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DosR antigen Rv1737c induces activation of macrophages dependent on the TLR2 pathway

Xiaoling Gao[a,1,*], Cong Wu[b,1], Wenhua He[b], Xiaoxia Wang[b], Yonghong Li[a], Yongxiang Wang[b], Yanjuan Jia[a], Rui Yuan[b], Haojie Li[b], Benzhong Zhang[b,*]

[a] The Institute of Clinical Research and Translational Medicine, Gansu Provincial Hospital, Lanzhou, China
[b] School of Public Health, Lanzhou University, Lanzhou, China

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ABSTRACT

Latent *Mycobacterium tuberculosis* (*Mtb*) infection (LTBI) is the main clinical manifestation after *Mtb* exposure. During the latent phase, *Mtb* retards the attempts of eradication by the host immune system. The dormancy survival regulator (DosR) is held as essential for *Mtb* persistence. Rv1737c is predominantly expressed by the *Mtb* in latent infection. However, the role of Rv1737c in the immune evasion is still largely unknown. In this study, we have characterized the Rv1737c functions in the recruitment and activation of macrophages, which play a cardinal role in the innate and adaptive immunity. For the first time, we have revealed that Rv1737c induced the tolerogenic phenotype of macrophages by upregulating the expression of indoleamine 2,3-dioxygenase 1 (IDO1). Rv1737c-activated macrophages upregulated interleukin (IL)-4, IL-10, and Foxp3 T cells proliferation in vitro. Furthermore, the interaction of Rv1737c with macrophages was found to depend on the Toll-like receptor 2 (TLR2) pathway. It augmented nuclear factor κB (NF-κB) phosphorylation and co-stimulatory molecule expression. Thus, this study provides a crucial insight into a strategy adopted by *Mtb* to survive in the host by inducing tolerogenic macrophage expansion.

1. Introduction

Tuberculosis, caused by the intracellular pathogen *Mycobacterium tuberculosis* (*Mtb*), remains a disease with one of the highest morbidity and mortality rates in the world. According to the World Health Organization, tuberculosis affects one-third of the population worldwide [1]. Latent tuberculosis infection (LTBI) is a special condition in which the host has no clinical symptoms after being infected with *Mtb*. The non-replicating or dormant state is widely recognized as the culprit for this scenario. Nonetheless, there is a risk of activation of the disease in 10–15% of these individuals. *Mtb* successfully tames the host immunity to survive in the host by rapidly altering its gene expression and releasing several factors [2]. However, when the host immune response is compromised, such as during co-infection with human immunodeficiency virus (HIV), or treated with immune depressants, dormant bacterium can resume growth to promote the disease activation [3]. In addition, the *Mtb* has evolved the ability of exacerbation to initiate the replication pathway, leading to the disease progression and bacteria dissemination [4]. Therefore, the latent *Mtb* infection is the main reservoir of prevalence of *Mtb* infection [5]. Consequently, it is of utmost importance to understand the intricate host-pathogen protein interaction, which may yield a novel assay to identify patients with the potential for progression. It also aids the development of a new therapeutic scheme for final limitation of tuberculosis.

Various mechanisms of *Mtb* adapted in hostile environment have been elucidated. One key aspect is the activation of dormancy of survival regulon (DosR), one of the bacterial transcription regulatory networks comprising approximately 48 genes expressed during latency [6]. The stress environment, such as the lack of essential nutrients, hypoxia, and respiratory inhibitory gases (NO and CO), forces bacteria

Abbreviations: Mtb, *Mycobacterium tuberculosis*; LTBI, latent tuberculosis infection; PTB, pulmonary tuberculosis; DosR, dormancy survival regulon; MFS, major facilitator superfamily; IPTG, isopropyl-β-thiogalactopyranoside; rRv1737c, recombinant protein Rv1737c; PMA, phorbol 12-myristate13-acetate; TLRs, Toll-like receptors; FITC, fluorescein isothiocyanate; PE, phycoerythrin; APC, allopycocyanin; PerCP-Cy5.5, peridinin chlorophylla protein cyanine 5.5 complexes; IDO1, indoleamine 2,3-dioxygenase 1

