# Endotoxin Tolerant Dendritic Cells Suppress Inflammatory Responses in Splenocytes via Interleukin-1 Receptor Associated Kinase (IRAK)-M and Programmed Death-Ligand 1 (PDL-1)

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Data Collection B
Statistical Analysis C
Data Interpretation D
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**Background:** Sepsis causes the highest mortality in non-cardiovascular intensive care units worldwide. Recent research has demonstrated that the late phase of sepsis, characterized as septic immunosuppression, is the central pathophysiological mechanism of immune dysfunction. Investigating the suppressive mechanism of immune cells may identify possible targets for therapy.

**Material/Methods:** We used LPS 2-hit model for dendritic cells (DCs) to establish endotoxin tolerance, and co-cultured with splenocytes. Co-culture responses and gene expressions were evaluated.

**Results:** Endotoxin tolerant DCs showed irresponsiveness in pro-inflammatory cytokine production and expressed negative regulator genes of inflammation. When co-cultured with splenocytes, suppression of inflammatory responses and T cells apoptosis were observed with elevated expression of IRAK-M and PDL-1, and interference and neutralization of these 2 molecules led to partly reversed suppression of inflammation.

**Conclusions:** Our research found direct regulation of endotoxin tolerant DCs to other immune cells and suggested a possible mechanism via IRAK-M and PDL-1. This may inform research on septic immunosuppression and suggests possible therapeutic targets for sepsis.

**MeSH Keywords:** Dendritic Cells • Immunosuppression • Sepsis

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## Background

Sepsis, characterized by the uncontrolled systemic inflammatory responses and multiple organ dysregulation, is the leading cause of death in non-cardiovascular intensive care units worldwide [1]. The early phase of sepsis presents with uncontrolled exaggerated inflammatory responses while the late phase presents with profound immunosuppression, leading to irresponsiveness to infection stimuli [2]. Recent research has proposed that septic immunosuppression, which causes severe secondary infection and multiple organ dysfunction, may be the central pathophysiological mechanism for the high mortality rate of sepsis [3]. And thus, uncovering the related mechanism may contribute to the development of effective therapy for sepsis.

A feature of septic immunosuppression is the irresponsiveness to the stimuli accompanied by elevated levels of anti-inflammatory cytokines [4,5], apoptosis of immune cells [6], and the intracellular activation of negative regulator of the inflammation [7]. Recent research reported that both CD4+ T cells [8] and dendritic cells (DCs) [9] were decreased in septic patients and the apoptosis as well as the cell counts of T cells correlations with the prognosis of sepsis in patients [10]. Further research proposed that, considering the antigen-presenting and cytokine production capacity of DCs, which is crucial for the activation and function of T cells [11], the suppression pf DCs might be the reason for T cells dysfunction. Meanwhile, regulatory T cells, the major negative regulator in immune tolerance and innate immunity, were also shown to be elevated when sepsis progresses [12,13], and thus worsen the immune irresponsiveness. However, although characteristics of single cell subsets during sepsis have been well discussed, the direct influence of DCs to other immune cells has been poorly reported.

In this paper, we used the LPS-endotoxin-tolerance (LPS-ET) model to study the septic immunosuppression [14], and to evaluate the influence of LPS-ET DCs to splenic T cells. We proposed that the DCs in the LPS-ET model would show irresponsiveness and altered cytokine production to secondary LPS stimuli. Further experiments showed that those irresponsive DCs, when co-cultured with splenocytes from healthy C57BL/6 mouse, led to a depression in Th1 cytokine production and increased CD4+ T cells apoptosis. In discovering the possible mechanism of these phenomenon, we reported that IRAK-M, the negative regulator in TLRs signaling, was activated in tolerant DCs and was negatively correlated with Th1 cytokine production of splenocytes. Downregulation of IRAK-M could thus partly reverse the immunosuppression to splenocytes and restore the Th1 cytokine production. These results indicated that the LPS-ET DCs with overexpressed IRAK-M could suppress the inflammatory responses of splenocytes and induce T cells apoptosis when co-cultured. These findings suggested that the T cells dysfunction in septic immunosuppression may

be due to the DCs irresponsiveness and upregulated IRAK-M, which thus could be proposed as a therapeutic target in DCs for the therapy of septic immunosuppression.

## Material and Methods

### Cell preparation

8–10 weeks old C57BL/6 mouse were purchased from Shanghai Research Center for Model Organisms and raised in specific pathogen-free (SPF) animal rooms. All animal experiments were approved by the Institutional Animal Care and Use Committee of Tongji University. Bone marrow-derived dendritic cells (BMDCs) were generated from the BM cells flushed from the femurs and tibias of C57BL/6 mice by 3 mL PBS and then cultured in 1640 cultured medium supplemented with 10% fetal bovine serum (FBS), 1% antibiotics (100 U/mL penicillin and 100 ug/mL streptomycin). BM cells were cultured in 6-cm dish at $2\times10^6$ cells/mL with recombinant GM-CSF (25 ng/mL) and IL-4 (10 ng/mL). Culture medium were half changed every 2 days with same concentration of cytokines and on day 6, cells were resuspended and seeded in a fresh dish. On day 8, cells were treated with LPS (1 ug/mL) for 48 h (LPS prime) to induce maturation of BMDCs. Matured BMDCs were then washed by PBS and treated with LPS (4 ug/mL) (LPS challenge) to induce the endotoxin tolerance (tolerant DCs).

