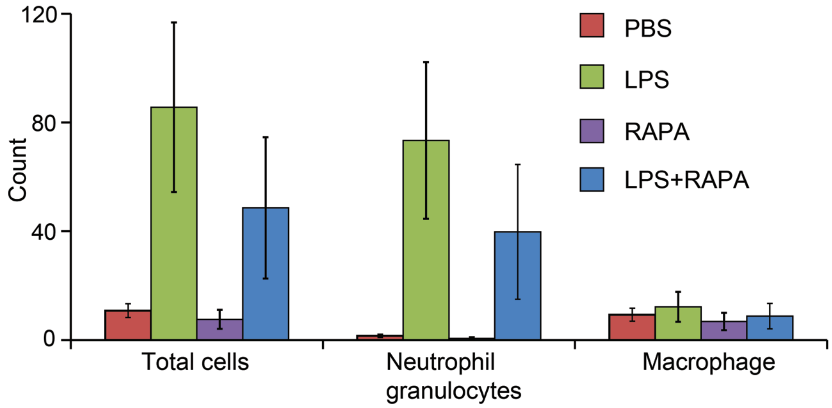

Figure 3. Count of total cells, neutrophil granulocytes and macrophages in mice treated with PBS, LPS, RAPA, or LPS + RAPA. The mice treated with LPS have a higher number of total cells and neutrophil granulocytes compared to mice treated with PBS. The lung tissue from mice treated with LPS + RAPA has a reduced number of total cells and neutrophil granulocytes than mice treated with LPS alone.

Isolation of spleen gδ T cells and lung DCs

We next isolated the gδ T cells and DCs from mice spleen and lung, respectively, with magnetic beads. While there were only 2.86% gδ T cells before magnetic bead isolation (Figure 4A), the purity of gδ T cells reached 95.2% after the isolation (Figure 4B). Purity of DCs in the lung tissue was also analyzed by flow cytometry, where the purity of DCs before and after magnetic bead isolation was 75.5 and 93.2%, respectively (Figure 4C-D). To further confirm the isolation of gδ T cells from spleen, the cells were stained with anti-CD3 (FITC-labeled) and anti-gδ (PE-labeled) and observed under a fluorescence microscope. The gδ T cells have a characteristic spherical shape and positive staining was observed under the microscope, indicating that the gδ T cells were successfully isolated (Figure 4E-H).

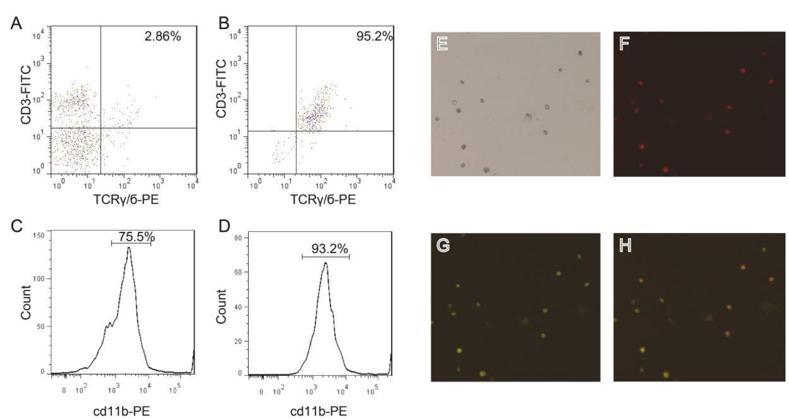

Figure 4. Flow cytometry assessment of gδ T cells before (**A**) and after (**B**) magnetic isolation, and CD11b dendritic cells before (**C**) and after (**D**) magnetic isolation. **E.** Phase contrast image of gδ T cells. Fluorescence image of TCRγ/6-PE-stained (**F**) and CD3-FITC-stained (**G**) gδ T cells. **H.** Merged image of **F** and **G**. The phase contrast and fluorescence images demonstrate the high purity of isolated gδ T cells.

