

**ORIGINAL ARTICLE**

Iran J Allergy Asthma Immunol  
June 2015; 14(3):321-330.

## **Transforming Growth Factor Beta-Induced Is Essential for Endotoxin Tolerance Induced by a Low Dose of Lipopolysaccharide in Human Peripheral Blood Mononuclear Cells**

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*Received: 24 September 2014; Received in revised form: 4 November 2014; Accepted: 11 November 2014*

### **ABSTRACT**

Our prior study found that transforming growth factor beta-induced (TGFB $\beta$ ) is an important negative regulator in TLR-induced inflammation. However, whether TGFB $\beta$  may affect inflammation during lipopolysaccharide (LPS)-induced endotoxin tolerance (ET) is still unclear.

This study aimed to investigate whether TGFB $\beta$  was involved in the mechanisms of ET in human through dampening nuclear factor-kappa B (NF- $\kappa$ B) mediated pathway. ET models of isolated healthy volunteers peripheral blood mononuclear cells (PBMCs) were established by pretreating with a low dose of LPS to observe the changes of TGFB $\beta$  expression during ET induction, compared with ten healthy controls. Moreover, a vector-based short hairpin RNA expression system was used to specifically inhibit TGFB $\beta$  expression to further explore its role in ET induction. The expression was analyzed by reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting. The responses to LPS were determined by the activation of NF- $\kappa$ B, the production of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and Nitric Oxide (NO), which were analysed by enzyme-linked immuno sorbent assay (ELISA).

The results showed that TGFB $\beta$  expression in the ET group obviously increased; ET also led to a hyporesponse of PBMCs to LPS with less activation of NF- $\kappa$ B, less production of TNF- $\alpha$  and NO, as well as more expression of TGFB $\beta$  than those of non-ET group. Moreover, the inhibitory effect was partly refracted in plasmid TGFB $\beta$  short hairpin RNA (pTGFB $\beta$ -shRNA) transfected PBMCs. Meanwhile, the absence of TGFB $\beta$  caused abnormal enhancement of inflammatory cytokine production and it was involved in ET induction through dampening NF- $\kappa$ B mediated pathway.

Therefore, TGFB $\beta$  may be a new target for the clinical treatment of inflammatory disorders.

**Keywords:** Endotoxin tolerance; Lipopolysaccharide; PBMCs; TGFB $\beta$

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## INTRODUCTION

Inflammation is a complex pathophysiological state, adopted primarily by innate immune cells in response to infection and/or tissue damage.<sup>1,2</sup> Innate immune cells like monocytes/macrophages detect and respond to “danger signals” (e.g. pathogens, tissue damage) through pattern recognition receptors (PRR) expressed on their surface. The family of toll-like receptors (TLRs) is the major PRR whose members play essential roles in innate immunity in various organs.<sup>3,4</sup> Peripheral blood mononuclear cells (PBMCs) are a critical component in the immune system with regard to first-line fighting against infections.<sup>5</sup>

TLR-mediated nuclear factor kappa B (NF- $\kappa$ B) activation induces inflammatory responses, and uncontrolled inflammation leads to extensive tissue damage and manifestation of pathological states like sepsis, autoimmune diseases, metabolic diseases and cancer.<sup>2</sup> To maintain the balance between activation and inhibition of the innate immune system, a variety of negative regulatory mechanisms control TLR-mediated cellular signaling.<sup>6,7</sup> One of the classic examples of such a protective mechanism is endotoxin tolerance (ET),<sup>2,8,9</sup> a phenomenon in which cells or organisms exposed to low concentrations of endotoxin (e.g. LPS) enter into a transient unresponsive state and are unable to respond to further challenges with endotoxin. It has been well recognized that induction of ET protects the host from cellular damage caused by hyperactivation of macrophages and likely represents a mechanism of immune cell adaptation to a persistent bacterial infection.

The gene transforming growth factor, beta-induced (*TGFBI*), a secreted protein, is induced by transforming growth factor- $\beta$  (TGF- $\beta$ ) in various human cell types.<sup>10</sup> Previous studies in our laboratory have implied that *TGFBI* was one of negative regulator genes in TLR-induced inflammation.<sup>11</sup> *TGFBI* was first identified in the human lung adenocarcinoma cell line A549 treated with TGF- $\beta$ .<sup>12-15</sup> Mutations or altered expression of this gene is believed to be responsible for the pathogenesis of human corneal dystrophy, angiogenesis, osteogenesis and inflammation.<sup>16,17</sup> In addition, over-expression of *TGFBI* was first detected in inflammatory processes associated with atherosclerosis,<sup>18</sup> wound healing,<sup>19</sup> diabetic angiopathy,<sup>20</sup> cyclosporine nephropathy,<sup>21</sup> and RA.<sup>22</sup> These observations demonstrated that *TGFBI* is involved with the modulation of inflammatory responses

and regulation of its expression might be a potential target for the treatment of inflammatory diseases.

