MEDICAL SCIENCE MONITOR

MOLECULAR BIOLOGY

e-ISSN 1643-3750
© Med Sci Monit, 2017; 23: 300-308
DOI: 10.12659/MSM.902081

Received: 2016.10.24
Accepted: 2016.11.30
Published: 2017.01.18

Toll-Like Receptor 4 Signaling in High Mobility Group Box-1 Protein 1 Mediated the Suppression of Regulatory T-Cells

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Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Source of support: Hubei Province Health and Family Planning Scientific Research Project (WJ2015MB172), the Science Foundation of Hubei University for Nationalities (MY2015b027)

Background: Treg cells play a central role in the suppression of immune response, and their suppressive capacity can be modulated by toll-like receptor (TLR) ligands. However, the detailed pathway of TLR ligand modulation is still unknown. The present study aimed to evaluate the effect of the high mobility group box-1 protein 1 (HMGB1) and lipopolysaccharide (LPS) on Treg cells through TLR4 signaling.

Material/Methods: Treg cells were purified from healthy human peripheral blood mononuclear cells (PBMCs) by magnetic-bead activity cell sorting (MACS), blocked by anti-TLR4 monoclonal antibody, and then incubated with different concentration of LPS or HMGB1. The level of gene expression of IL-1β, IL-10, IFN-γ, and TGF-β were detected using quantitative real-time polymerase chain reaction (qPCR) and enzyme-linked immunosorbent assay (ELISA), and the proliferation of Treg cells after treating by LPS and HMGB1 was analyzed by flow cytometry. The NF-κB expression in Treg cells was examined by Western blotting.

Results: LPS treated CD4 CD25 Treg cells directly increased the expression of IL-1β and IL-10 and decreased the expression of IFN-γ and TGF-β. However, HMGB1 treatment resulted in a marked decreased expression of IL-1β, IL-10, IFN-γ, and TGF-β. The proliferation of $CD4^+$ T cells was significantly inhibited by Treg cells in the LPS treatment group, but weaken in the HMGB1 treatment group. These data suggest that HMGB1 and LPS stimulation could downregulate the expression NF-κB p65 in cytoplasmic proteins and increase the expression in nuclear proteins, thus leading to modulation of IL-1β, IL-10, IFN-γ, and TGF-β expression; moreover, the suppressive function of Treg cells could be regulated by TLR4.

Conclusions: TLR4 signaling in HMGB1 mediated the suppressive function of Treg cells through the activation of the NF-κB pathway.

MeSH Keywords: High Mobility Group Proteins • T-Lymphocytes, Regulatory • Toll-Like Receptors

Full-text PDF: http://www.medscimonit.com/abstract/index/idArt/902081

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Background

The innate immune system utilizes pattern recognition receptors (PRRs) to recognize conserved pathogen-associated molecular patterns (PAMPs) on pathogens. Toll-like receptors (TLRs) are evolutionarily conserved receptors discovered to be important for defense against microbial infection; they can recognize highly conserved pathogen-associated microbial patterns which are exclusively expressed by microbial pathogens [1–3]. To date, 10 human and 12 murine TLRs have been characterized [4,5]: TLR1 to TLR10 in humans, and TLR1 to TLR9, TLR11, TLR12, and TLR13 in mice. TLRs are type I transmembrane proteins characterized by an extracellular domain containing leucine-rich repeats (LRRs) [6,7]. TLR4 has been studied extensively, and different TLRs have been shown to recognize different PAMPs [8]; for example, TLR4 recognizes lipopolysaccharide (LPS) of Gram-negative bacteria and TLR5 recognizes flagellin [9], TLR11 identify toxic plasma and non-pathogenic *E. coli* pili proteins [10,11]. Moreover, TLRs also recognize an endogenous ligand released from damaged or necrotic tissue, such as heat shock protein 60, 70, and the high mobility group protein. It is worth noting that different TLRs activations may lead to differentiation of different types of T lymphocyte subsets (Th1, Th2, Th17, and Treg).

$CD4^+CD25^+$T regulatory T (Treg) cells have been shown to mediate immunosuppression, and their identification represents a milestone in the field of immunology [12,13]. Recent studies have suggested that TLR ligands can directly modulate the suppressive capacity of Treg cells [1]. TLR4 mainly regulates Treg cells in graft rejection, autoimmune diseases, infectious diseases, and cancers [14]. Furthermore, TLR4 binding with LPS has been shown to enhance the suppression of Treg cells [1].

High mobility group box-1 protein 1 (HMGB1) was identified as a gene transcription regulator. While recent reports have shown that HMGB1 plays an important role in the innate immune system, it also has the potential to mediate Th1 polarization and activate antigen presenting cells. Although HMGB1 can modulate the suppressive capacity of Treg cells directly, whether TLR4 is essential for HMGB1 suppression on Treg cells still needs to be elucidated [14]. In the present study, we found that HMGB1 and LPS stimulated Treg cells could be regulated by TLR4 through the NF-κB pathway.

Material and Methods

Isolation and purification of Treg cells

Human peripheral blood mononuclear cells were obtained from peripheral blood of healthy adult donors. After Ficoll-Paque density gradient centrifugation, Treg cells were isolated from the mononuclear cells using human Treg cell MACS kit (BD Biosciences) according to the manufacturer's instructions. Treg cells were suspended in 2 mL RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), and then examined by FACS calibur flow cytometer (BD Biosciences).