IRAK-M siRNA interference was achieved by using IRAK-M siRNA or control siRNA (Genechem, China) for 24 h before co-culturing. PDL-1 antibodies were added to co-cultured system using anti-PDL-1 antibody (Abcam, USA) according to the manufacturer's instructions.

### Enzyme-linked immunosorbent assays (ELISAs)

Briefly, culture medium from BMDCs, or BMDCs-T cells co-culture system were centrifuged (3000 rpm, 5 min) and the supernatants were collected. Mouse IL-12, TNF-α, IL-6, IL-10, TGF-β, and PDL-1 using detected by ELISA kit (R&D Systems, USA) according to the manufacturer's instructions and the results were measured by Microtiter Plate Reader (TECAN, Switzerland) at 450 nm. Three wells were included in the measurements.

### Flow cytometry and apoptosis analysis

CD4+ T cells were marked by using Anti-Mouse-CD4-APC (R&D Systems, USA). Apoptosis were analyzed by using Alexa Fluor 488 Annexin V-FITC (BD, USA). All samples were washed 3 times after staining with antibodies, and then subjected to FACS LSR II (BD Bioscience, USA) and further analyzed by FlowJo software (Tomy Digital Biology Co., Ltd., Japan). At least 3 duplicates were measured for each group.
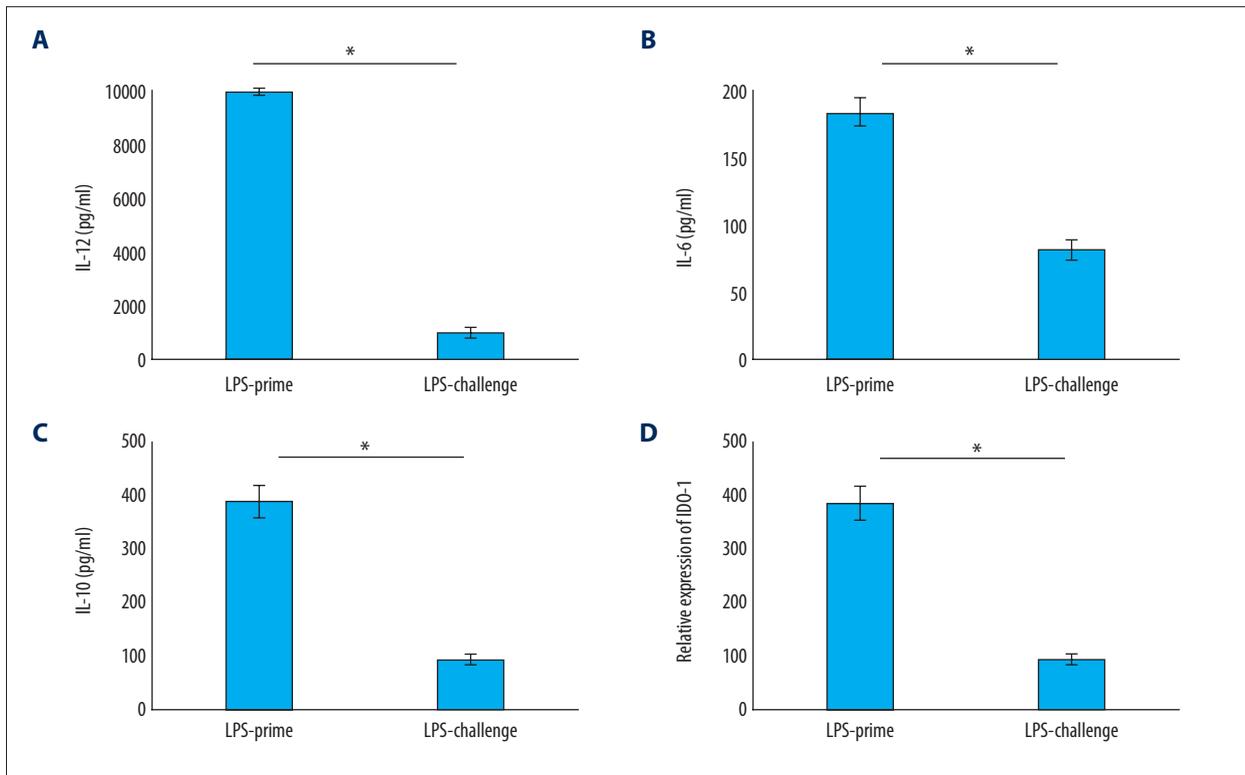
**Figure 1.** Cytokine production and gene expression in BMDCs after LPS prime and challenge. The concentrations of (**A**) IL-12, (**B**) IL-6, and (**C**) IL-10 in supernatant of culture medium analyzed using ELISA assay, and (**D**) IDO-1 expression analyzed by qPCR (n=3). The expression of the error bars represents ±S.D. (* *P*<0.05); n=biological replicates.