ELISA analysis of cytokine production

After isolating the gδ T cells and DCs, we studied the effect of RAPA on the interaction between gδ T cells and DCs by ELISA. Four treatment groups were analyzed: PBS, LPS, RAPA, and LPS + RAPA. For each treatment group, three types of cell samples were studied: gδ T cells only, DCs only, and co-culture of gδ T and DCs. Figure 5A shows the ELISA analysis of IFN-γ production in different cell types. We found that LPS can induce the production of IFN-γ in gδ T cells (but not DCs) and this production is significantly increased when gδ T cells and DCs are co-cultured (Figure 5A). However, the production of IFN-γ is reduced to normal levels when RAPA is added with LPS (Figure 5A). These results indicate the effect of RAPA in modulating IFN-γ production in the interaction between gδ T cells and DCs. Similar results were obtained in ELISA analysis of IL-4 production, where LPS can enhance the production of IL-4 in co-culture of gδ T cells and DCs. However, this production is reduced to normal levels when RAPA is added with LPS (Figure 5B). For TNF-α, we found that both gδ T cells and DCs can produce this cytokine when they are cultured alone and its production is maintained in co-culture (Figure 5C). A similar trends was observed in the production of IL-12 (Figure 5D). There was an increase in the levels of TNF-α and IL-12 after treatment with LPS in co-culture of gδ T cells and DCs only, but this increase was reversed after the addition of RAPA (Figure 5C-D).

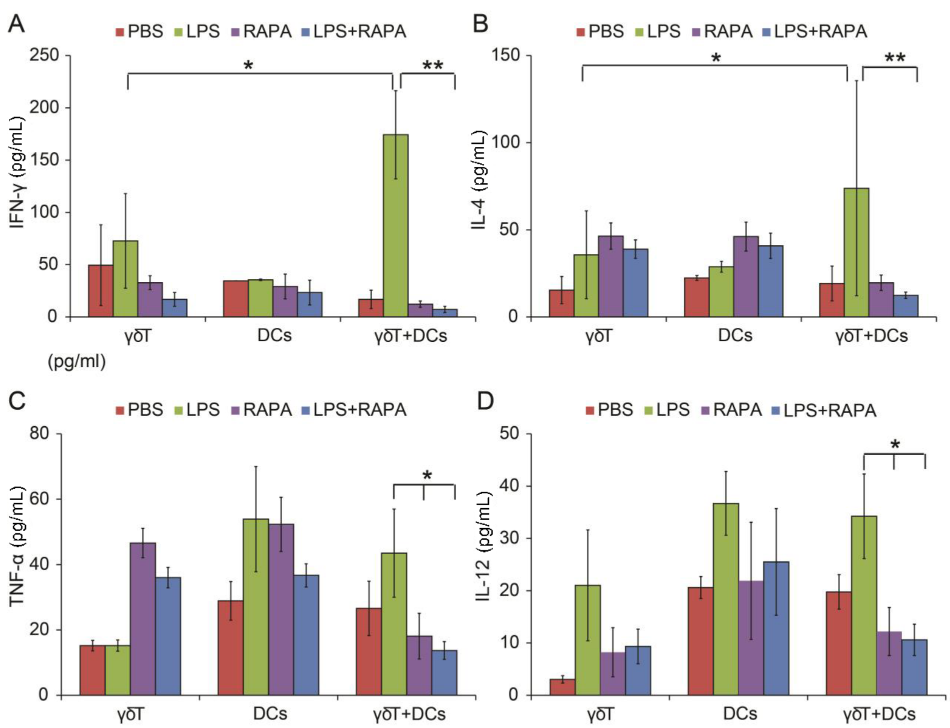

Figure 5. Cytokine production in co-culture of gδ T cells and DCs from mice treated with PBS, LPS, RAPA or LPS + RAPA. Levels of IFN-γ (**A**), IL-4 (**B**), TNF-α (**C**), and IL-12 (**D**) are shown. The co-culture of gδ T cells and DCs from mice treated with LPS + RAPA demonstrates reduced levels of IFN-γ and IL-4 compared to LPS alone, showing the effect of RAPA in the interaction between gδ T cells and DCs. (* $P < 0.05$, ** $P < 0.01$).

