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*Original Article*

# Mannose-capped Lipoarabinomannan from Mycobacterium tuberculosis induces IL-37 production via upregulating ERK1/2 and p38 in human type II alveolar epithelial cells

Zhen Huang[1,2], Gao Wei Zhao[3], Chun Hai Gao[4], Xiu Wen Chi[5], Tao Zeng[6], Yan Wei Hu[1], Lei Zheng[1], Qian Wang[1]

*[1]Laboratory Medicine Center, Nanfang Hospital, Southern Medical University, Guangzhou, Guangdong 510515, P.R. China; [2]Department of Laboratory Medicine, Longgang Central District Hospital , Shenzhen, Guangdong 518116, P.R. China; [3]Linyi Blood Center, Linyi, Shandong276000, P.R. China; [4]Department of Laboratory Medicine, People's Hospital of Linyi, Linyi, Shandong 276003, P.R. China; [5]School of Nursing, Guangdong Medical College, Dongguan, Guangdong 523808, P.R. China; [6]School of Laboratory Medicine, Guangdong Medical College, Dongguan, Guangdong 523808, P.R. China*

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**Abstract:** The major surface lipoglycan of Mycobacterium tuberculosis (M. tb), mannose-capped lipoarabinomannan (ManLAM), is an immunosuppressive epitope of M. tb. Interleukin (IL)-37, is a newly identified anti-inflammatory cytokine, which reduces systemic and local inflammation. However, the correlation between ManLAM and IL-37 remains unknown. Therefore, in this study, we investigate the possible role and relative molecular mechanism of ManLAM in IL-37 production of human type II alveolar epithelial cells by using A549 cell line. Here, we report that M. tb induced IL-37 mRNA and protein expression in a time-dependent manner. We next fractionated components of M. tb using chloroform: methanol (C:M) and water. In sharp contrast to the C:M phase, water phase was mainly responsible for the production of IL-37. Since ManLAM is the major component of water phase, we found that ManLAM induced IL-37 mRNA and protein expression in a time and dose-dependent manner, while this activity was almost totally abolished by the ERK1/2 (U0126) and p38 (SB203580) inhibitor. ManLAM stimulation significantly induced ERK1/2 and p38 phosphorylation in A549 cells, as well as cell surface TLR2 expression. After interfering TLR2 expression, ERK1/2 and p38 phosphorylation levels were markedly decreased, and also IL-37 production. Though ManLAM also promoted TLR4 expression on A549 cells, TLR4 interference showed no influence on ManLAM-induced IL-37 production. Our results indicate that ManLAM induces IL-37 production in human type II alveolar epithelial cells via up-regulating TLR2/p38 or ERK1/2 pathway, and this provide an important evidence to explain the pathological role of ManLAM that contribute to the persistence of M. tb.

**Keywords:** ManLAM, tuberculosis, IL-37, TLR2, ERK1/2, p38

## Introduction

Tuberculosis (TB), usually attacks the lungs, resulting in the pulmonary TB (PTB) [1]. Mycobacterium tuberculosis (M. tb), the etiologic agent of TB, is a ubiquitous and extraordinarily aggressive human pathogen which infects an estimated one-third of the world's population and causes millions of TB-associated deaths yearly [2]. Mannose-capped lipoarabinomannan (ManLAM), as a kind of lipoglycan, is a major cell wall component and virulence factor of M. tb [3]. It has long been known to have both inhibitory and stimulatory effects on host immunity [4]. Its function has been described to inhibit phagosome-lysosome fusion in macrophages, dendritic cells maturation, $CD4^+$ T-cell activation and recruitment [5-8]. As for the influence of cytokines production, ManLAM increases interleukin (IL)-10 production by dendritic cells (DCs), and depresses its IL-12 cytokine production [7].

IL-37, as a new member of IL-1 family, recently, it has been proved to be a natural suppressor of innate immunity and inflammatory responses in autoimmune diseases and tumors [9-11], and also plays a pivotal role in regulating adaptive immunity by inducing regulatory T cells and impairing activation of effector T-cell responses [12]. Importantly, in bacterial diseases, IL-37 functions as a broad spectrum inhibitor of the innate response to infection-mediated inflammation, and could be considered to be therapeutic in reducing the pulmonary damage [13].