### Real-time RT-PCR

Cell total RNA was extracted using Total RNA Purification Kit (Sigma, USA) and then reverse transcribed. Gene transcription were quantified on 7900HT Fast Real-time PCR system (Life Technology Corporation, USA) using SYBR green dye and normalized with GAPDH. Primer sets were used: IRAK-M: F-5'-ACGTCACTGAAGGCACTGAG-3', R-5'-TTTGACTGAGAGGCGGTCAC-3'. GAPDH: F-5'-GAGAGTGTTTCCTCGTCCCGTAG-3', R-5'-GCCTCACCCCATTTGATGTTAGT-3'. Three duplicates were measured for each group.

### Western blot

Samples were resolved on SDS-PAGE gel. Proteins were transferred onto a PVDF membrane (Millipore, Germany) by conventional methods. Western blot was probed with indicated antibodies and the immunoreactive protein were visualized by using BeyoECL Plus kit (Beyotime, China).

### Statistical analysis

All of the measurements were collected in triplicate for each independent preparation. The results were statistically analyzed using Student's *t*-test or ANOVA. The SPSS software, version 16.0, was used for all of the statistical analyses, and differences with a *P* value less than 0.05 were considered statistically significant (* *P*<0.05).

## Results

### LPS prime and challenge confers BMDCs endotoxin tolerance

Although LPS endotoxin tolerance has been widely studied in monocyte-macrophages models, this model used in DCs remains unclear as approaches vary in inducing tolerant DCs maturation [15–17] and little research has reported on a standard method for inducing endotoxin tolerance in BMDCs [15–17]. Thus, we established a new protocol for the induction of endotoxin tolerance in DCs and evaluated its main features. On day 6, DCs were treated with 1 ug/mL LPS for 48 h for