In the present study, we detected the *TGFBI* expression patterns in the human peripheral blood mononuclear cells (PBMCs) under ET condition by using short hairpin RNA (shRNA) approach to support our new view that *TGFBI* negatively regulates TLR signaling and participates in the mechanisms of ET in PBMCs through dampening NF- $\kappa$ B mediated pathway. These are the first known results to show that *TGFBI* is an early responsive negative regulator of TLR signaling in PBMCs, which may contribute to the first line of defense during inflammation.

## MATERIALS AND METHODS

### Reagents and Antibodies

The reagents and antibodies used in our experiments are as follows: purified *Escherichia coli* LPS (Sigma, St Louis, MO, USA), Lipofectamine 2000 (Invitrogen, California, CA, USA), Nuclear Extraction Kit, TransAM<sup>®</sup> NF- $\kappa$ B p65 Transcription Factor Assay Kit (Active Motif, Carlsbad, CA, USA).

### PBMC Isolation and Induction of Endotoxin Tolerance

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats of fifty healthy volunteers by centrifugation on Ficoll-Hypaque Plus (TBD) as described before<sup>[11]</sup> and grown in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS). All reagents for cell culture were endotoxin-free. Once seeded, adherent cells were left untreated (non-endotoxin tolerance, NETT) or stimulated with 10 ng ml<sup>-1</sup> LPS (endotoxin tolerance, ETT) for 24h, washed twice with warm RPMI-1640 and kept in complete medium for 2h through the time of recovery phase.<sup>23</sup> Then, cells were restimulated with 100 ng ml<sup>-1</sup> LPS for periods of time ranging from 1 to 24 h. PBMCs from ten healthy volunteers were included as healthy controls.

### Plasmid Constructs for *TGFBI* shRNA

A vector-based shRNA expression system, the pGenesil vector (Genesil, China) containing the cDNA of GFP and kanamycin resistance gene, was used to endogenously express shRNA in PBMCs. The control RNA interference (RNAi) vector (Genesil, China) was constructed by the insertion of a scrambled sequence

that did not show significant sequence homology to rat, mouse or human gene sequences. We selected the target regions in the exons 311 bp and 325 bp boundary of TGFBI cDNA (NM000358) according to the Tushul's principle and designed DNA oligonucleotides for the in vitro transcription. The target sequences for TGFBI were 5'- TGT GCA GAA GGT TAT TGG CAC TAA T-3' and 3'-ATT AGT GCC AAT AAC CTT CTG CAC A-5'. The DNA sequences were cloned into the BamHI/HindIII restriction site of the pGenesil vector. All inserted sequences were identified with restriction endonuclease digestion and sequencing. The double-stranded shRNA molecules were prepared by brief boiling and slow cooling, and stored at -80°C.

### Cell Transfection and Treatment

PBMCs were plated in six-well plates and transfected transiently with either pTGFBI-shRNA or pGenesil-1 using Lipofectamine 2000 reagent as recommended by the manufacturer. Twenty-four hours after transfection, the medium was replaced with fresh medium and the cells were treated and re-stimulated in a manner similar to their non-transfected counterparts.

### RNA Extraction and Real time-Polymerase Chain Reaction (PCR)

Total RNA was extracted from different samples using Trizol reagent according to the manufacturer's instructions. Total cellular RNA was extracted from whole cells and 3µg of RNA was treated with RNase free DNase (Takara, Otsu Shiga, Japan) for the synthesis of first-strand cDNA using a RevertAid FirstStrand cDNA Synthesis Kit (Fermentas, Hanover, USA) according to the manufacturer's protocol. All primers used were flanked by intron-exon junctions using the national center for biotechnology information (NCBI) blast tool and Primer 5 software. Specific primer sequences of TGFBI, TNF- $\alpha$  and  $\beta$ -actin were as follows and the sizes of production were 170bp, 484bp, 155bp, respectively, TGFBI: forward: 5'-GTGGACGTGCTGATCATCTT-3', reverse: 5'-TCCTGGCTGGTTACAGATAC-3'; TNF- $\alpha$ : forward: 5'-GAAAGCATGATCCGGGACG-3', reverse: 5'-TCTGGTAGGAGACGGCGATG-3';  $\beta$ -actin: forward: 5'-ACTTAGTTGCGTTACACCCTTTC-3', reverse: 5'-CTGTACACCTTCACCGTTCC-3'. All PCR products were electrophoresed on 2% agarose gels. The relative expression of mRNAs were assessed by taking

the ratio of the intensity of the DNA bands of TGFBI or TNF- $\alpha$  to  $\beta$ -actin band using the Bio-Image analysis system (Bio-Rad Gel Doc 2000, USA) and expressed as arbitrary units.