It has been demonstrated that IL-37 mainly expresses on epithelial cells and can be induced by several toll-like receptor (TLR) ligands, like TLR2 and TLR4 [14-16]. Furthermore, intracellular signaling cascades analysis reveals that the expression of IL-37 can be dampened by the extracellular signal-regulated kinase 1 and 2 (ERK1/2) and p38 inhibitors [17]. As another important inhibitory cytokine, enhanced IL-10 production also can be induced by TLRs stimulation and the activation of MAPK family components, ERK1/2 and p38 in DCs [18, 19]. Furthermore, ManLAM is a ligand of TLRs and regulates multiple host immune responses through ERK1/2 and p38 activation, like promoting IL-8 and iNOS production with presence of IFN-γ [4, 20-24]. Thus, we speculated that ManLAM from M. tb could induce IL-37 production via binding to TLR2 and promoting the activation of p38, ERK1/2 in human type II alveolar epithelial cells.

In the present study, we assessed whether ManLAM purified from M. tb were able to induce IL-37 production by human type II alveolar epithelial cells, namely A549 cells, and examined the relative molecular pathways involved in IL-37 production. Our results showed that ManLAM induced IL-37 production in a time and dose-dependent manner, more importantly, this effect depended on the unregulated expression of TLR2, as well as enhanced p38 and ERK1/2 phosphorylation.

## Materials and methods

### *Cell line and bacteria*

The human type II alveolar epithelial cell line A549 (Institute of Biochemistry and Cell Biology, China) was maintained in RPMI-1640 (Hyclone, USA) supplemented with 10% fetal calf serum (FBS, Gibco) at 37°C. M. tb H37Rv (strain American Type Culture Collection (ATCC) 93009) was purchased from the Beijing Biological Product Institute (Beijing, China) and maintained on Lowenstein-Jensen medium and harvested while in the log phase of growth. Bacilli were washed in PBS containing 0.05% Tween-80 and triturated uniformly before use [25].

### *ManLAM preparation*

ManLAM was extracted and purified from delipidated cells as previously described [26]. Purified ManLAM were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, silver staining.

### *Real-time PCR*

Total RNA was extracted from A549 cells using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA was reversely transcribed into cDNA using First Strand cDNA Synthesis Kit (Toyobo). cDNA was quantified using specific primers using a real-time RT-PCR system (Step-one Real-Time PCR system, Applied Biosystems) with the SYBR Green real-time PCR Master Mix kit (Toyobo). Amplification of the IL-37 gene was performed with the following primers: forward, 5'-CAGCCTCTGCGGAGAAAGGAAGT-3', and revers, 5'-GTTTCTCCTTCTTCAGCTGAAGGGATGGAT-3' [27]. β-actin [28], forward (5'-TCGTCGACAACGGCTCCGGCATGT-3') and reverse (5'-CATTGTAGAAGGTGTGGTG-3'), was used for housekeeping gene control.

### *Western blot*

IL-37 was detected using monoclonal antibody [11] and a horseradish peroxidase-labeled goat anti-mouse secondary antibody (Santa Cruz Biotechnology). Phosphorylated and total p38 and ERK1/2 MAPKs were examined by Western blot analysis using MAPK antibody kits (Cell Signaling Technology). β-actin was set as endogenous control. Bands were visualized with Fluor S-MultiImager MAX system (Bio-Rad Laboratories) and quantified by image analysis software (Quantity One, Bio-Rad Laboratories).

### *ELISA*

The level of IL-37 was measured by ELISA following the manufacturer's instructions respectively. Human IL-37 ELISA reagent Kits were purchased from Cusabio (Wuhan, China), all of the samples were measured in triplicate.