Levels of IFN-γ, IL-4, TNF-α, and IL-12 mRNA in gδ T cell and DC co-culture

After assessing the production of IFN-γ, IL-4, TNF-α and IL-12 by ELISA, we analyzed the levels of IFN-γ,IL-4, TNF-α and IL-12 mRNA by RT-PCR (Figure 6A-D). In accordance with the production of IFN-γ in Figure 5A, we found that LPS can modulate the levels of IFN-γ mRNA, when gδ T cells and DCs are co-cultured and this is reversed by the addition of RAPA. This effect is not observed when the two types of cells are cultured alone (Figure 6A). While we observed the modulatory effect of RAPA in increasing TNF-α mRNA levels in DCs (Figure 6C), we did not observe any effect on its levels in co-culture of gδ T cells and DCs. We did not observe any significant change in TNF-α, IL-4, and IL-12 mRNA levels when gδ T cells and DCs were co-cultured (Figure 6C-D).

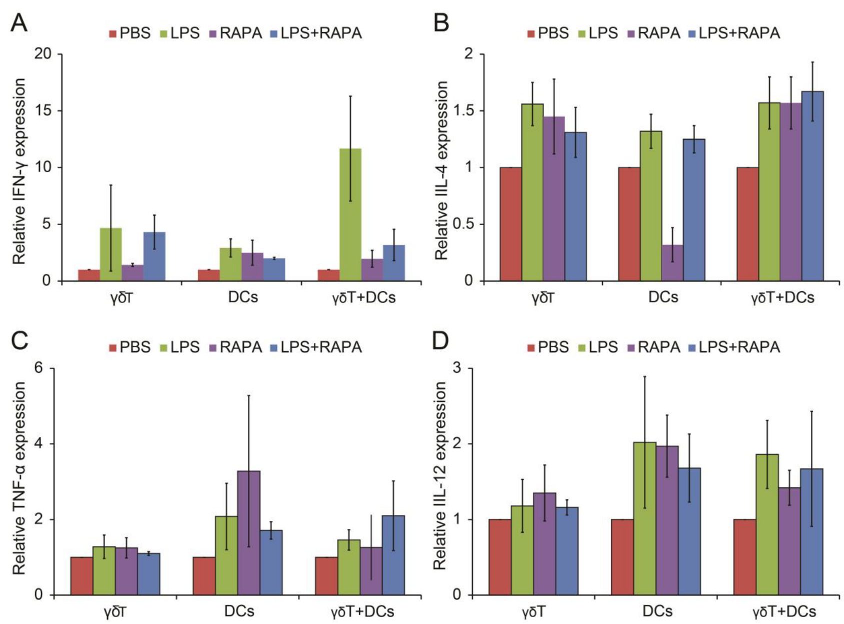

Figure 6. Expression of cytokines in co-culture of gδ T and DCs from mice treated with PBS, LPS, RAPA or LPS + RAPA. mRNA levels of IFN-γ (**A**), IL-4 (**B**), TNF-α (**C**), and IL-12 (**D**) are shown. The co-culture of gδ T cells and DCs from mice treated with LPS has higher production of IFN-γ compared to treatment with LPS + RAPA.

DISCUSSION

gδ T cells play a significant role in balancing the immune system by connecting the adaptive and innate immune response. They also play an important role in treating infectious diseases and cancer (Holtmeier and Kabelitz 2005; Li et al., 2012). While gδ T cells recognize adjuvant through non-MHC TCRs on their surface (Cao and He, 2005), there is a report that that these cells can recognize adjuvant in a pathway that does not involve TCRs (e.g. TLRs (Martin et al., 2009), CD226 (Gertner-Dardenne et al., 2009; Toutirais et al., 2009), or natural killer antibody (NKRs) (Das et al., 2001)).