### Western Blotting

Expression of the TGFBI protein in the supernatant of PBMCs was confirmed by Western blotting. PBMCs were plated and grown in RPMI-1640 with 10% FBS for 24 h, and then transferred to serum-free medium and maintained for another 24 h. The medium was then harvested and trichloroacetic acid (TCA) was added to a final concentration of 10%. It was then incubated at room temperature (RT) for 30 min, centrifuged at 13,000 rpm at 4°C for 30 min, and the supernatant was aspirated. The pellet was washed three times at acetone and then air dried. Fifty microliters of laemmli sample buffer was added to the pellet and boiled for 5 min. It was then resolved on SDS-PAGE. The gels were transferred onto PVDF membrane and incubated serially with monoclonal antihuman TGFBI (R&D Systems, Minneapolis, MN, U.S.) followed by sheep anti-mouse IgG conjugated with horseradish peroxidase as secondary antibody (Amersham Biosciences, Piscataway, NJ, U.S.). Multiple clones were chosen for the study, and similar results were observed with each. The results shown in this manuscript are representatives of the findings.

### ELISA

Supernatants were collected after stimulation, and levels of TNF- $\alpha$  and NO were analyzed with commercial ELISAs (R&D Systems), following the manufacturer's protocols.

### Analysis of NF- $\kappa$ B Transcriptional Activity

The NF- $\kappa$ B DNA binding activity in PBMCs was detected using the Trans-AM NF- $\kappa$ B p65 ELISA Kit according to the manufacturer's instructions. Briefly, 5 µg nuclear extract added to a 96-well plate to which oligonucleotide containing NF- $\kappa$ B consensus-binding site had been immobilized. The NF- $\kappa$ B complex bound to the oligonucleotide was detected by adding a specific mAb for p65 subunit. A secondary horseradish peroxylase-conjugated mAb was added and developed with tetramethylbenzidine substrate. After an optimal development time, the reaction was stopped using H<sub>2</sub>SO<sub>4</sub> 0.5 mol l<sup>-1</sup>, and absorbance was measured at 450 nm.

### Statistical Analysis

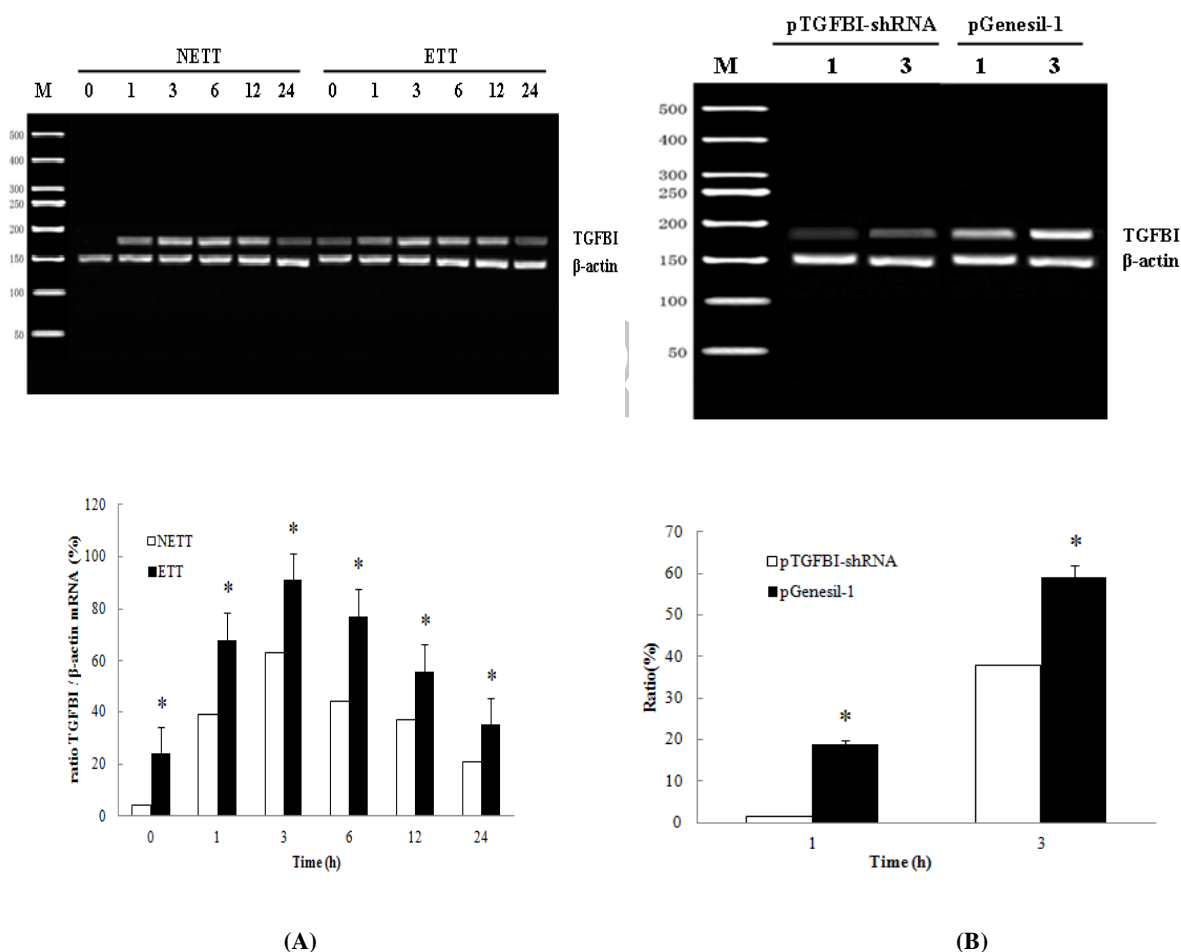
All data were shown as mean±standard deviation (SD). Statistical significance was determined by Student *t* test using the SPSS 13.0 software package. The level of statistical significance was set at  $p<0.05$ .