maturation (LPS-prime). Then cells were washed with PBS 3 times and DCs were re-challenged with 4 ug/mL LPS for another 24 h (LPS-challenge). We found that after the LPS challenge, cytokine production of IL-12, which is regarded as a marker for its immune responses [16], was significantly decreased in the supernatant of culture medium, nearly irresponsive to the LPS stimuli (Figure 1A). To further prove its tolerance feature,
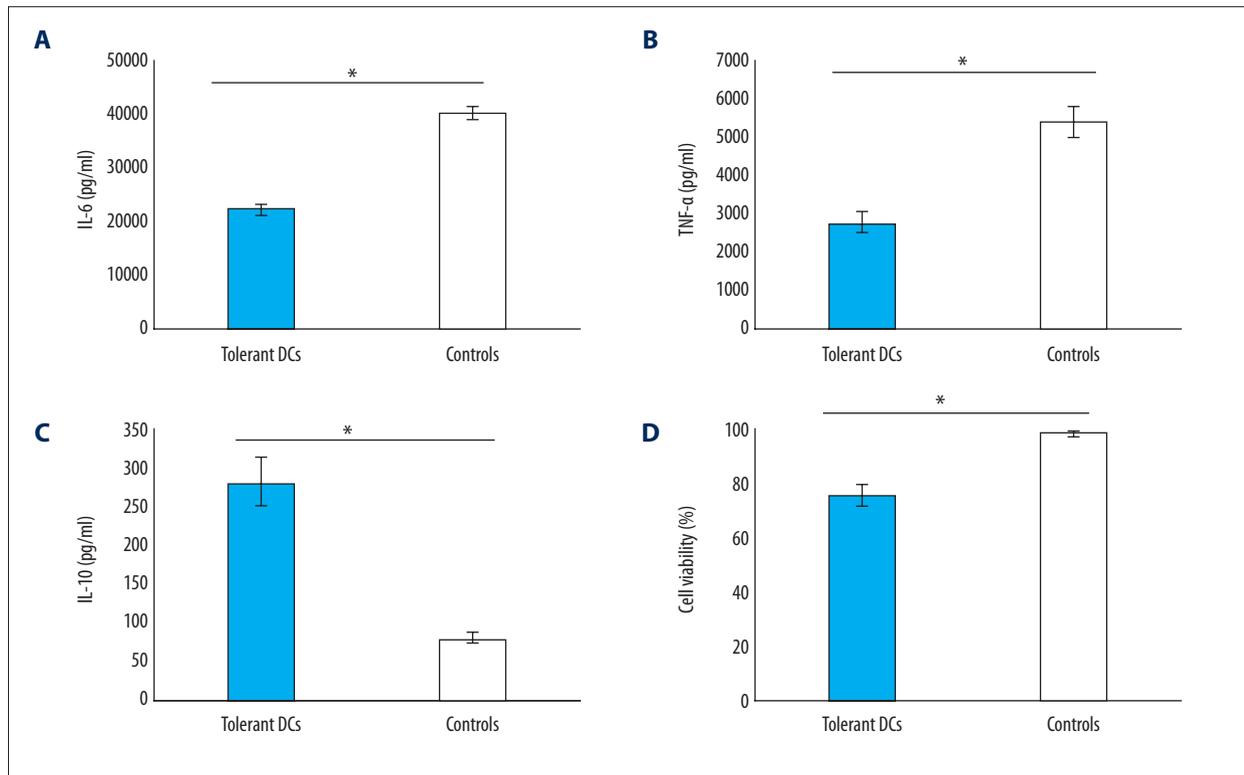
**Figure 2.** Cytokine production and cell viability in co-culture system. Tolerant DCs after LPS prime and challenge were co-cultured with splenocytes from healthy C57BL/6 mice, unprimed DCs were regarded as controls. The concentrations of (**A**) IL-6, (**B**) TNF-α, and (**C**) IL-10 in supernatant of co-culture medium analyzed using ELISA assay (n=3). Cell viability analyzed by CCK-8 kit shown with controls group as 100% viability (n=3). The expression of the error bars represents ±S.D. (* *P*<0.05); n=biological replicates.
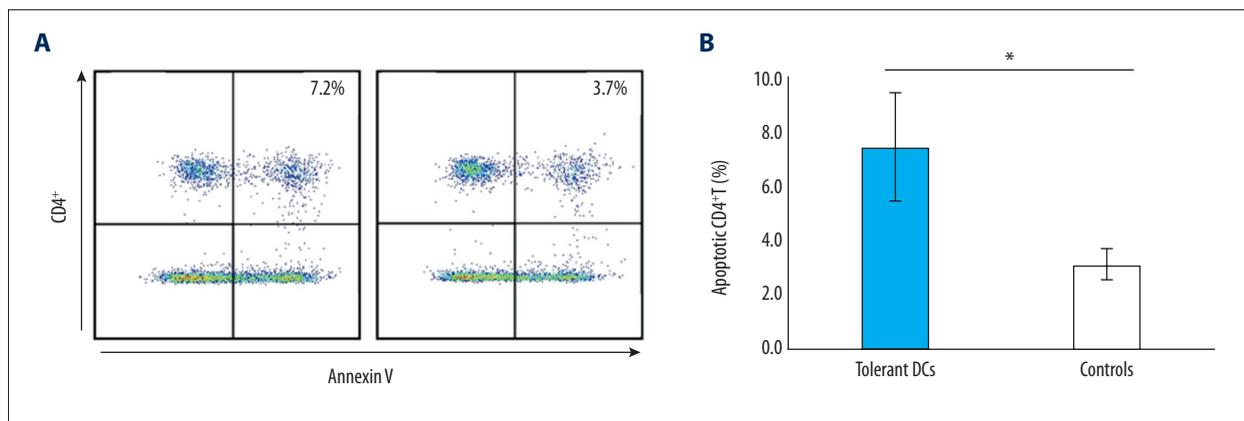


**Figure 3.** Analysis of apoptosis of CD4+ T cells. Representative of (A) flow cytometry dot plots and (B) graphs show the apoptotic CD4^{+} T cells in co-cultured system (n=3). The error bars represent ±S.D. (* *P*<0.05); n=biological replicates.

we determined the level of IL-6 and IL-10 in the supernatant and evaluated the expression of IDO1, which has previously been proven to correlated with DCs tolerance [15]. We found that IL-6 was decreased while IL-10 was highly elevated (Figure 1B, 1C). As for the IDO1 expression, compared with LPS-unprimed DCs, LPS-primed DCs showed elevated expression both in mRNA and protein levels (Figure 1D). Thus, these