Cell culture and stimulation

Isolated Treg cells were counted and cultured in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin). After pre-incubation with anti-TLR4 antibody, anti-TLR4 isotype control, and mouse anti-human TLR4 blocking antibodies (20 ng/mL) for two hours at 37°C, different concentrations of LPS (0 μg/mL, 0.1 μg/mL, 1 μg/mL, and 10 μg/mL) or HMGB1 (0 μg/mL, 0.01 μg/mL, 0.1 μg/ml, and 1 μg/mL) were added, incubated with different time points (4, 8, 16, 24, 32, 48, or 72 hours). Meanwhile, 20 U/mL IL-2 was added into the wells. Following the culture and stimulation procedure, the supernatant was collected and used to determine the content of IL-1β, IL-10, IFN-γ, and TGF-β.

Cytokine assays

Supernatants were measured using commercial ELISA kits (eBioscience) following the protocols provided by the manufacturer. The color reaction was terminated by adding 50 μL of 2N H_2SO_4. Absorbance was read in a microplate reader (Bio-Tek, USA) at the wavelength of 450 nm.

RNA isolation and quantitative PCR

Total RNA was prepared using TRIzol LS reagent according to the manufacturer's instructions. For reverse transcription, cDNA was synthesized using Revert AidTM First Strand cDNA Synthesis Kit (Fermentas) following the manufacturer's guidelines. Quantitative PCR (qPCR) reactions (EcoTM, Illumina) were performed following the protocol of the kit (Applied Biosystems). The reaction step was two minutes at 50°C, followed by 10 minutes at 95°C, and 40 cycles of 30 seconds at 95°C and 30 seconds at 60°C.

Co-cultures and proliferation assay

$CD4^+$ T cells (>97% pure) were obtained by magnetic-bead activity cell sorting (MACS) and cultured in medium containing 10% FBS at 37°C in 5% CO_2 incubator. To determine cell proliferation, carboxyfluorescein succinimidyl ester (CFSE) dye (Invitrogen) was added to the T cells following the manufacturer's protocol. Co-cultures were set up using 2×10^5 $CD4^+$ T cells and 2×10^5 pretreated Treg cells. Cells were cultured for five days, and then analyzed separately via flow cytometry (FACalibration, BD, USA).

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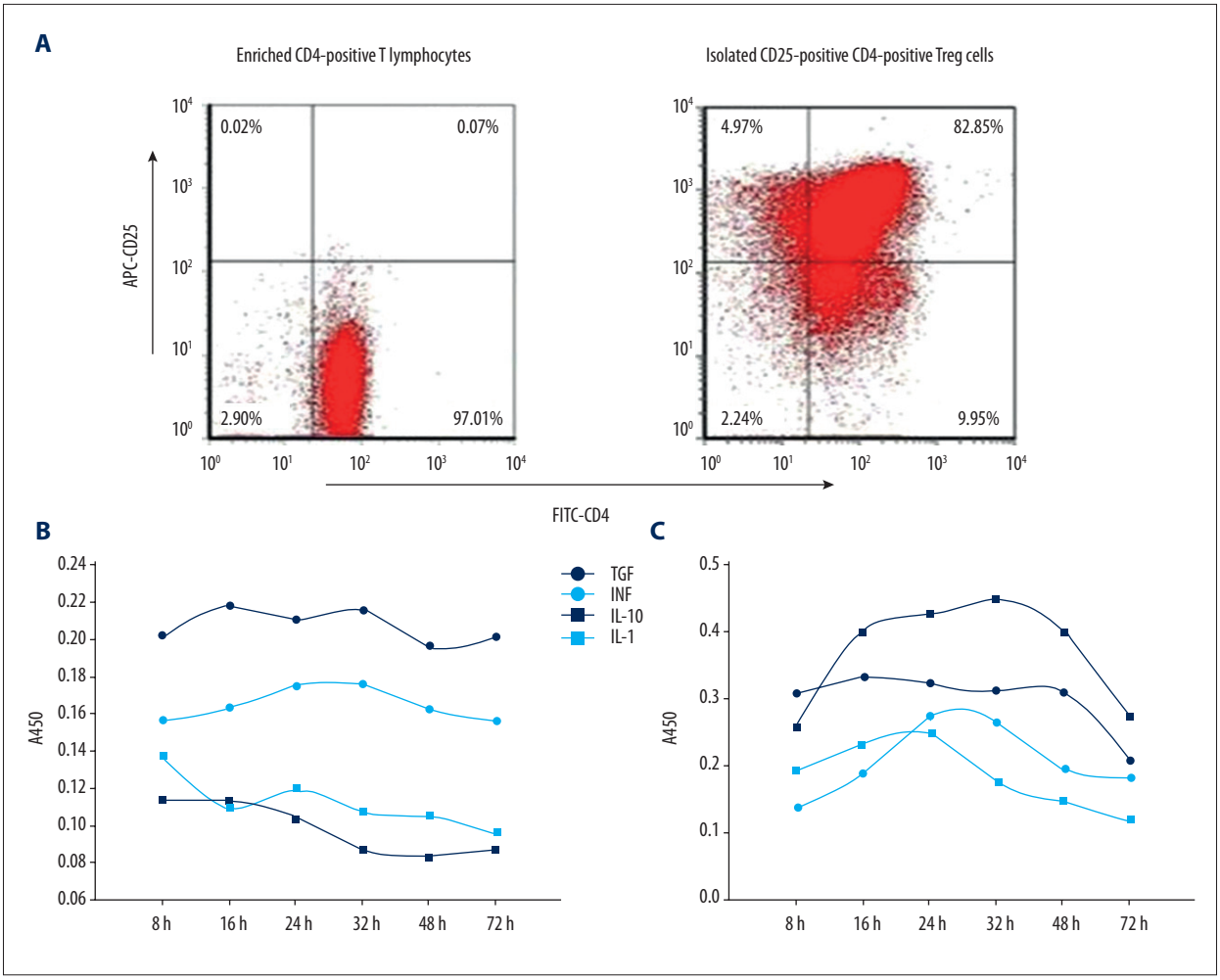