## RESULTS

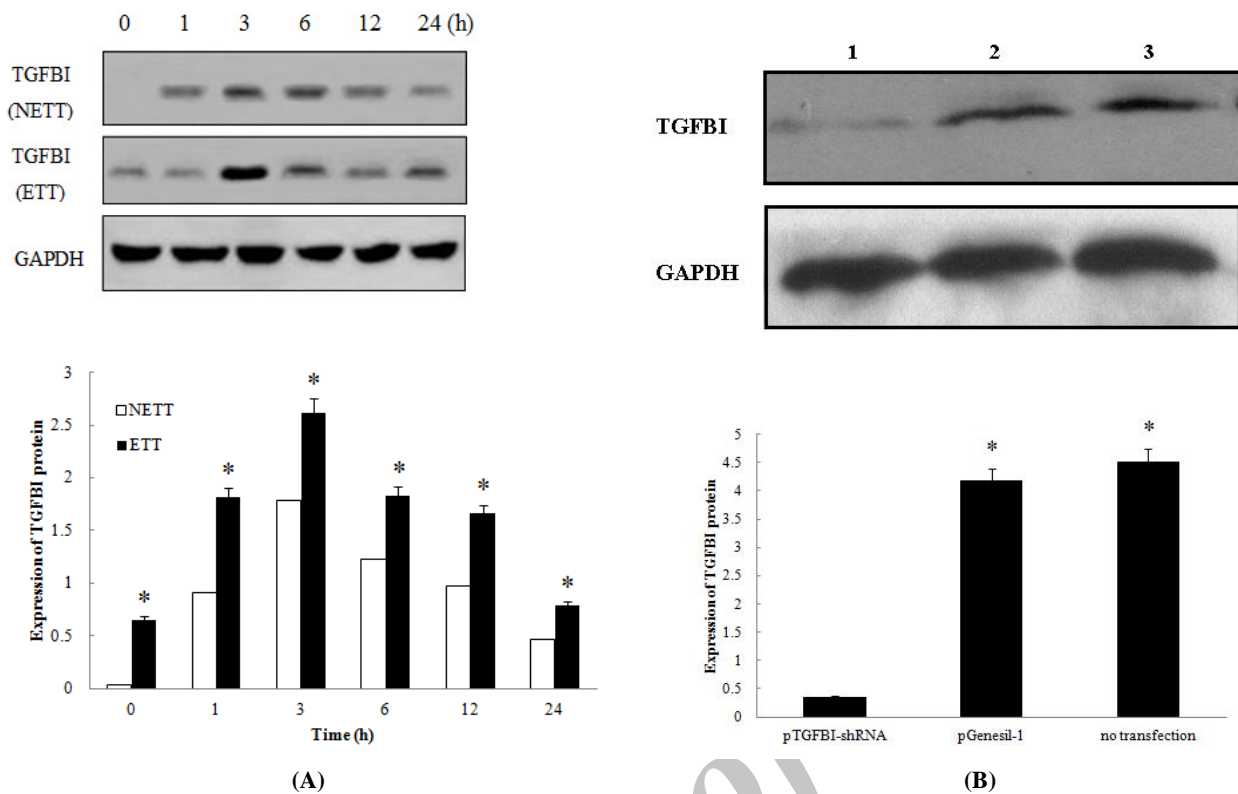
### The Expression of TGFBI mRNA and Protein in PBMCs