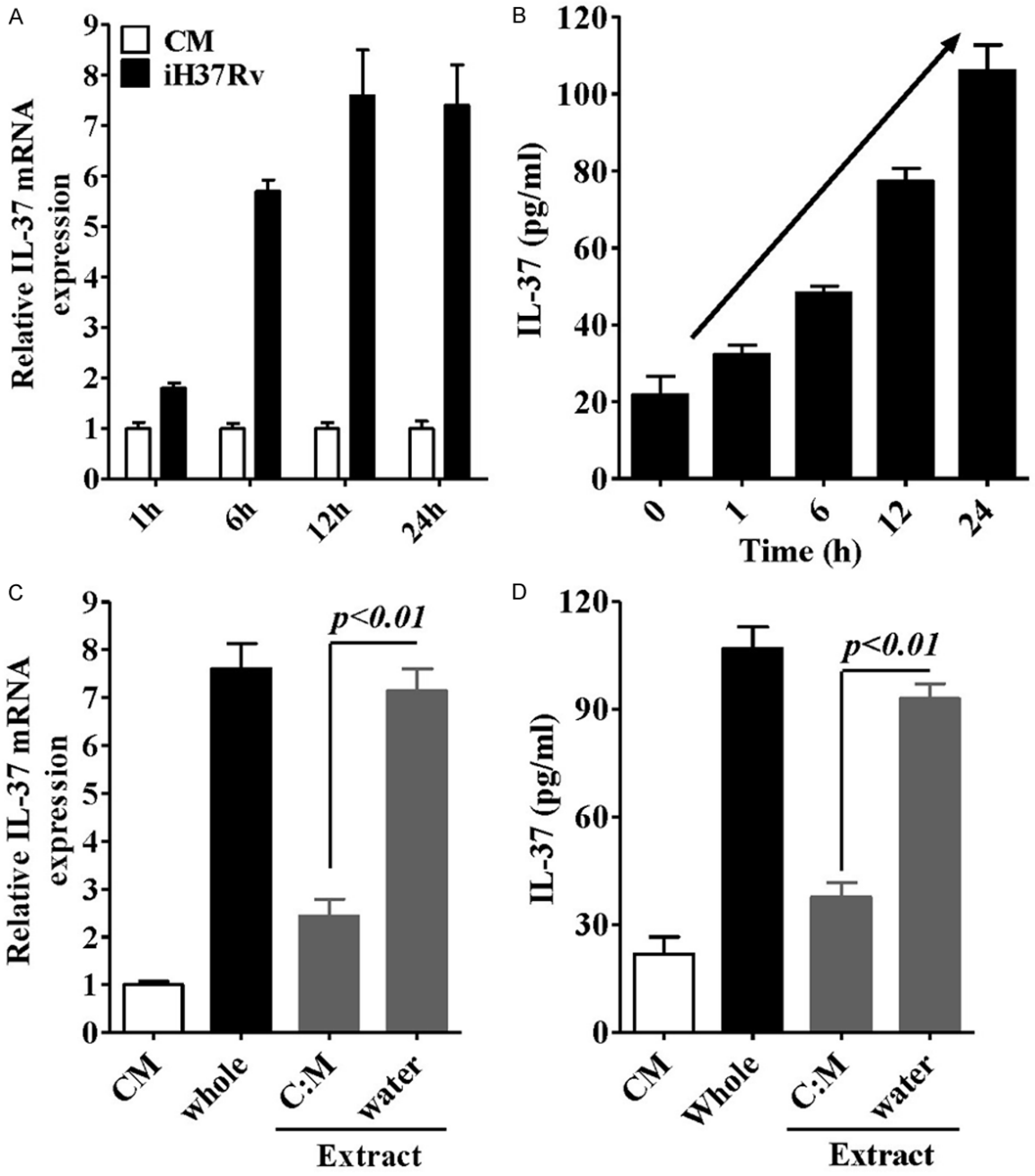


**Figure 1.** Analysis of the effects of inactivated H37Rv (M.tb strain) on the expression of IL-37 in A549 cells. Real-time PCR (A) and ELISA (B) analysis of IL-37 mRNA or protein expression induced by iH37Rv at different incubation periods. Real-time PCR (C) and ELISA (D) analysis of IL-37 mRNA or protein expression induced by plate-coated water extract or (C) M extract from iH37Rv for 24 h. Data are expressed as mean ± SD.

*siRNA transfection*

siRNA to TLR2 or TLR4 or control siRNA were obtained from life technologies [29, 30]. A549 were transfected with 60 pmol siRNA per $5\times10^5$ cells and 3 μl Lipofectamine 2000 (Invitrogen, Carlsbad, CA) in serum-free Dulbecco's modified Eagle's medium for 4 h, rinsed, and then placed in fresh RPMI-1640 supplemented with 10% FBS.