* Corresponding authors at: The Institute of Clinical Research and Translational Medicine, Gansu Provincial Hospital, Lanzhou 730000, Gansu, China (X. Gao). School of Public Health, Lanzhou University, Lanzhou 730000, Gansu, China (B. Zhang).
E-mail addresses: GAOXL008@hotmail.com (X. Gao), zhangbzh@lzu.edu.cn (B. Zhang).
[1] Xiaoling Gao and Cong Wu are listed as co-first authors.

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to modify its DosR expression for survival [7–9]. DosR proteins have been divided into several functional categories, including nitrogen metabolism, cell wall synthesis, redox balance, host–pathogen interaction, and hypothetical proteins. Studies have demonstrated that DosR antigens Rv1733c, Rv2029c, Rv2627c, and Rv2628 can induce strong interferon-γ (IFN-γ) responses in TST^+ (tuberculosis skin test) individuals [10], although the pathophysiological functions of these proteins have not yet been clarified. How *Mtb* employs its latency-related antigens in manipulating the immune response remains broadly undefined.

Rv1737c, one of the DosR-encoded proteins, is predominantly expressed in the latent phase of infection. Recently, a systemic review summarized some potential antigens applied for TB infection detection. Rv1737c is one of the most frequently tested proteins, with the highest discriminatory potential of active TB vs. exposed one (30473692). To understand the immune functions of Rv1737c, we obtained the recombinant protein by genetic engineering and characterized the role of recombinant proteins Rv1737c (rRv1737c) in activation and polarization of macrophages. Our data indicated that rRv1737c interacted with macrophages through the Toll-like receptor 2 (TLR2)-dependent pathway, resulting in the nuclear factor κB (NF-κB) phosphorylation and cytokine production. The rRv1737c-loaded macrophages polarized into the indoleamine 2,3-dioxygenase 1 (IDO1) expressed subset. This indicated that rRv1737c contributed to the establishment of immune suppression in LTBI. Our study explored the molecular events to strength our understanding of the immune responses during LTBI.

2. Materials and methods

2.1. Mice and cell lines

C57BL/6 female mice (6–8 weeks) were purchased from Charles River Laboratories (Beijing, China). The animal handling was in compliance with the guidelines set by the Committee on Animal Care and Use (Ethic Approval File: syll2013308). The THP-1 cells were purchased from the National Center for Cell Science (Shanghai, China).

2.2. Isolation and culture of mouse peritoneal macrophages

C57BL/6 mice were given 20 μg of rRv1737c intraperitoneally. Phosphate-buffered saline (PBS) was used as the control. After 24 h, mice were euthanized, and intraperitoneal lavage was performed with 10 ml of PBS containing 5% bovine serum albumin (BSA). Cell numbers were counted after staining with Trypan blue. Cell classification was detected under a microscope after Wright staining. The cells were suspended and cultured in 6-well plates (1 million cells per well) with RPMI-1640 (Biological Industries, Israel) complete medium containing 10% fetal bovine serum (FBS, Biological Industries, Israel) at 37 °C and 5% CO_2 for 4 h. The unattached cells were removed. The rest of the cells were stimulated with different concentrations of rRv1737c (100 ng/ml, 500 ng/ml, and 1000 ng/ml) for 24 h.

2.3. THP-1 cell induction and treatment

The 80–90% confluent THP-1 cells were collected and resuspended in 6-well plates (2 million cells/well) with RPMI-1640 complete medium and treated overnight with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma, USA) to induce macrophage-like differentiation [11]. PMA-differentiated THP-1 cells were treated with various concentrations of rRv1737c (100, 500, and 1000 ng/ml) or LPS/Pam3CSK4 (100 ng/ml, InvivoGen, USA). In an experiment designed to block TLR2 signaling, PMA-differentiated THP-1 cells were pretreated for 1 h at 37 °C with anti-TLR2 (1:100) (Biolegend, USA) or an immunoglobulin G1 (IgG1) isotype control Ab (1:100) (Biolegend, USA), then treated with rRv1737c. The PBS group was used as a mock control. Culture supernatants were collected after 24 h and stored at −80 °C until detection.