Figure 1. (**A**) The purity of negatively sorted CD4+ T cells was (97.01±2.65%). The purity of positively sorted CD4+ CD25+ Treg cells was (84.52±2.10%). (**B**) Purified CD4+ CD25+ Treg cells were treated with 1 μg/mL HMGB1 for different times (8, 16, 24, 32, 48, or 72 hours); cytokines in the supernatant were detected by ELISA. (**C**) Purified CD4+ CD25+ Treg cells were treated with 10 μg/mL LPS for different times (8, 16, 24, 32, 48, or 72 hours); cytokines in the supernatant were detected by ELISA.

Western blotting

Treg cells were collected and washed with cold PBS after treatment with HMGB1 and LPS. Total protein was extracted using a protein extraction kit according to the manufacturer's instruction. Protein concentration of fractions was quantified with bicinchoninic acid kit (Biyuntian, China). Equal amounts of denatured protein samples were separated by 12% SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were incubated with appropriate dilution of the specific primary antibody against NF-κB (1: 1,000) and β-actin (1: 500) at 4°C overnight, washed three times with PBST, then incubated for 45 minutes with HRP-labeled secondary antibody (1: 6,000), and then washed with PBST; the exposure was performed after adding substrate. All immunoblots were detected by enhanced chemiluminescence (Amersham Biosciences).

Statistical analysis

Data are represented as mean ± standard deviation (SD), and were analyzed with one-way ANOVA. Unpaired Student's *t*-test was used to evaluate significant differences between groups. A *p* value of 0.05 or less was considered statistically significant.

Results

IL-1β, IL-10, IFN-γ, and TGF-β expression decreased in CD4+ CD25+ Treg cells after HMGB1 treatment

Treg cells were isolated in two steps by MACS kit according to the manufacturer's instructions. The purity of T cells (more than 82%) was assessed by FACS calibur flow cytometer (Figure 1A). As HMGB1 is a common endogenous ligand

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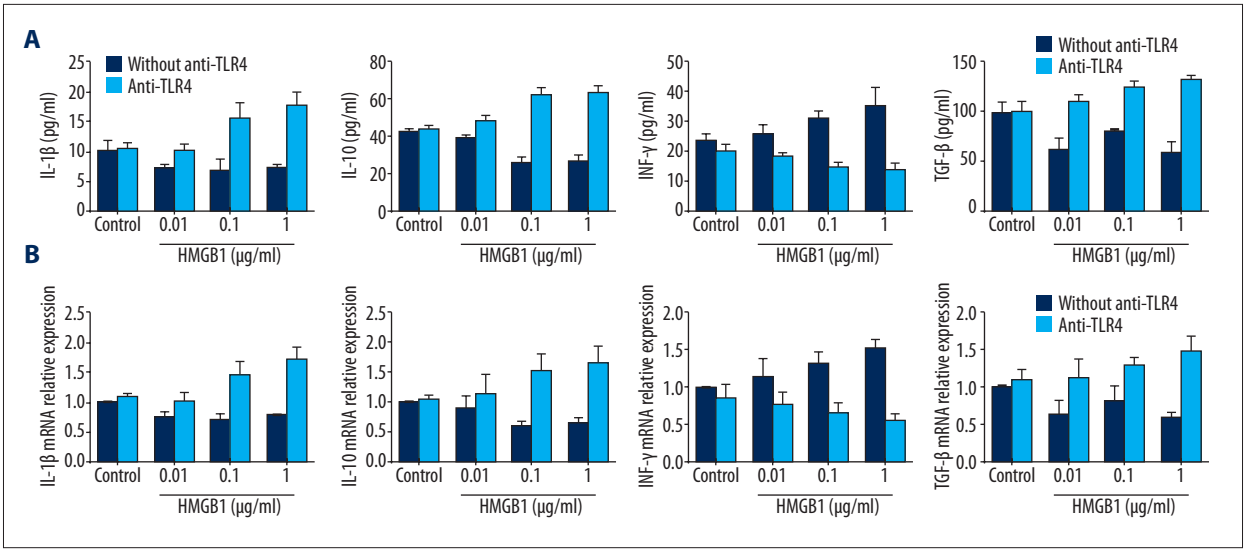

Figure 2. Quantization of cytokines produced after HMGB1 (0.1 μg/mL and 1 μg/mL for 16 hours) treatment in the non-anti-TLR4 group and the anti-TLR4 group. Cells cultured without HMGB1 were used as controls. (**A**) Cytokines in the supernatant were detected by ELISA, and (**B**) their expression was also detected by quantitative PCR.