After re-stimulation with  $100 \text{ ng ml}^{-1}$  LPS, the expression of TGFBI mRNA was rapidly up-regulated in PBMCs of ETT group, peaking at 3 h and about 150% higher than that of NETT group (Fig. 1(A)). Although PBMCs were transfected with pTGFBI-

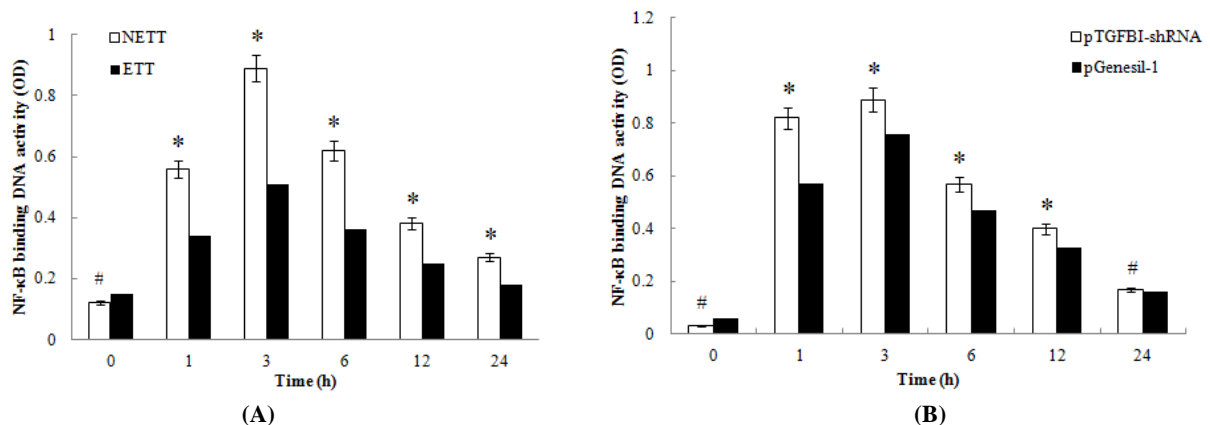
shRNA after pretreatment by LPS, the expression of TGFBI mRNA was significantly lower compared with PBMCs transfected with pGenesil-1 (Fig. 1(B)). The results of western blotting showed that TGFBI protein expression in PBMCs of NETT group was lower than that of ETT group, which difference was statistically significant. (Fig. 2(A)). Meanwhile, the expression of TGFBI in PBMCs transfected with pTGFBI-shRNA was much lower than PBMCs transfected with pGenesil-1 and no transfection group, which statistically significant differences forcefully indicated that pTGFBI-shRNA specifically inhibited TGFBI expression induced by LPS pretreatment. However, there was no significant differences between pGenesil-1 and no transfection group (Fig. 2(B)).



**Fig. 1. (A)** The TGFBI mRNA in PBMCs isolated from NETT and ETT group after  $100 \text{ ng ml}^{-1}$  LPS challenge shown in lanes 1–12 at 0, 1, 3, 6, 12 and 24 h intervals. Gels were photographed and digitized, and optical densities of TGFBI mRNA bands were normalized using optical densities of the  $\beta$ -actin mRNA bands, \*  $P<0.01$  vs. NETT. **(B)** The TGFBI mRNA isolated from pTGFBI-shRNA and pGenesil-1 group after  $100 \text{ ng ml}^{-1}$  LPS rechallenge shown in lanes 1–4 at 1 and 3 h. Densitometry of TGFBI mRNA was performed as described in the legend to (A), \*  $P<0.01$  vs. pTGFBI-shRNA.



**Fig. 2.** (A) The TGFBI protein in isolated PBMCs from NETT and ETT groups after LPS restimulation were shown in lanes 1-6 at 0, 1, 3, 6, 12, 24h. Densitometry of TGFBI protein was performed as described in the legend to Fig. 1, \* $P < 0.01$  vs. NETT. (B) The TGFBI protein isolated from PBMCs transfected with pTGFBI-shRNA, pGenesil-1 or no transfection at 24 h after LPS rechallenge were shown in lanes 1-3. Densitometry of TGFBI protein was performed as described in the legend to (A), \* $P < 0.01$  vs. pTGFBI-shRNA.



**Fig. 3.** Time course of NF- $\kappa$ B DNA-binding activity was determined by ELISA. Optical density values ( $A_{450}$ , nm) were corrected for background levels. (A) NF- $\kappa$ B activity of PBMCs after 100 ng ml<sup>-1</sup> LPS rechallenge at the indicated hours. \* $P < 0.01$  vs. ETT; # $P > 0.05$  vs. ETT. (B) NF- $\kappa$ B activity of PBMCs transfected with pTGFBI-shRNA or pGenesil-1 after 100 ng ml<sup>-1</sup> LPS challenged at 0, 1, 3, 6, 12, 24h. \* $P < 0.01$  vs. pGenesil-1; # $P > 0.05$  vs. pGenesil-1.