2.4. Western blot analysis

After 24 h of stimulation with different concentrations of rRv1737c, total protein was extracted from PMA-differentiated THP-1 cells and mouse peritoneal macrophages according to a published study [12]. The cells were washed twice with pre-chilled PBS and pelleted by centrifugation at 800 g for 5 min. Cell pellets were treated with 100 μL of radioimmunoprecipitation assay (RIPA, containing 1 mM of phenylmethylsulfonyl fluoride (PMSF)) (Sigma, USA), shaken at 4 °C for 15 min, and centrifuged at 14,000 rpm for 15 min, and the protein concentration was determined by BCA protein assay (Thermo Scientific, USA). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). Membranes were blocked with 5% BSA in tris-buffered saline containing 0.1% Tween-20 (TBST) buffer for 1 h and immunoblotted with primary antibodies overnight, such as anti-β-actin (Cell Signaling Technology, USA), anti-TLR2 (human/mouse) (Cell Signaling Technology, USA), anti-NF-κB (p65, p-p65) (Cell Signaling Technology, USA), respectively. The secondary Ab was goat anti-rabbit IgG (Cell Signaling Technology, USA). Target proteins were visualized using ECL reagent (Thermo Scientific, USA). Band intensity was quantified using ImageJ software.

2.5. Cytokine detection by enzyme-linked immunosorbent assay (ELISA)

PMA-differentiated cells were cultured in 24-well plates and incubated with various concentrations of rRv1737c. The levels of cytokines (tumor necrosis factor α (TNF-α), IL-1β, IL-6, and IL-8) in supernatants were measured by ELISA kits (Elabscience, Wuhan, China). The experimental procedure was followed by the manufacturer's instructions. The levels of cytokines were determined by absorbance at 450 nm by a microplate reader.

2.6. RNA isolation and real-time quantitative reverse transcription polymerase chain reaction (RT-PCR)

PMA-differentiated THP-1 cells were treated with various doses of rRv1737c protein, and total RNA was extracted using Trizol (Invitrogen Carlsbad, USA). RNA was converted into cDNA by PrimeScript™ RT Master Mix Kit (TaKaRa®, China), and then amplified using SYBR Green qPCR Master Mix (TaKaRa®, China). Glyceraldehyde-3-phosphate

Table 1
The primer sequences of target genes.

Gene Name	Forward (5′-3′)	Reverse (5′-3′)
Rv1737c	CGGGATCCATGAGAGGGCAAGCGGCCAAT	CCCAAGCTTCCTGGACGCCTCCTCACTCA
TLR2	GGCCAGCAAATTACCTGTGTG	AGGCGGACATCCTGAACCT [13]
TLR4	AGTTGATCTACCAAGCCTTGAGT	GCTGGTTGTCCCAAAATCACTTT
IDO1	CTTTGCTCTGCCAAATCCACA	ATCCCAGAACTAGACGTGCAAG
iNOS	ATTCACTCAGCTGTGCATCG	TCAGGTGGGATTTCGAAGAG
GAPDH	GGAAGGTGAAGGTCGGAGTC	TGAGGTCAATGAAGGGGTCA

dehydrogenase (GAPDH) was applied as the internal reference gene. The difference of relative expression of mRNA of target gene to GAPDH was calculated as $2^{-\triangle\triangle Ct}$. All primer sequences are shown in Table 1.