results indicated that BMDCs after LPS prime and challenge presented endotoxin tolerance features that not only were irresponsiveness in pro-inflammatory cytokine production but also activated negative regulator of TLRs.
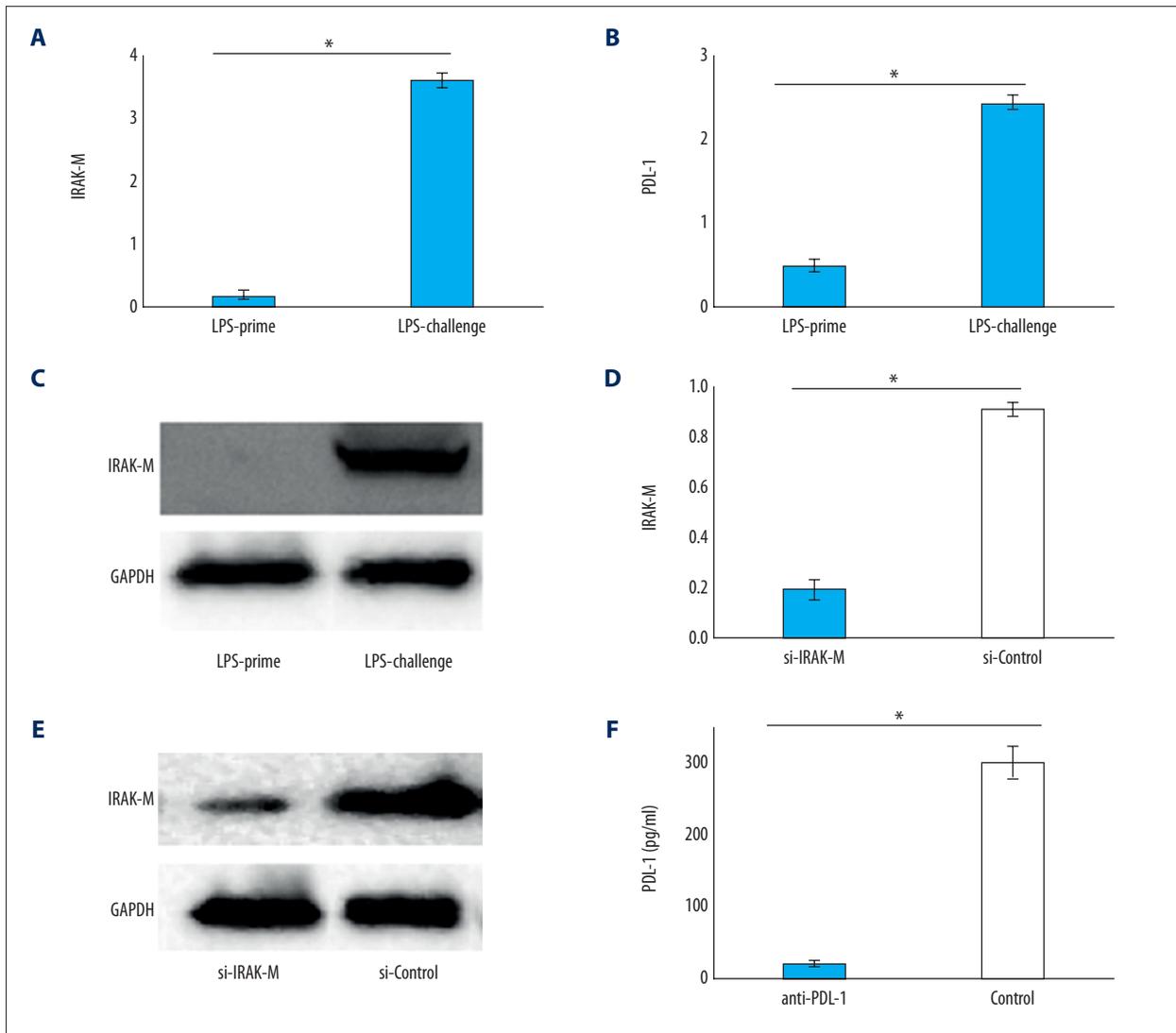
**Figure 4.** Expression of IRAK-M and PDL-1 before and after the IRAK-M and PDL-1 inhibition of BMDCs. (**A, C**) Quantitative PCR analysis and western blot of IRAK-M expression of LPS primed or challenged DCs (n=3). (**B**) The expression of PDL-1 in LPS primed or challenged DCs (n=3). (**D**) IRAK-M mRNA and (**E**) protein expression after the interference using siRNA or si-RNA-controls to IRAK-M (n=3). (**F**) PDL-1 concentration of ELISA after using anti-PDL-1 antibody to neutralize supernatant PDL-1 (n=3). The expression of the error bars represents ±S.D. (* $P<0.05$); n=biological replicates.

### Tolerant DCs hindered pro-inflammatory responses of splenocytes and induce T cells apoptosis

After the establishment of the tolerance model in DCs, we studied the effects of the tolerant DCs on splenocytes in co-culture system. Normally, LPS could be recognized by TLRs of DCs or macrophages, initiate pro-inflammatory signaling, and thus activate adaptive immune responses [18]. Therefore, we used LPS in our co-culture system and collected the supernatant of the culture medium. We found that compared with unprimed DCs, the supernatant from the tolerant DCs co-culture system presented a decreased in Th1 cytokines, including IL-6, and TNF-α, while Th2 cytokines, such as IL-10, were