### NF- $\kappa$ B Activation

After LPS stimulation, NF- $\kappa$ B activation was increased in both ETT and NETT groups at 3h, and in both of the groups then gradually decreased from 3h up to 24h; but this tendency was discounted in ETT group compared with NETT group, in which the peak value were just 57.3% of NETT group (Fig. 3(A)). Meanwhile, after rechallenge with 100ng ml<sup>-1</sup> LPS, NF- $\kappa$ B activation showed the tendency of reducing after raising, and the result of NF- $\kappa$ B activation analysis indicated that the response to restimulation of LPS in PBMCs transfected with pTGFBI-shRNA was stronger than that of control group transfected with pGenesil-1 group, which the difference was statistically significant, suggesting that TGFBI suppression obviously attenuated ET development (Fig. 3(B)).

### The Expression of TNF- $\alpha$ and NO Level in PBMCs

One of inflammatory cytokines, TNF- $\alpha$ , has emerged as a useful marker of tolerant monocytes. Since NF- $\kappa$ B family members are well known to control the expression of the TNF- $\alpha$ , the mRNA and protein levels were also quantified using RT-PCR and ELISA. The results showed that the expression of TNF- $\alpha$  mRNA was rose in the NETT group after LPS stimulation, while those parameters were discounted in ETT group compared with NETT group (Fig. 4).

After LPS restimulation, the TNF- $\alpha$  level of both ETT and NETT groups gradually rose, and the increase in ETT group was much lower than that of NETT group and the difference was statistically significant, in which the peak value was only 36.9% of NETT group (Fig. 5(A)). At the same time, the NO level of both

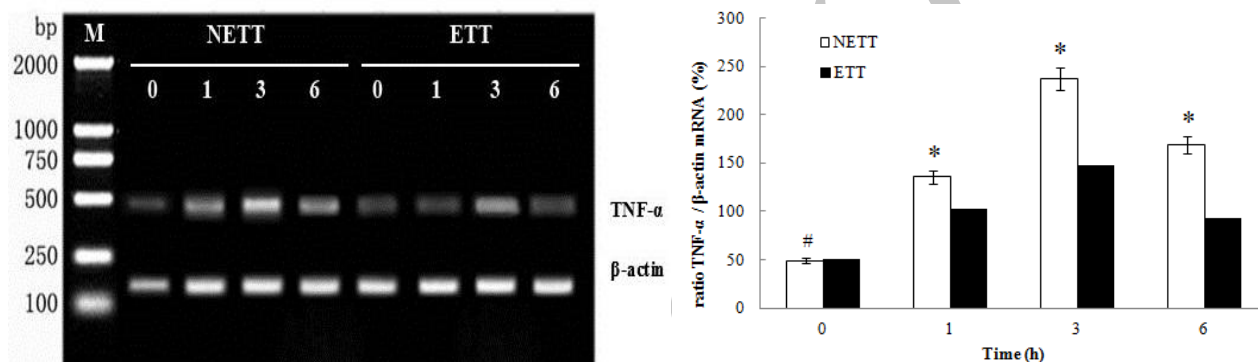


Fig. 4. The TNF- $\alpha$  mRNA in PBMCs isolated from NETT and ETT groups were shown in lanes 1–8 at 0, 1, 3 and 6 h. Densitometry of TNF- $\alpha$  mRNA was performed as described in the legend to Fig. 1. \* $P$  < 0.01 vs. ETT; # $P$  > 0.05 vs. ETT.