*Flow cytometry*

All staining reactions were performed at 4°C on cells first exposed to Fc receptor mAb (Human TruStain FcX™, Biolegend) in order to reduce

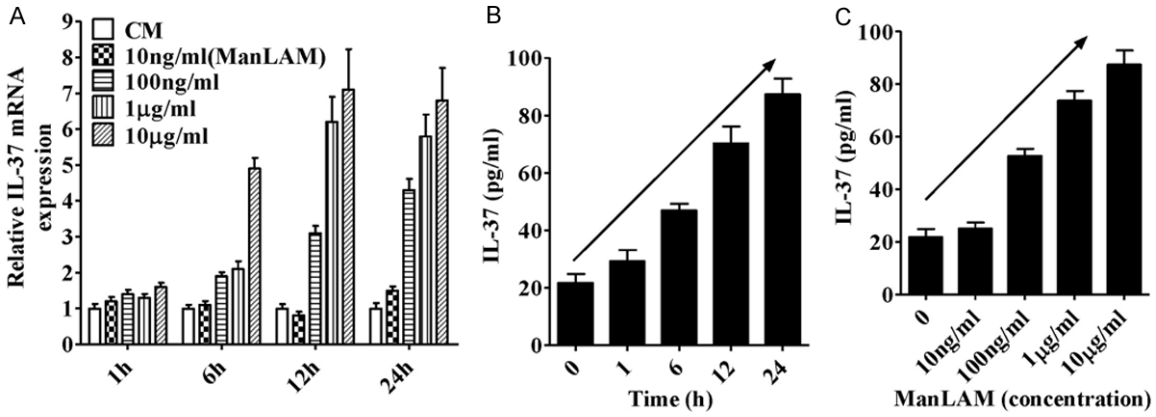


**Figure 2.** Analysis of the effects of ManLAM extracted from H37Rv on the expression of IL-37 in A549 cells. (A) Real-time PCR analysis of IL-37 mRNA expression induced by ManLAM at different concentrations for various periods. ELISA analysis of IL-37 production induced by ManLAM at different concentrations (C) for various periods (B). Data are expressed as mean ± SD.

nonspecific binding. PE labelled anti-TLR2 (TL2.1) and APC labelled anti-TLR4 (HTA125) were purchased from Biolegend. Cells were analyzed with a BD Accuri C6 flow cytometer (BD).

*Statistical analysis*

Data were expressed as mean ± SD or median (range) and analyzed by Graphpad Prism V.5.00 software (GraphPad Software, San Diego CA, USA). Differences among different groups were tested by one-way ANOVA followed by Neuman-Keuls post hoc test. A two-side *p* values under 0.05 were considered statistically significant.

## Results

*Mycobacterium tuberculosis stimulation increases IL-37 expression in A549 cells*

Human type II alveolar epithelial cell line A549 cells were stimulated with inactivated M. tb strain H37Rv at a ratio of 10:1 (bacteria to cells), IL-37 mRNA and protein expression were detected at different time-period. As showed in **Figure 1A**, RT-PCR revealed that the IL-37 mRNA expression level in A549 cells was significantly increased after 12 h of H37Rv stimulation (an approximate 7.6-fold enhancement). Furthermore, protein production analysis also identified that H37Rv promoted IL-37 production in a time-dependent manner (**Figures 1B** and **3A**). These findings suggest that the IL-37 as an inhibitory cytokine might play a role in the response of type II alveolar epithelial cells to M. tb infection.

*H37Rv cell wall component ManLAM mainly respond for the H37Rv-induced IL-37 expression*

We next fractionated components of H37Rv using lipophilic and hydrophilic solvents, such as chloroform: methanol (C:M) and water, The IL-37 expression activity for each of the extracts was assessed in a plate-coated form [4]. We found that only the water phase demonstrated a stimulatory activity for IL-37 expression (**Figure 1C** and **1D**), which showed no significantly difference with whole bacteria stimulation, in sharp contrast to the C:M phase that only showed minor increase for IL-37 expression. These results suggest that the hydrophilic components of mycobacteria are candidates for the IL-37 expression.