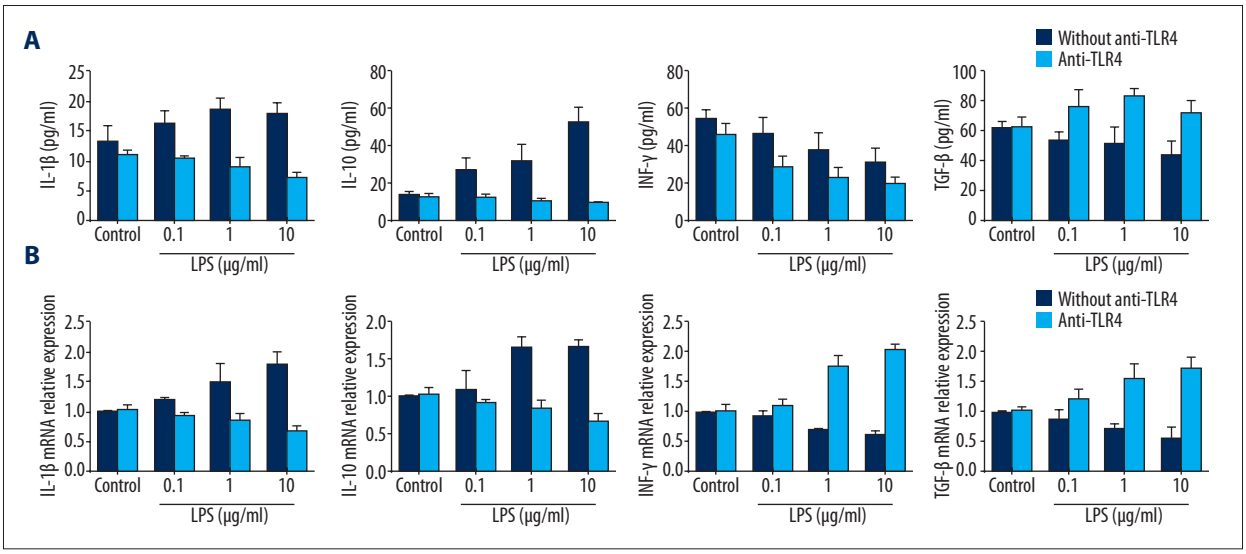

Figure 3. Quantization of cytokines produced after LPS (0.1 μg/mL, 1 μg/mL, and 10 μg/mL for 24 hours) treatment in the non-anti-TLR4 group and the anti-TLR4 group. Cells cultured without HMGB1 were used as controls. (**A**) Cytokines in the supernatant were detected by ELISA, and (**B**) their expression was also detected by quantitative PCR.

of TLR4, to find the optimal stimulation time, we examined the levels of IL-1β, IL-10, IFN-γ, and TGF-β in Treg cells after stimulation by 1 μg/mL HMGB1 at different time points (4, 8, 16, 24, 32, 48, and 72 hours). Supernatants were collected for ELISA (the standard curve for the different protein detections are shown in Supplementary Figure 1, the data are shown in Figure 1B). IL-10 and TGF-β were significantly increased at 16 hours of treatment, and IFN-γ was significantly increased at 24 hours of treatment, while IL-1β was decreased at 8 hours to 72 hours of treatment. We used HMGB1 treatment for 16 hours with different concentrations of HMGB1 (0.01 μg/mL, 0.1 μg/mL, and 1 μg/mL) to act on the non-anti-TLR4 group and the anti-TLR4 group of Treg cells. The supernatants and cells were collected separately for ELISA (Figure 2A) and mRNA expression analysis (Figure 2B). Treg cells without HMGB1 incubation were used as the control. After stimulation for 16 hours, in the non-anti-TLR4 group, the RNA level and protein content of IL-1β, IL-10, and the TGF-β of HMGB1-treated Treg cells decreased significantly compared with the control ($p<0.05$), while in the anti-TLR4 group, they are higher than that of the corresponding control ($p<0.05$). The change trends of RNA level and protein content of IFN-γ were the opposite, and they are