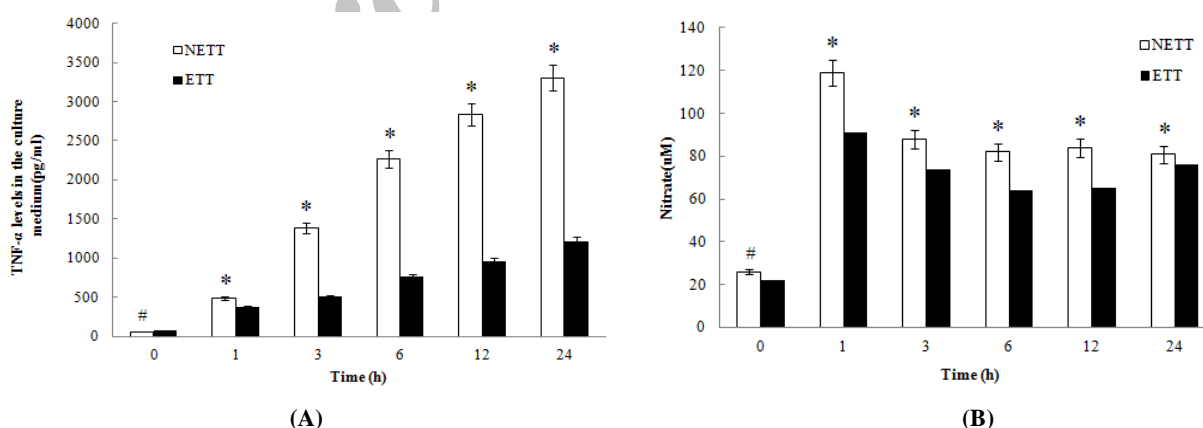
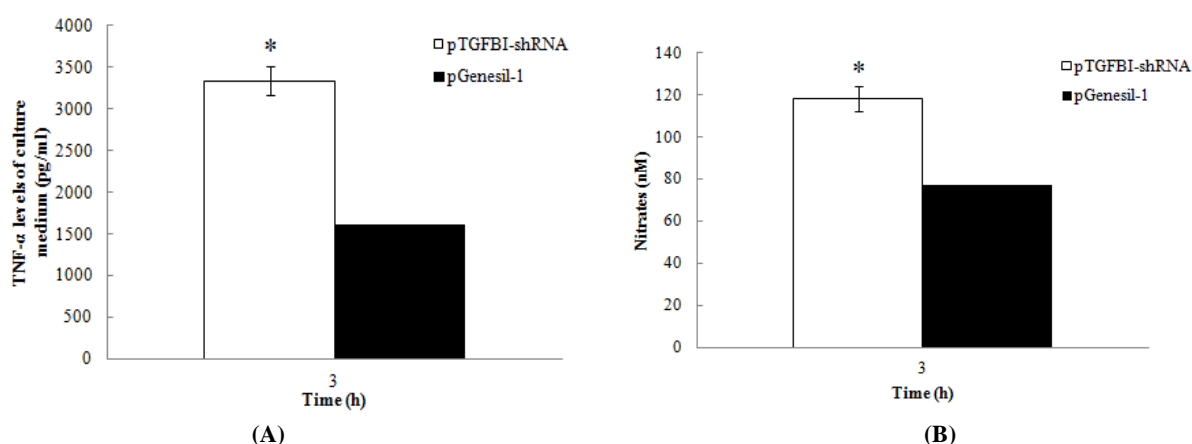


Fig. 5. Changes of (A) TNF- $\alpha$  and (B) NO levels in culture medium of NETT and ETT groups of PBMCs after 100 ng ml<sup>-1</sup> LPS rechallenge for the indicated hours. \* $P$  < 0.01 vs. ETT; # $P$  < 0.05 vs. ETT.



**Fig. 6. Changes of (A) TNF- $\alpha$  and (B) NO levels in culture medium of pTGFBI-shRNA and pGenesil-1 groups at 3 h after a subsequent challenge with 100 ng ml<sup>-1</sup> LPS, \* $P < 0.01$  vs. pGenesil-1.**

ETT and NETT groups increased initially and decreased afterwards, and both of them maintained at some level at 24h; compared with NETT group, the tendency was discounted in ETT group compared with NETT group, in which the peak value was only 76.5% of NETT group (Fig. 5(B)).

After transfection, the results of TNF- $\alpha$  and NO levels from supernatant of PBMCs in pTGFBI-shRNA group were both significantly higher than pGenesil-1 group at 24h after 100ng ml<sup>-1</sup> LPS restimulation, indicating that the response in PBMCs transfected with pTGFBI-shRNA were stronger compared with the control group transfected with pGenesil-1 group (Fig. 6(A) and (B)).

## DISCUSSION

LPS tolerance, demonstrated by hyporesponsiveness to a second stimulation with LPS after a preceding LPS treatment, is an essential phenomenon for regulation of host innate immune responses.<sup>24</sup> However, the detailed mechanisms involved with TLR-related tolerance induction remains unknown.

As PBMCs play a sentinel role, allowing the host efficiently to sense and adapt to the presence of danger signals such as LPS,<sup>25</sup> it is reasonable to speculate that PBMCs' functions are regulated by TLR signaling, and that several different negative regulators of TLR signaling may contribute to restore immune balance under physiological and pathological conditions. Recently, the discovery of TLR4 as the major receptors for LPS has prompted a resurgence of interest in ET

mechanisms.<sup>26,27</sup>

TGFBI was originally implicated as a regulator of cell adhesion and migration. More recently, some of the inflammatory disease states associated with increased TGFBI expression are rheumatoid arthritis,<sup>28</sup> atherosclerosis,<sup>29</sup> cyclosporine nephropathy<sup>30</sup> and stab wound healing.<sup>31</sup> However, the physiological role of TGFBI is still largely unknown. Previous reports have noted that TGFBI plays important roles in various pathophysiological phenomena, such as cellular growth, differentiation, adhesion, migration, and angiogenesis.<sup>32,33</sup> Furthermore, its expression levels are elevated in inflammatory conditions during both normal<sup>34-36</sup> and abnormal conditions.<sup>37,38</sup> This indicates that the identification of the cell type and the stimuli that are responsible for its expression are important for the regulation of pathologic conditions where TGFBI is involved. Another study also demonstrated that TGFBI plays a suppressive role in the development of mesothelioma and breast cancer cells, possibly through inhibitions of cell proliferation.<sup>39</sup> Thus, TGFBI may be a potential therapeutic tool for treatment of several immune and inflammatory diseases.