When stimulated by adjuvant, gδ T cells are able to secrete multiple types of immunomodulatory cytokines, including IL-2, IFN-γ, and IL-4 (Huang et al., 2013). There have been studies reporting that gδ T cells can produce a high level of IFN-γ but a low level of IL-4 in mice infected with *Listeria monocytogenes* (Tramonti et al., 2008; Price and Hope, 2009). Another study reported that gδ T cells can produce low levels of IFN- γ but high levels of IL-4 *in vitro* in cells infected with *Nippostrongylus* (Tam et al., 2001). These studies show that gδ T cells can secret different cytokines when faced with varying stimulants, therefore controlling the type of immune response generated (Champagne, 2011). DCs are one of the most important antigen presenting cells that integrate innate and adaptive immunity. They are also one of the most important cells to protect lung tissue from being damaged by external adjuvant, bacteria, and viruses. Although gδ T cells have been reported to regulate DCs and alveolar cells in resolving *S. pneumoniae*-induced inflammation (Kirby et al., 2007), there are few studies reporting these types of effects in a LPS-induced lung injury model in mice.

RAPA is a pharmacologic drug developed from filamentous bacteria (Dumont and Su, 1996). RAPA exhibits potent immunosuppressive functions by binding to the intracellular membrane-bound mammalian target of rapamycin (mTOR) complex of immune cells (T cells) and DCs (Fischer et al., 2011). In DCs, RAPA is able to inhibit the production of cytokines and reduce the expression of MHC class II and co-stimulator molecules (Fischer et al., 2011). gδ T cells in spleen and lung belong to the same class, as spleen is one of organs critical for immune function and contains a higher ratio of immune cells compared to other organs. It is therefore ideal to isolate gδ T cells from spleen in order to examine their interactions with DCs from lung.

In this study, we first confirmed the effect of LPS on lung damage by counting the number of cells and neutrophil granulocytes in bronchoalveolar lavage fluid and paraffin sections of lung tissues, both of which indicate that LPS induced inflammation in the lung. Mice treated with LPS + RAPA had fewer cells and neutrophil granulocytes than mice treated with LPS only. These results indicate that RAPA can reduce the inflammation induced by LPS.

After demonstrating the effect of RAPA *in vivo*, we isolated gδ T cells and DCs from spleen and lung tissues, respectively. Our characterization indicates that a high purity of gδ T cells and DCs are obtained by positive selection with magnetic beads. Immune staining of gδ T cells further confirmed that the cells were successfully isolated. To analyze the effect of RAPA in modulating the interaction between gδ T cells and DCs, ELISAs were performed. Our study found that the co-culture of DCs and gδ T cells from LPS-injected mice produce high amounts of IFN-γ and this is significantly reduced upon simultaneous treatment with RAPA (Figure 5A). It has been reported in the literature that gδ T cells stimulated with LPS can produce a high level of IFN-γ (Price and Hope, 2009). Our study also found that the co-culture of gδ T cells and DCs enhances the production of IFN-γ, which is consistent with what has been reported (Gertner-Dardenne et al., 2009). Our study also found that RAPA can reduce the production of this cytokine. More importantly, RT-PCR further confirmed that this modulation is processed through the enhancement of IFN-γ mRNA levels. However, while we found that RAPA modulates IL-4 production co-culture of DCs and gδ T cells, this result was not confirmed by RT-PCR analysis (Figure 5B and B). We also did not observe a modulatory effect of RAPA with TNF-α and IL-12.

In this study, we studied the effect of RAPA in modulating the interaction between gδ T cells and DCs *ex vivo* in a LPS-induced lung injury mouse model. Our study found that LPS induces obvious damage to lung tissue. Co-culture of gδ T cells and DCs from LPS- or LPS + RAPA-treated cells demonstrate that RAPA can modulate the secretion of IFN-γ by enhancing its mRNA level. Future studies will be performed to determine the mechanism by which DCs and gδ T cells interact with each other, including how DCs are activated during co-culture with gδ T cells and the molecular pathway of DC and gδ T cell interactions.

Conflicts of interest

The authors declare no conflict of interest

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