2.7. Detection of surface markers by flow cytometry

PMA-differentiated THP-1 cells were stimulated with rRv1737c (100 ng/ml, 500 ng/ml, 1000 ng/ml) for 24 h, and cells were collected and incubated with APC mouse anti-human CD40, PerCP-Cy™5.5 mouse anti-human CD86, PE mouse anti-human CD80, FITC mouse anti-human MHC-II (BD, CA, USA), and Alexa 688-conjugated anti-human CD282 (TLR2) (Biolegend, USA). The mouse peritoneal lavage fluid was collected as described previously. Mouse peritoneal macrophages were incubated with FITC rat anti-mouse CD40, PerCP-Cy™5.5 hamster anti-mouse CD80, APC rat anti-mouse CD86, and PE rat anti-mouseF4/80 (BD, CA, USA). Cells were fixed in 4% paraformaldehyde for 20 min at 4 °C and resuspended in 1x PBS solution containing 5% BSA. The cells were run by BD FACS Calibur (BD Biosciences, CA, USA), and data were analyzed by FlowJo software.

2.8. Cell co-culture

rRv1737c-activated T cells were obtained from splenocytes of C57BL/6 mice, which were immunized intraperitoneally three times over a 2-week period with 20 μg of rRv1737c formulated with cholera toxin subunit B (CTB) adjuvants (Absin Bioscience Inc., Shanghai, China). Sorting of CD4 T Cells by BD FACS Aria™ II (BD Biosciences, CA, USA). Mouse peritoneal macrophages were isolated from C57BL/6 mice as previously described. Purified CD4 T cells and macrophages were co-cultured in RPMI-1640 complete medium at a ratio of 1:5/1:10 for 48 h at 37 °C and 5% CO_2 with or without rRv1737c. Cells were stained with rat anti-mouse CD4-APC, rat anti-mouse IL-4-PE, rat anti-mouse IL-10-PE, or rat anti-mouse Foxp3-PE (BD Biosciences, CA, USA) and analyzed by flow cytometry.

2.9. Statistical analysis

Data were examined by one-way analysis of variance (ANOVA) and Student's *t*-test. $p < 0.05$ was considered as significant. All figures were created using GraphPad Prism 5 software. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

3. Results

3.1. rRv1737c induced peritoneal macrophage enrichment in mice and promoted TLR2 expression on peritoneal macrophages

Firstly, in this study, we obtained the recombinant plasmid pET30a-Rv1737c by molecular cloning (Supplementary Fig. S1A). The plasmid was transformed into *Escherichia* (*E.*) *coli* BL21 (DE3), and recombinant protein expression was induced. The results of SDS-PAGE confirmed that the recombinant protein was expressed and purified (Supplementary Fig. S1B) and its molecular weight was consistent with the expected (43 kDa). Anti-His tag was used to identify its specificity (Supplementary Fig. S1C).

To explore the role of the recombinant protein in immune response, C57BL/6 mice were treated with rRv1737c (20 μg) intraperitoneally. The peritoneal lavage fluid was collected after 24 h. The cell sorting was conducted under a microscope after Wright staining. The data showed that most of the peritoneal recruited cells are macrophages after rRv1737c pretreatment (Fig. 1A).

The ability of macrophages to express major histocompatibility complex (MHC), costimulatory molecules makes them potent cells to modulate the adaptive immune response. rRv1737c induced significant increases in costimulatory molecules, named CD40, CD80, and CD86, while no changes were observed in MHC-II (Fig. 1B). This data suggested that the rRv1737c-recruited macrophages showed no changes in antigen-presenting function, and even its costimulatory markers have been upregulated.

We further detected TLR expression on peritoneal recruited macrophages to identify the potential pattern recognition receptors which may be attributed to the rRv1737c recognition. The dramatically dose-dependent increases in the expression of TLR2 on peritoneal macrophages from the rRv1737c-treated mice was demonstrated (Fig. 1C). These results suggested that the rRv1737c promotes TLR2 expression and induces macrophage activation.