In the present study, in order to survey the expression change of TGFBI under ET induction in PBMCs, we pretreated PBMCs isolated with low dose of LPS for 24h. The data showed that ET induction leads to a hypo-response of PBMCs to LPS rechallenge as assessed by the levels of cellular NF- $\kappa$ B activity, as well as inflammatory mediator production, accompanied with up-regulated TGFBI expression, indicating that TGFBI might be involved in ET

induction in PBMCs. Interestingly, the pro-inflammatory cytokine TNF- $\alpha$ , an accepted marker for demonstrating ET, was consistently reduced in our study, consistent with numerous other studies.<sup>40,41</sup> Besides, we found that NO level was opposite to the expression of TGFBI mRNA, which indicated that TGFBI may in part inhibit the signal transduction of LPS. These results suggested that TGFBI may play a crucial role to regulate excess and uncontrolled inflammatory responses induced by TLR signaling in PBMCs.

To further survey the possible role of TGFBI under ET induction in PBMCs, we silenced gene TGFBI in PBMCs by RNA interference technique. Though primary LPS stimulation apparently attenuated the response of PBMCs to the second LPS stimulation, the inhibitory effect was partly refracted in pTGFBI-shRNA transfected cells than the control group transfected with pGenesil-1, indicating that silence of TGFBI caused abnormal enhancement of inflammatory reaction. Here, our results for the first time clearly identified the effect of TGFBI under ET induction in PBMCs and that it may play the role through dampening NF- $\kappa$ B mediated pathway on TLR signaling. To our knowledge, NF- $\kappa$ B is critical for inflammatory responses by regulating the expression of genes such as cytokines and chemokines that drive inflammation.<sup>42</sup> There is indirect evidence linking TGFBI to NF- $\kappa$ B signaling, since reverse signaling mediated by cell surface B-cell-activating factor (BAFF, a member of TNF superfamily) also induced the expression of TGFBI through signaling pathway that requires the activation of NF- $\kappa$ B,<sup>43</sup> which further supports that the activation of NF- $\kappa$ B is required for the induction of TGFBI expression. Meanwhile, our data showed that after LPS restimulation, the activity of NF- $\kappa$ B reduced, compared with the downregulation of TNF- $\alpha$  at NO level, which suggested that the inappropriate activation of NF- $\kappa$ B induced by LPS was the key of excess inflammatory responses and inflammatory damage, and the reaction of endotoxin could be reduced by endotoxin tolerance of organism or cells. However, it is not possible to speculate whether NF- $\kappa$ B is recruited directly to the promoter or it works indirectly through interaction with other transcription factors, since the promoter for TGFBI has not been defined.

It remains to be determined whether ET involves a single critical signaling pathway or sequential

multiple changes in signaling events during tolerance induction.<sup>44</sup> Here, we indeed found that although ET was significantly attenuated, it was not completely abrogated by specific silence of TGFBI using RNA interference. The possible reason is that up-regulation of TGFBI expression is not the unique mechanism and obviously there are other additional regulatory factors and mechanisms to control the response to LPS. In recent reports, it has been indicated that IRAK-M is one of vital inducible negative regulators of TLR signaling.<sup>45</sup> Besides, ET has also been suggested to be associated with decreased Gi protein content and activity, decreased protein kinase C activity, and reduced NF- $\kappa$ B-induced gene transactivation.<sup>46,47</sup>

In the present study, we aimed to clarify the mechanism of TGFBI in PBMCs under ET induction; however, its function remains poorly understood and additional investigations are necessary to elucidate the precise role of TGFBI under ET induction. In summary, we showed that ET could be induced in PBMCs by pretreatment with a low dose of LPS accompanied with up-regulation of TGFBI and the silence of TGFBI by RNA interference obviously attenuated the inhibitory response. Therefore, our results provide new insights into the negative feedback regulation of TLR signaling that maintains the innate immune system in PBMCs.

## ACKNOWLEDGEMENTS

This work was supported by Guangdong Province Major Projects of Key Areas {YS no. (2005)162}, and Major Technology Program Funded Projects, Guangzhou (2011Y1-00017-3).

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