elevated (Figure 2A–2C). Moreover, as another key characteristic of septic immunosuppression, apoptosis of CD4+ T cells were further evaluated in the co-culture system. As the results in Figure 2D show, the general cell viability of tolerant DCs co-culture system was decreased. In addition, there was an observable increase in apoptotic CD4+ T cells (CD4+ Annexin V-FITC+) in tolerant DCs co-culture system (Figure 3A, 3B). These results indicated that tolerant DCs could hinder the pro-inflammatory responses in splenocytes from healthy mice and induce apoptosis in T cells.
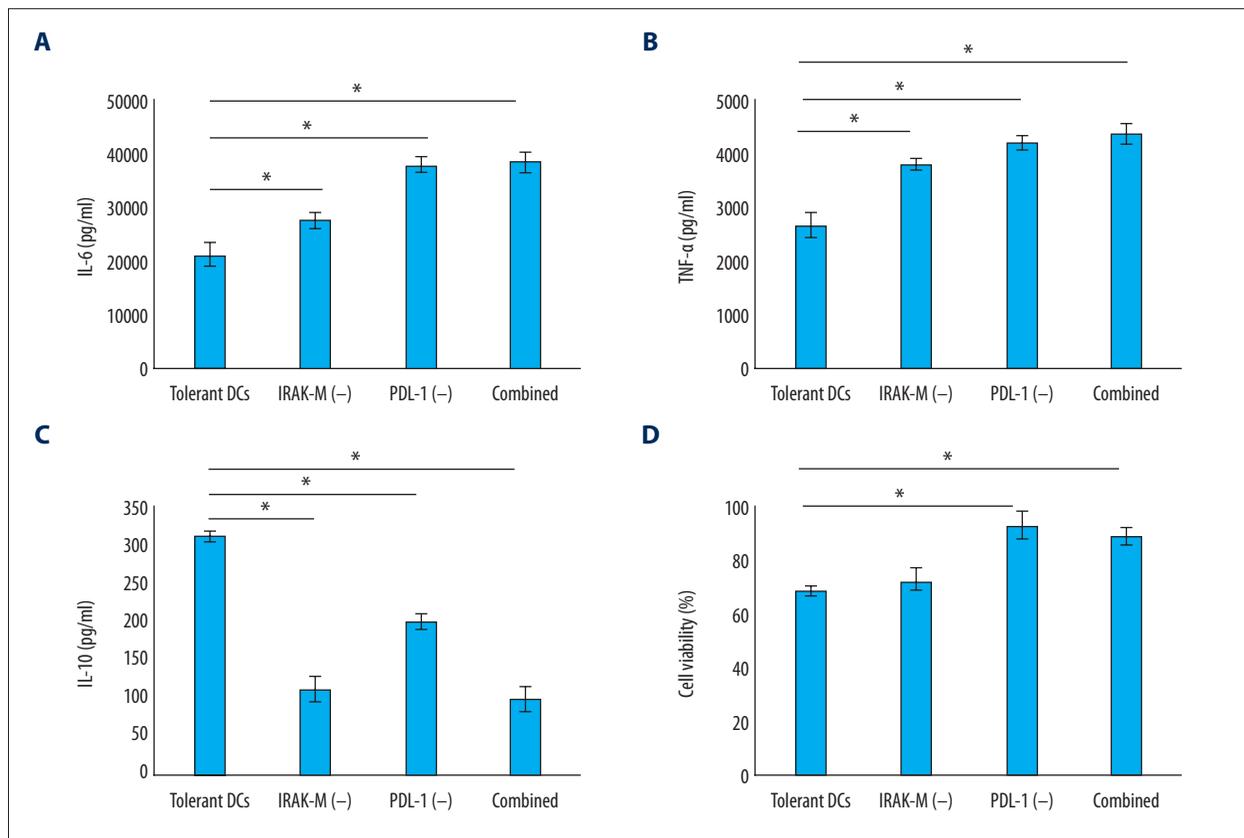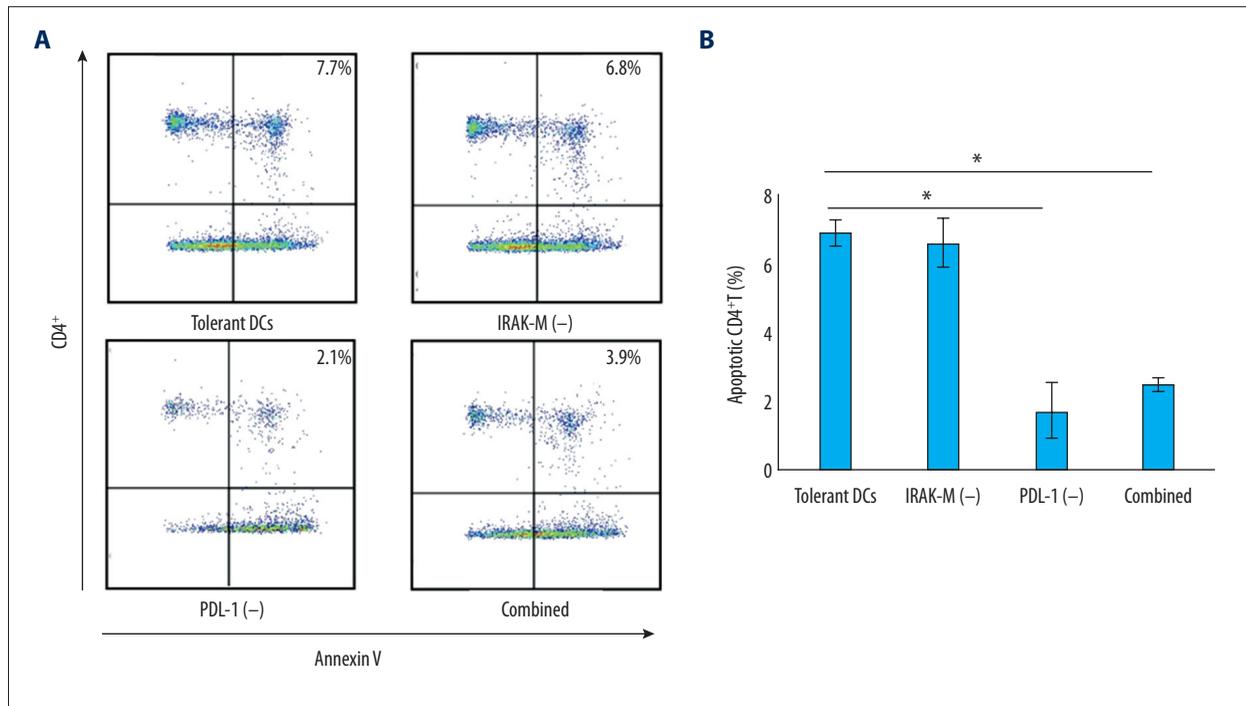
**Figure 5.** Cytokine production and cell viability in co-culture system after IRAK-M interference and PDL-1 neutralization. Cytokine production and cell viability of tolerant DCs and splenocytes co-culture system after IRAK-M interference and PDL-1 antibody neutralization respectively or combined in co-culture system. The concentrations of (**A**) IL-6, (**B**) TNF-α, and (**C**) IL-10 in supernatant of co-culture medium analyzed using ELISA assay (n=3). (**D**) Cell viability analyzed by CCK-8 kit shown with controls group as 100% viability (n=3). The expression of the error bars represents ±S.D. (* $P<0.05$); n=biological replicates.