3.2. rRv1737c induces TLR2 expression and NF-κB phosphorylation in THP-1 cells

To further explore the role of rRv1737c in macrophage activation, the THP-1 cell line was selected, and the rRv1737c has no effect on THP-1 cells viability (Supplementary Fig. S2). TLR2 expression level on PMA-differentiated THP-1 cells following rRv1737c treatment was analyzed. TLR2 mRNA level in PMA-differentiated THP-1 cells was significantly increased, while no changes were observed in TLR4 expression (Fig. 2A). The flow cytometry results also confirmed that the surface expression of TLR2 was significantly increased when treated with rRv1737c (Fig. 2B). The increased TLR2 expression, as well as the phosphorylated NF-κB (p-p65), the key transcriptional factor in TLR2 signaling, showed a dose-dependent increase in rRv1737c-treated cells (100 ng/ml, 500 ng/ml, and 1000 ng/ml), compared with the PBS group (Fig. 2C).

To further detect the relationship of the phosphorylation of NF-κB with TLR2 activation, the TLR2 signaling was blocked with specific antibody. As shown in Fig. 2D, the phosphorylation of NF-κB was blanched without TLR2 activation. This suggested that rRv1737c mediated phosphorylated NF-κB (p-p65) dependent on TLR2 signaling pathway.

3.3. rRv1737c induces TLR2-dependent macrophage activation

To further evaluate the effects of rRv1737c on macrophage activation, we analyzed the co-stimulatory marker expression (CD40, CD80, CD86, and MHC-II) by exposing PMA-differentiated THP-1 cells to low/high doses of rRv1737c (100 ng, 1000 ng/ml) for 24 h. The cell surface marker expression was detected by flow cytometry. A significantly increased expression of CD40, CD80, and CD86 was observed as compared to control cells (Fig. 3A). Specifically, CD40 expression increased from low-dose rRv1737c stimulation and kept increasing with higher doses. The expression CD80 has not significantly changed at low-dose rRV1737c exposure, only with the increasing trend, while CD86 only increased at the high-dose pretreatment (1000 ng/ml). The CD40 and CD80 expression on macrophages decreased when the TLR2 signal pathway was blocked (Fig. 3B). However, the CD86 and MHC-II expression were not affected by TLR2 signal activation, suggesting that other signal pathways might be involved in macrophage activation. The data demonstrated the various sensitivity of costimulatory markers on macrophages to rRv1737c treatment. The upregulation of CD40 and CD86 depend on the TLR2 activation by rRv1737c.

3.4. rRv1737c induced production of inflammatory factors though TLR2

To detect the proinflammatory functions, PMA-differentiated THP-1 cells were treated with rRv1737c (1000 ng/ml). The cytokines (TNF-α, IL-1β, IL-6, and IL-8) in the supernatant were measured by ELISA. As shown in Fig. 4A, the release of TNF-α, IL-1β, IL-6, and IL-8 was significantly higher than those of the untreated group. When TLR2 was blocked, the secretion of proinflammatory cytokines dramatically decreased (Fig. 4A). Further, the cytokine production was partially dependent on TLR4 signaling, even when no dramatically increased TLR4 was detected (Fig. 4B). The results suggested that rRv1737c induced the