Among mycobacterial hydrophilic components, ManLAM constitutes the most abundant hydrophilic lipoglycan [31], we thus further evaluated the ability of ManLAM in inducing IL-37 expression by A549 cells. As shown in **Figure 2A**, ManLAM upregulated IL-37 mRNA level in a dose-dependent manner, and the maximum up regulation of IL-37 (an approximate 7.1-fold enhancement) was observed in the presence of 10 μg/ml of ManLAM at the 12 h incubation period. More importantly, IL-37 protein production induced by ManLAM showed time and dose-dependent manner (**Figure 2B** and **2C**), and showed no apparent difference with H37Rv treatment (**Figure 3A**). These findings indicate that ManLAM is the main component of H37Rv

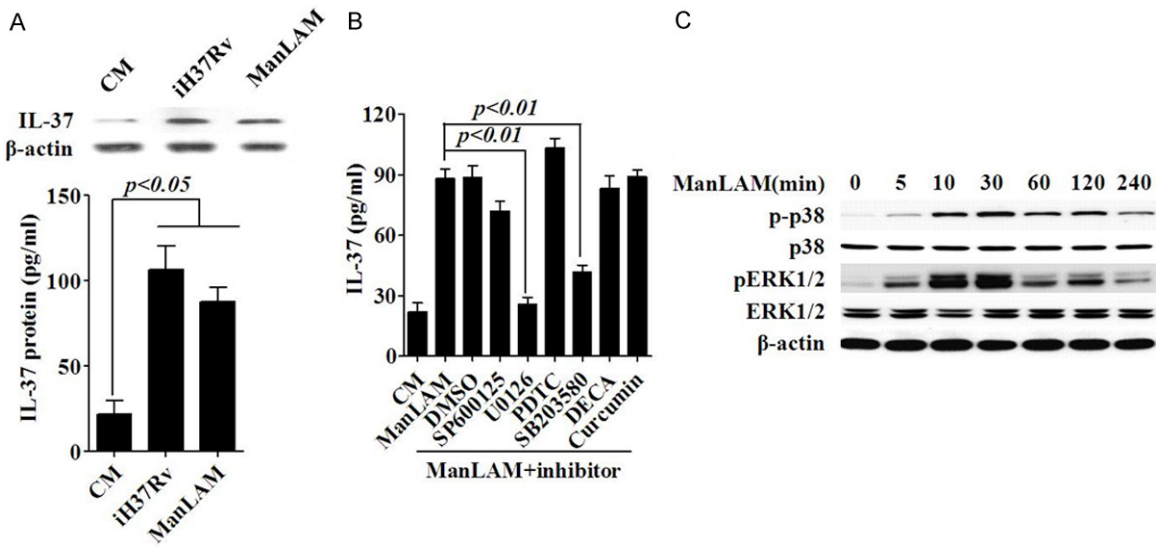


**Figure 3.** p38 MAPK and ERK1/2 are involved in ManLAM-induced IL-37 production. A. IL-37 production induced by medium (CM), iH37Rv or ManLAM inA549 cells for 24 h were examined by western blotting. B. ELISA analysis of the effect of various signal proteins' inhibitors on the production of IL-37 in A549 cells induced by ManLAM for 24 h. C. Western blot analysis for detection of ERK1/2, p38, and β-actin activation in A549 cells stimulated by ManLAM. Data are expressed as mean ± SD.

with the ability to induce IL-37 production in human type II alveolar epithelial cells.

*p38 and ERK1/2 activation involved in ManLAM induced IL-37 production*

To further understand the mechanism by which ManLAM induced the expression of IL-37, inhibitors target to multiple important signal proteins were investigated, like JNK or MAPK pathways. A549 cells were pre-treated with various inhibitors (Cell Signaling Technology) for 30 min, and then, the treated cells were stimulated with ManLAM for 24 h. The results showed that p38 and ERK1/2 inhibitors (SB203580 and U0126) markedly abolished the production of IL-37 induced by ManLAM (**Figure 3B**), moreover, ManLAM significantly promoted the phosphorylation of p38 and ERK1/2 MAPKs in A549 cells. These results suggest that the p38 MAPK and ERK1/2 pathways may be involved in the upregulation of ManLAM-induced IL-37.

*TLR2 involves in ManLAM-induced IL-37 production*

The TLR ligands lipopolysaccharide (LPS) and $Pam_3CSK_4$ were highly effective in inducing IL-37 [16, 32, 33], it is worth mentioning that ManLAM is also a ligand of TLRs [4, 34]. In A549 cells, ManLAM stimulation significantly induced TLR2 and TL4 expression, more importantly, after knockdown TLR2 expression, ManLAM-induced IL-37 production was almost completely abrogated, as well as the phosphorylation of p38 and ERK1/2. While TLR4 knockdown only showed negligible influence in ManLAM-induced IL-37 production (**Figure 4A** and **4B**). Thus, TLR2 deficiency resulted in decreased IL-37 production presumably due to the downregulation of its downstream signal proteins p38 and ERK1/2 activation (**Figure 4C**). Collectively, these results suggest that ManLAM enhances IL-37 production in human type II alveolar epithelial cells mainly by regulating TLR2 expression as well as p38 and ERK1/2 phosphorylation.