## Immunosuppressive effects of tolerant DCs was mediated through IRAK-M activation

To further discover the possible mechanism of immunosuppressive effects of tolerant DCs, we focused on a widely-researched molecule, IRAK-M, which is an important negative regulator in TLRs signaling [18–21]. In analyzing the IRAK-M expression in tolerant DCs, we found that, accompanied with the establishment of endotoxin tolerance, the expression of IRAK-M was highly elevated (Figure 4A, 4C). In addition, we also found that PDL-1, the important suppressive regulator to T cells, was also over-expressed in tolerant DCs (Figure 4B). This result may well explain the apoptosis and irresponsiveness of T cells in co-culture system and thus indicates 2 important mechanisms, through IRAK-M and PDL-1, that tolerant DCs use to regulate splenocytes responses.

## Immunosuppression partly reversed in IRAK-M downregulated and PDL-1 neutralized tolerant DCs

In order to prove that IRAK-M and PDL-1 play major roles in tolerant DCs in inducing immunosuppressive effects to splenocytes, we used si-RNA to interfere with the IRAK-M expression and PDL-1 antibodies to neutralize the effects respectively as well as combined, in tolerant DCs. After the IRAK-M siRNA interference and PDL-1 neutralization, expression of IRAK-M in tolerant DCs was depressed and PDL-1 concentration in supernatant was decreased (Figure 4D, 4E). We then used inhibitory protocol (IRAK-M or PDL-1 inhibition or combined) to analyzed cytokine production and T cells apoptosis. As the results showed, after single interfere of IRAK-M, the Th1 cytokine production of splenocytes was partly restored with Th2 cytokine levels decreased (Figure 5A–5C), while no significant change was observed in cell viability or T cells apoptosis (Figures 5D and 6A, 6B). On the other hand, single PDL-1 neutralization also restored Th1 cytokine production with higher concentration but with an equivalent decrease in Th2 cytokines compared with single IRAK-M interference (Figure 5A–5C), and T

**Figure 6.** Analysis of apoptosis of CD4+ T cells after IRAK-M interference and PDL-1 neutralization. Representative of (**A**) flow cytometry dot plots and (**B**) graphs show the apoptotic CD4+ T cells in co-cultured system after IRAK-M interference and PDL-1 neutralization respectively or combined (n=3). The error bars represent ±S.D. (* *P*<0.05); n=biological replicates.

cells apoptosis was significantly reduced when cell viability increased (Figures 5D and 6A, 6B). When interference in IRAK-M and PDL-1 were combined, the cytokine production was furthered elevated in Th1 cytokines but Th2 cytokines still showed equivalent change (Figure 5A–5C) and T cells apoptosis decreased when cell viability increased (Figures 5D and 6A, 6B). These results indicated that the immunosuppressive effects of tolerant DCs was mainly through IRAK-M and PDL-1 signaling with PDL-1 mechanism showing more promising outcomes. However, these 2 important mechanism both suggest possible therapeutic targets for the immunological treatment of sepsis.