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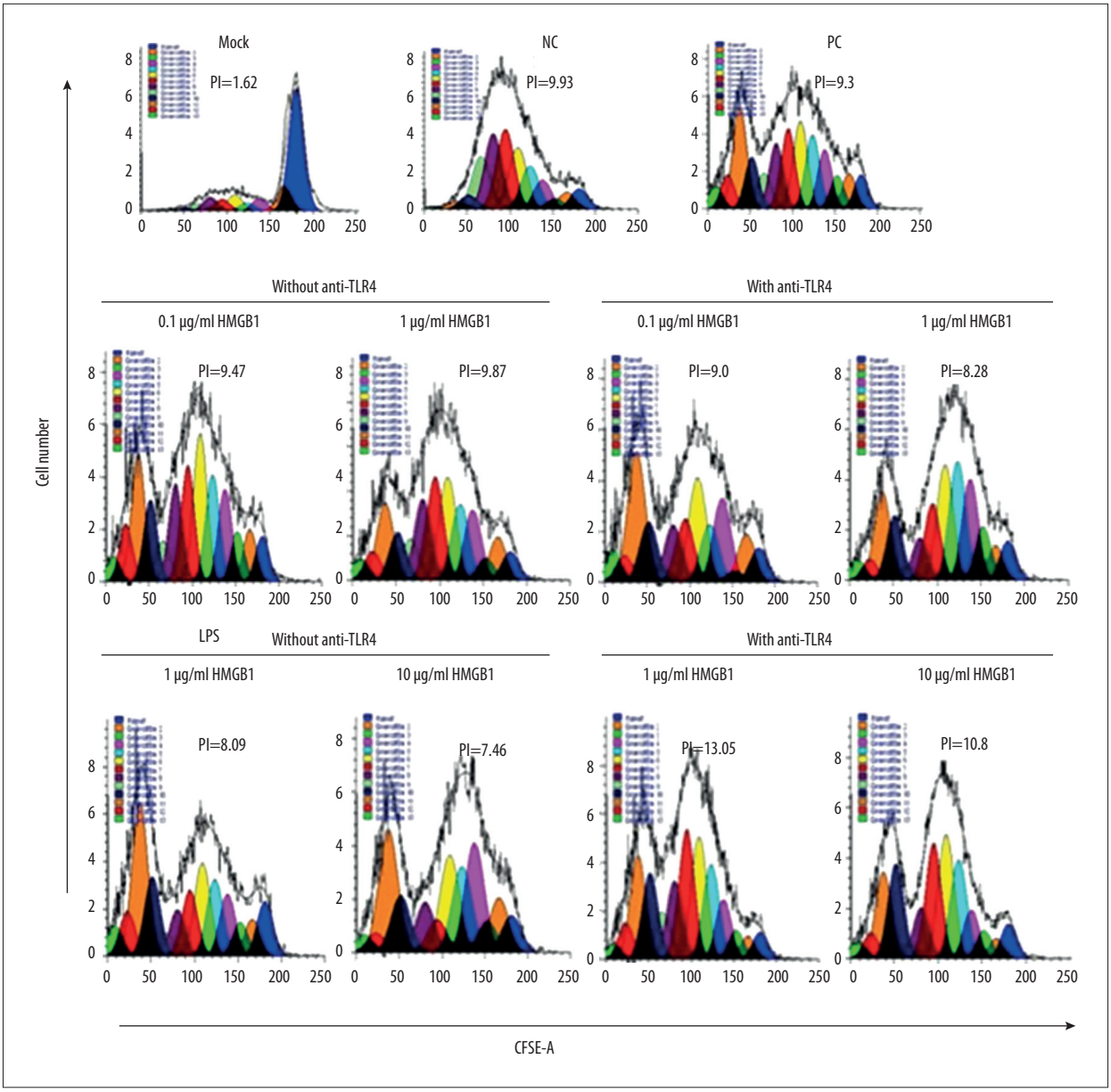

Figure 4. Proliferation of CD4+ T cells in different culture systems detected by flow cytometry.

significantly higher than that of the control in the non-anti-TLR4 group ($p<0.05$) and much lower than that of the corresponding control in the anti-TLR4 group ($p<0.05$).

IL-1β, IL-10, IFN-γ, and TGF-β expression increased in Treg cells treated with LPS

LPSs are typical exogenous TLR4 ligands. Similar to HMGB1, the best stimulation time of 10 μg/mL LPS was 24-hour detected by ELISA (shown in Figure 1C). The standard curve was created to calculate protein concentration (shown in Supplementary Figure 2). Both the non-anti-TLR4 group and the anti-TLR4 group were incubated with different concentrations of LPS (0.1 μg/mL, 1 μg/mL, 10 μg/mL), and the group without LPS incubation was used as the control. After stimulation for 24 hours, both supernatants and cells were collected for ELISA or qPCR. As shown in Figure 3, the RNA level and protein content of IL-1β and IL-10 increased significantly compared with the control ($p<0.05$), while in the anti-TLR4 group, they were lower than that of the corresponding control group ($p<0.05$). The RNA level and protein content of IFN-γ and TGF-β decreased significantly compared with the control group ($p<0.05$), while in the anti-TLR4 group, only the RNA level of IFN-γ and both the RNA and protein content of TGF-β were clearly higher than that of the corresponding control ($p<0.05$). In addition, the protein content of IFN-γ was still lower than that of the corresponding control ($p<0.05$).

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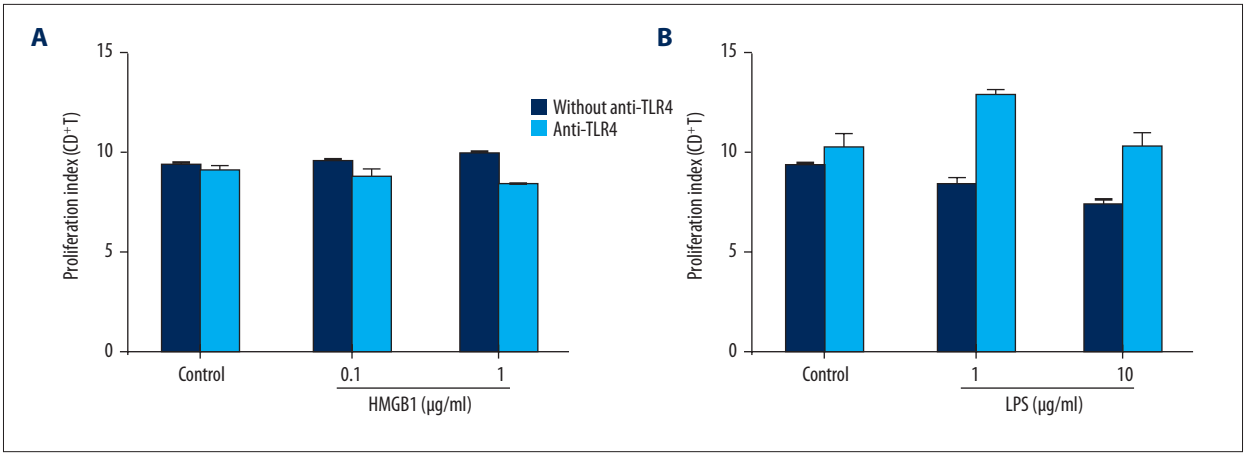

Figure 5. CD4+ CD25+ Treg cells treated with HMGB1 (**A**) and LPS (**B**) could inhibit the proliferation of CD4+ T cells. Data were shown as the mean ±SD.