## Discussion

In this study, we firstly demonstrate that a M. tb virulence factor, ManLAM, is capable of inducing IL-37 production in human type II alveolar epithelial cells. This activity was accomplished by the promotion of TLR2 expression, and also the upregulation of p38 and ERK1/2 phosphorylation. Since the first description of IL-37 [35], IL-37 serve as a fundamental inhibitor of innate immunity [16], its mRNA and protein have been detected in inflammatory and autoimmune dis-

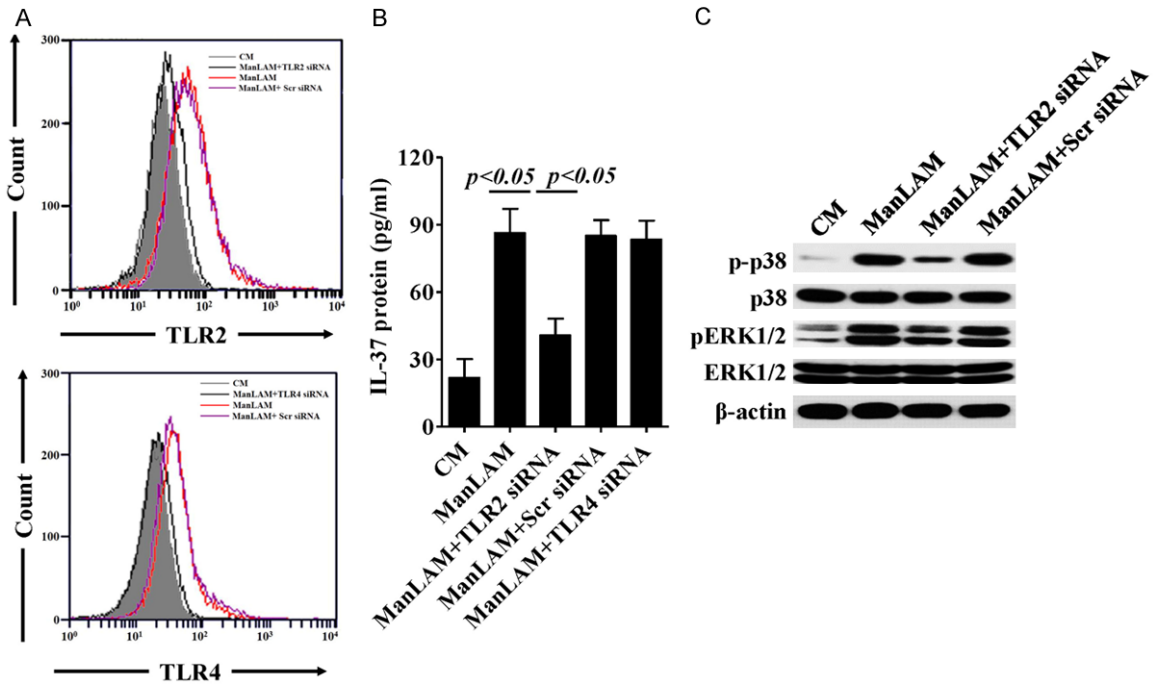


**Figure 4.** TLR2 involves in ManLAM-induced IL-37 production in A549 cells. (A) Flow cytometry analysis of TLR2 or TLR4 expression in A549 cells stimulated by ManLAM for 24 h, TLR2 or TLR4 expression in A549 cells were intervened by its specific siRNA before ManLAM stimulation, respectively. (B) ELISA analysis of IL-37 production in cell supernatant of A549 cells same treated as (A). (C) Western blot analysis for detection of ERK1/2, p38, and β-actin activation in A549 cells stimulated by ManLAM, with or without the presence of TLR2 siRNA. Data are expressed as mean ± SD. Scr, scramble; CM, control medium.

eases, as well as tumors [11], such as rheumatoid arthritis [16], atopic dermatitis [36], inflammatory bowel disease [15] and systemic lupus erythematosus [37]. Nowadays, the inhibitory activity of IL-37 has been expanded in adaptive immunity [12] and fungi infection [13], especially, IL-37 reduces the pulmonary damage in invasive aspergillosis infection.