## Discussion

Previous research and treatment focusing on systemic inflammatory responses have shown unsatisfied outcomes and prognosis [22,23]. Recent studies have proven that the complex immunosuppressive status induced by anti-inflammatory feedback has been the key pathophysiological mechanism leading to poor prognosis and high mortality of sepsis [24,25]. The immunosuppressive status was usually characterized by irresponsiveness to pathogen stimuli [26], elevation of anti-inflammatory cytokines [19], deceased cell counts of immune cells [27], and intracellular expression of negative regulatory genes [19,26]. In discussing immunosuppression, the endotoxin tolerant (ET) model has provided a controllable and

stable scenario to investigate molecular mechanism and cellular interactions with comparable signaling and characteristics to septic immunosuppression [19]. However, though the ET model has been widely studied in macrophages, monocytes, and other immune cells [28–30], limited research has reported on the ET model in DCs. Because DCs are important antigen presenting cells with the ability to support T cells maturation and functions, it is crucial to investigate its features and cellular interactions in immunosuppression. In our research, we conducted a new protocol for inducing the ET model in bone marrow-derived DCs (BMDCs) and determined its cytokine production and gene expressions. Our results showed that after LPS prime for maturation and LPS challenge for inducing tolerance, DCs showed irresponsiveness to stimuli and suppressed cytokine production.

To further investigate the molecular mechanism of endotoxin tolerance in DCs, we evaluated the expression of IRAK-M and PDL-1 in tolerant DCs. As a widely-studied molecule in TLRs, IRAK-M has been proven to be a central molecule in regulating the immune homeostasis, mainly via suppressing the NFκB signaling [19]. However, previous results in IRAK-M have been from studies mostly conducted in monocyte-macrophage models [28–30] while expression in DCs has been poorly illuminated in studies. In this paper, we have proven the elevated expression of IRAK-M in tolerant DCs and proven its relation to the regulatory function of DCs to other immune cells. Moreover,

we also determined expression of PDL-1, which is regarded as a central molecule in suppression of immune responses and inducing cell apoptosis [31], in tolerant DCs. We found PDL-1 was also elevated in tolerant DCs. These results further proved that this ET model is well-established for DCs with comprehensive characteristics as immunosuppression, and thus indicating an applicable tolerant DCs model for further research.

In order to analyze the regulatory function of tolerant DCs to other immune cells, we used tolerant DCs and splenocytes co-culture system with LPS stimuli and determined general viability, cytokine production, and T cells apoptosis. To our knowledge, this is the first report that tolerant DCs can suppress the inflammatory responses of splenocytes and induce CD4+ T cells apoptosis. These results hold well for explaining, though not fully accounting for, the enhanced apoptosis of T cells and low Th1 cytokine production in septic immunosuppression [3]. In discovering the effects of tolerant DCs after the interference of IRAK-M and PDL-1, we found both molecules, when expression-depressed or neutralized, showed reversal effects of immunosuppression, while PDL-1 neutralization presented more obvious effects. Although both interferences combined led to the best reverse of immunosuppression, immune responses were still suppressed to some extent. It may be interpreted that the tolerant DCs exercise immunosuppressive effects through multiple molecules and mechanisms, and IRAK-M and PDL-1 may be 2 molecules among those involved.

Septic immunosuppression has attracted much attention and numerous efforts have been devoted to discovering specific mechanism and therapeutic targets [6]. As multiple immune cells participate in immunoregulation, in-depth understanding of particular immune cells may provide important indications for immune therapy. In our research, we proposed that endotoxin tolerant DCs, with irresponsiveness to stimuli and expression of negative regulator genes, hindered inflammatory responses of splenocytes. This suppression could be partly reversed by downregulating IRAK-M and neutralize the PDL-1 molecules. This may provide indications for immunosuppressive regulation and important target for immune therapy of DCs in septic immunosuppression.

## Conclusions

Our research established a well-proven model for endotoxin tolerant DCs and its direct regulation of other immune cells through a co-culturing system. The suppression effects and induction of T cells apoptosis of tolerant DCs were proven to be correlated with the expression of IRAK-M and PDL-1, as interference and neutralization of these 2 molecules were shown to partly reversed the phenomenon. The present study identified an important feature that may be involved in septic immunosuppression and our study results suggest possible roles for DCs regulations, as well as possible therapeutic targets for DCs as immune therapies.

### Conflicts of interests

None.

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