HMGB1 weakened the Treg cells and inhibited the ability of CD4+ T cell proliferation; whereas LPS had the opposite effect

Co-cultures of CD4+ T cells and pretreatment of Treg cells were carried out for five days, and then the proliferation index (PI) was determined using CFSE labeling. The results suggested that CD3/CD28 antibody could activate CD4+ T cells and significantly promote proliferation. When activated CD4+ T cells were co-cultured with Treg cells, which were isolated autologously, proliferation of CD4+ T cell were weaken (shown in Figure 3). PI values of T cells were changed after Treg cells were treated with different concentrations of HMGB1 and LPS. As shown in Figure 4, Treg cells could significantly inhibit the proliferation of CD4+ T cells, and there was a significant difference compared with the positive control which was activated by CD3/CD28 antibody ($p<0.05$). In the non-anti-TLR4 group, HMGB1 treated Treg cells were co-cultured with CD4+ T cells. The PI of CD4+ T cells that were analyzed by flow cytometry (FCM) (Figure 5) were slightly higher than the untreated control ($p<0.05$); while in the anti-TLR4 group, the PI of CD4+ T cells were significantly lower than the control ($p<0.05$). However, when LPS treated Treg cells were co-cultured with CD4+ T cells, the PI of CD4+ T cells was much lower than the untreated control in the non-anti-TLR4 group ($p<0.05$); while in anti-TLR4 group, the PI of CD4+ T cells was significantly higher than the mock and the positive control ($p<0.05$).

HMGB1 and LPS regulated Treg cell function via activation of NF-κB

To further explore the potential signaling pathway after HMGB1 and LPS treatment, the protein levels of NF-κB in cytoplasmic and nuclear fractions of Treg cells were assessed after treatment with HMGB1 (1 μg/mL for 16 hours) or LPS (1 μg/mL for 24 hours). As shown in Figure 6, after using 1 μg/mL HMGB1 and LPS to activate CD4+ CD25+ Treg cells, in the non-anti-TLR4 group, the level of NF-κBp65 in cytoplasm protein was lower than in the control; while in nucleoprotein, it was higher than the corresponding control. No significant differences existed in the anti-TLR4 group.

Discussion

Treg cells are critically important for maintaining balanced immune responses. They can effectively suppress the immune response in occurrences of self-immune diseases, cancer, and other diseases [15–17]. However, the suppressive mechanisms, as well as signal transduction of Treg cells, remain poorly understood [14]. Previous reports have suggested that TLR signaling may directly or indirectly regulate the immunosuppressive function of Treg cells in immune responses [18]. Moreover, TLR4 signaling has been implicated in the regulation of Treg cell functions [19].

Both Treg and CD4+ CD25− T cells express TLR4 [1]. Activation of TLR4 expressed in CD4+ CD25− T cells promotes the suppressive function of Treg [2]. But, it is still unknown whether TLR4 expressed on the membrane of Treg cells can regulate Treg cell function. In addition, it is unclear if there are any differences in Treg cell regulation by TLR4 endogenous and exogenous stimulation. To address these issues, further studies are needed to study the mechanism of HMGB1 and LPS in Treg cell immunosuppression modulation. In our experiment, we isolated and purified Treg cells and co-cultured them with low, middle, and high concentrations of HMGB1 [14,20] and LPS [21,22], then used ELISA and PCR for analysis. We found that LPS treated Treg cells directly increase the expression of IL-1β and IL-10 and decrease the expression of IFN-γ and TGF-β. However, HMGB1 treatment resulted in a marked decrease in the expression of IL-1β, IL-10, IFN-γ, and TGF-β. Although we found that IFN-γ from media and mRNA levels were not consistent after LPS treatment, this result may have been due to the qPCR analysis, which is generally more sensitive than ELISA,