In mycobacterium tuberculosis (M. tb) infection, M. tb is typically restricted in lung and causes pathological injury. Recently, alveolar epithelial cells were identified as important mediators of the immune response to respiratory pathogens. Several in vitro studies have demonstrated that type II alveolar pneumocytes are capable of internalizing M. tb, and once inside the type II alveolar cells, the bacteria have been shown to replicate extensively [28, 38], we thus speculated that M. tb may facilitate its replication by promoting IL-37 production of alveolar cells.

Human type II alveolar cell line A549 cells were stimulated with inactivated M. tb strain H37Rv for different time period. From the results, we found that H37Rv surely promoted the mRNA expression and protein production of IL-37. As for the first to report M. tb has the ability to induce IL-37 production, we further want to identify which component was attributed to H37Rv-induced IL-37.

H37Rv cells were fractionated as two components by using lipophilic and hydrophilic solvents, such as chloroform: methanol (C:M) and water, we found that only the water phase demonstrated an apparent stimulatory activity for IL-37 production. LPS has been reported to induce IL-37 in A549 cells [16], although mycobacteria do not possess LPS, the cell wall of M. tb contains a complex lipid glycoprotein called ManLAM which shares many physicochemical properties with LPS [4, 21, 39]. Among mycobacterial hydrophilic components, ManLAM constitutes the most abundant hydrophilic lipoglycan, which has been shown to suppress host immune system [40], one of the key events is the production of immunosuppressive cytokine IL-10.

In A549 cells, ManLAM induced IL-37 mRNA and protein expression in time and concentration-dependent manner. Furthermore, though

IL-37 production is slightly lower in ManLAM-treated A549 cells, there is no significant difference in IL-37 production between H37Rv and ManLAM after 24 h incubation, this slight decrease may own to other inhibitory components of H37Rv, like mycolylarabinogalactan peptidoglycan (mAGP) complex [41] and LprG [42], which need to be further identified. IL-37 inhibits pro-inflammatory cytokines production, like IL-1β, IL-6 and TNF, in autoimmune diseases and fungi infection [13, 16, 43], and also the effects of ManLAM or H37Rv induced IL-37 on various pro-inflammatory cytokines could be determined in future studies.

The MAPK signaling pathway is one of the major signaling pathways in inflammation. As for IL-37 production, it can be inhibited by p38 and ERK1/2 inhibitors in THP1 cells [17]. ManLAM not only activated p38 MAPK and MEK/ERK phosphorylation to arrest mycobacterial phagosome maturation [44, 45] but also inhibited pro-inflammatory cytokines production, like IL-8 [23], and stimulated IL-10 production [46]. Our experimental results showed that ManLAM promoted p38 and ERK1/2 phosphorylation in A549 cells, and also inhibitors specific to these two proteins inhibited ManLAM-induced IL-37 production (**Figure 4A** and **Figure 4B**). However, JNK inhibitor SP600125 also inhibited IL-37 production and further detailed investigation is required to address the mechanism of IL-37 upregulation induced by ManLAM.

ManLAM can be recognized by TLR2 and TLR4 [21, 47], which have been found to mediate IL-37 production [16]. Furthermore, TLR ligands also promote IL-10 production by dendritic cells (DC) through p38 and ERK1/2 MAPK pathways [19, 48]. After stimulated with ManLAM for 24 h, TLR2 and TLR4 expression in cell surface of A549 cells all significantly enhanced, while only TLR2 knockdown decreased ManLAM-induced IL-37 production. More importantly, TLR2 interfere also markedly reduced p38 and ERK1/2 phosphorylation level with the stimulation of ManLAM.

In conclusion, this study was the first to demonstrate that ManLAM is the main cell component of M. tb with the ability to induce IL-37 in human type II alveolar cells, which is mediated by upregulating TLR2 expression, ERK1/2 and p38 MAPK phosphorylation. These findings have revealed previously unrecognized immunoregulatory effects of ManLAM from M. tb.

## Disclosure of conflict of interest

None.

**Address correspondence to:** Dr. Qian Wang, Laboratory Medicine Center, Nanfang Hospital, Southern Medical University, 1023 Shatai South Road, Guangzhou 510515, Guangdong, P. R. China. E-mail: 1505700787@qq.com

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