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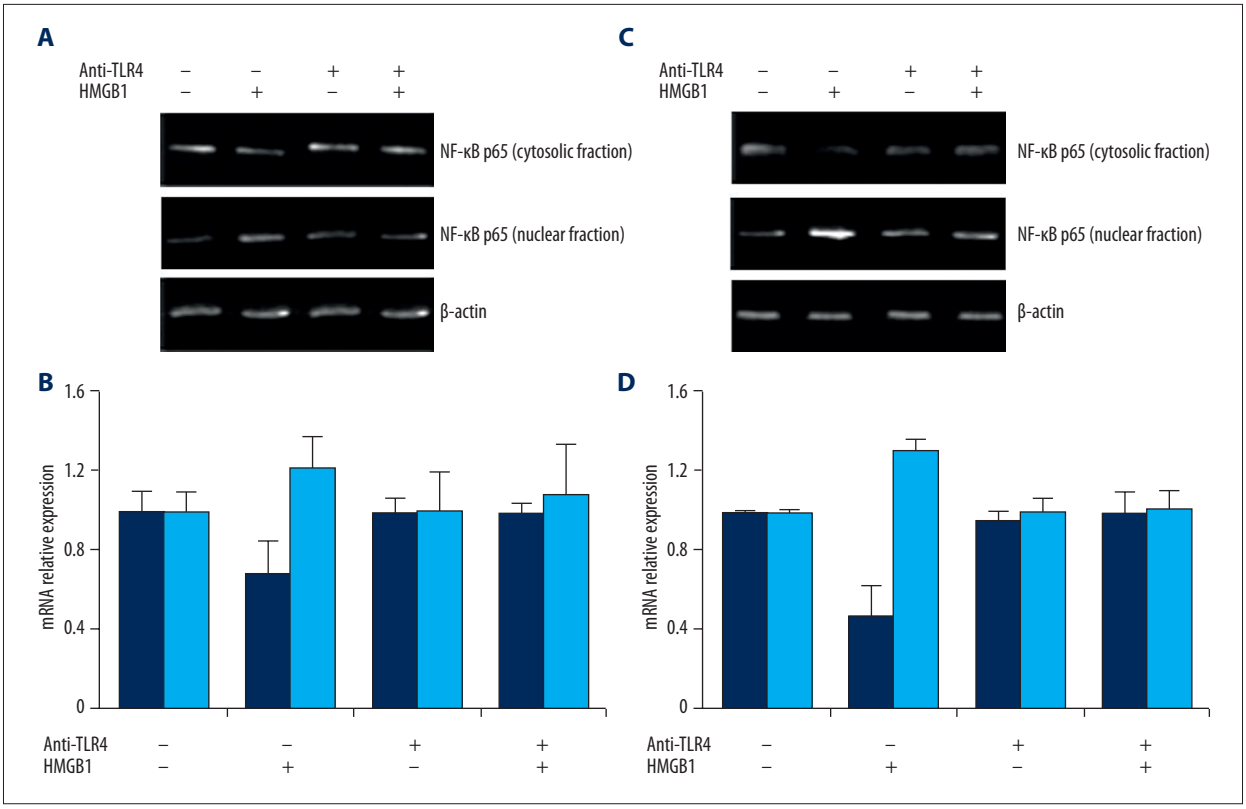

Figure 6. NF-κB p65 expression of CD4⁺ CD25⁺ Treg cells treated with HMGB1 and LPS.

given that the protein translation process and their secretion is slower than mRNA transcription. To further analyze whether these potential effects were associated with TLR4 signaling, neutralizing antibody against TLR4 (anti-TLR4 mAb) was used in our experiments. When TLR4 was blocked, the opposite effects occurred. This suggested that both LPS and HMGB1 could regulate Treg cell function via TLR4.

Another study showed that Treg cells can directly inhibit the proliferation of $CD4^+$ $CD25^-$ T cells and secretion of cytokines, as well as possibly act through inhibiting the stimulatory capacity of antigen-presenting cells to indirectly inhibit the activation of $CD4^+$ $CD25^-$ T cells. To investigate the effect of Treg cells on proliferation of $CD4^+$ $CD25^-$ T cells in different microenvironments, we co-cultured $CD4^+$ T cells and Treg cells pretreated with HMGB1 or LPS for five days. The results showed that the proliferation of $CD4^+$ T cells was significantly inhibited by Treg cells. Moreover, the inhibition effect was increased when Treg cells were pre-incubated with LPS, but it was reduced when Treg cells were pre-incubated with HMGB1.

TLR4 and LPS recognition initiates two separate signaling pathways through the recruitment of MyD88 or TRIF, which eventually induces the production of pro-inflammatory cytokines and type I IFN by activation of NF-κB, MAPK, and IRF3 [6,23]. MyD88 is a universal adaptor for all TLRs (except for TLR3), mediated by the NF-κB signal pathway to induce inflammatory cytokines production [2]. To further explore the mechanisms underlying the effects of HMGB1 and LPS on TLR4 signaling, the protein levels of NF-κB in cytoplasmic and nuclear fractions of Treg cells were assessed after treatment with HMGB1 (1 μg/mL for 16 hours) or LPS (1 μg/mL for 24 hours). Our data suggest that HMGB1 and LPS stimulation could downregulate the expression NF-κB p65 in cytoplasmic protein and increase it in nuclear protein, thus leading to modulation of IL-1β, IL-10, IFN-γ, and TGF-β expression. Moreover, the suppressive function of Treg cells could be regulated by TLR4. The different effects of HMGB1 and LPS treatment may be due to the effect these two signaling molecule can have on the regulate Treg cell suppressive function in different stages, in that LPS can induce the early inflammation factors expression after infected by bacteria, whereas HMGB1 can induce late inflammation process after host cell damage and/or necrosis leading to its release.

Conclusions

Our data suggest that the membrane molecule TLR4 in Treg cells plays different roles in different microenvironments, and regulates the suppressive function of Treg cells. HMGB1 treated Treg cells directly increased the expression of pro-inflammatory cytokine IFN-γ and inhibited its suppressive function.

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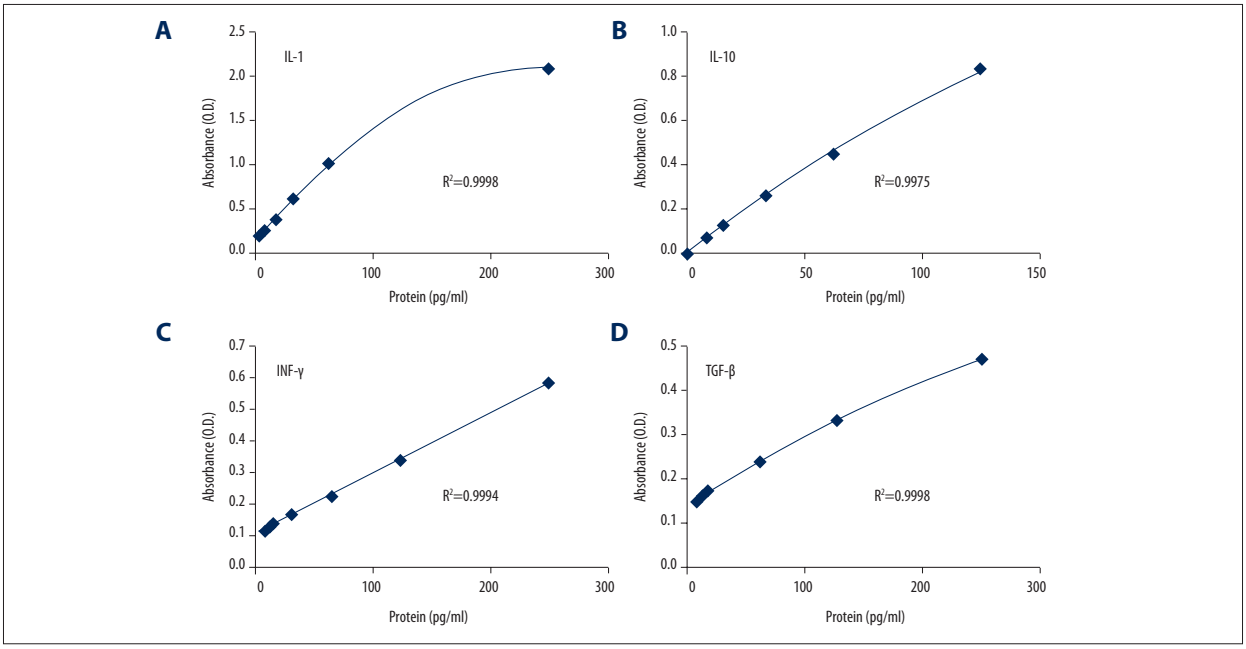
However, LPS mainly upregulated the expression of IL-1β and IL-10, and promoted the suppressive capacity of Treg cells. Both effects are related to the activation of NF-κB. In conclusion, TLR4 had an effect on the suppressive function of regulatory T cells through the activation of the NF-κB pathway. TLR4 could regulate Treg cell suppressive function; and thus it is expected to provide a new target for the treatment of diseases.

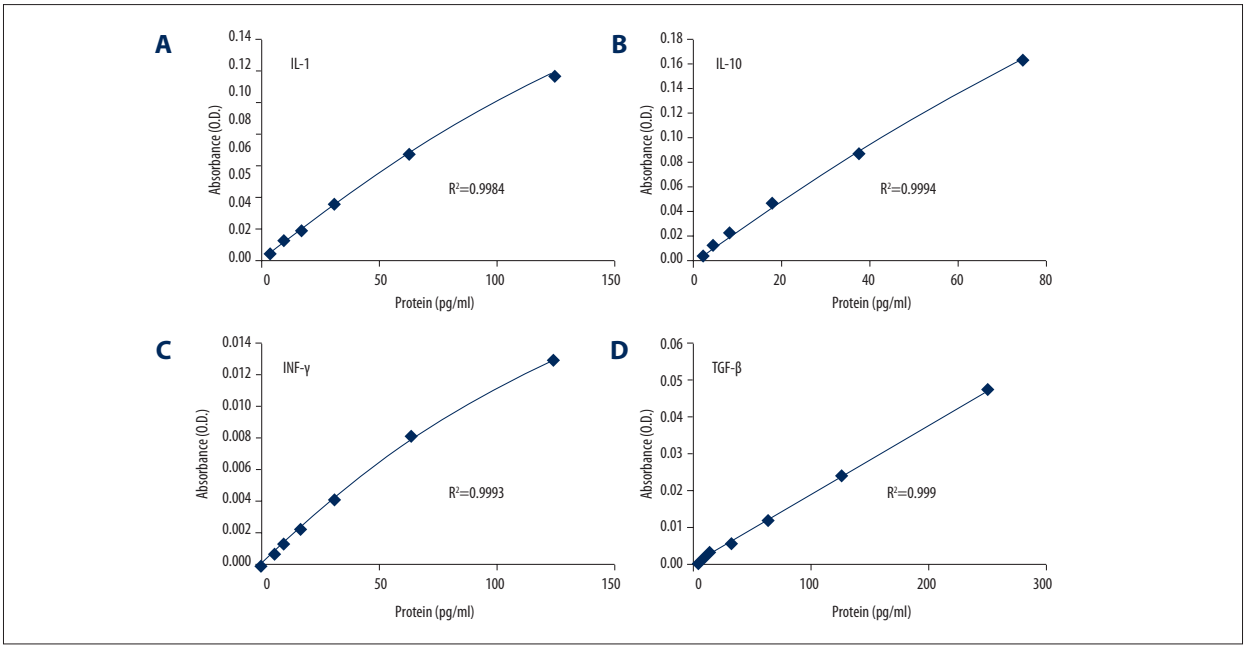
Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Supplementary Figures



Supplementary Figure 1. The standard curve was created to calculate protein concentration after HMGB1 treatment for 16 hours.



Supplementary Figure 2. The standard curve was created to calculate protein concentration after LPS treatment for 24 hours.

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