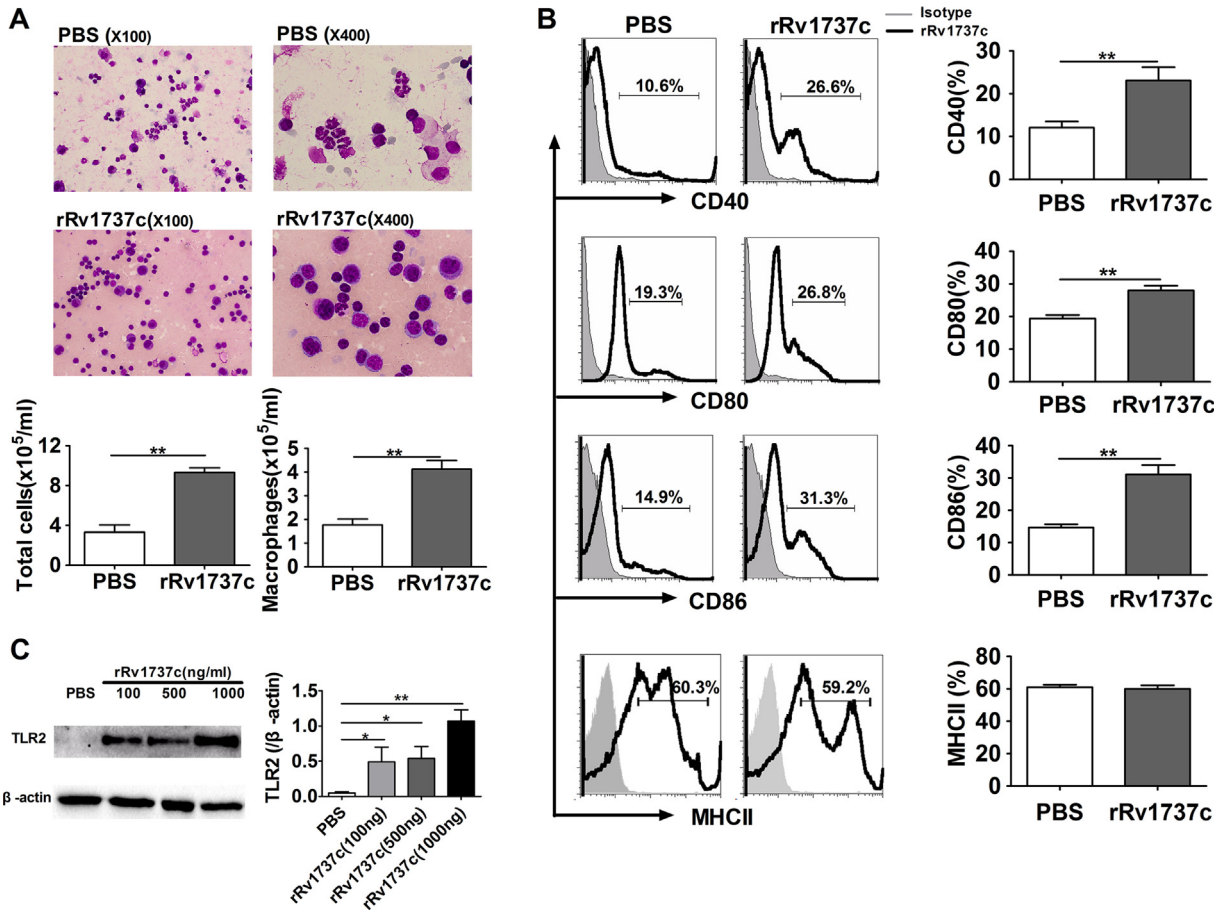

Fig. 1. rRv1737c stimulates peritoneal macrophage recruitment and activation in C57/BL6 mice and induces TLR2 expression in mouse macrophages. C57/BL6 mice (n = 5) were treated with rRv1737c (20 μg) through intraperitoneal injection. In 24 h post injection, sacrificed mice were intraperitoneally lavaged with PBS (contains 5% BSA). Cytospin was used to prepare the cell slides for cell count and differentiation. The lavage cells were counted and co-stained with F4/80-PE, CD80-Percp-Cy5.5, CD86-APC, CD40-FITC, or MHCII-FITC for analysis of surface marker expression using flow cytometry. The remaining cells were incubated in 6-well plates for 4 h to remove unattached suspended cells, and then the total protein was extracted 24 h after stimulation with rRv1737c (100 ng, 500 ng, 1000 ng/ml) for Western blot analysis. A, The total cell number and macrophages in the peritoneal lavage fluid. B, The expression of surface markers (CD40, CD80, CD86, and MHCII) on macrophages; macrophages were defined as F4/80-positive cells. C, Expression of TLR2 in mouse macrophages stimulated by different concentrations of rRv1737c. One representative experiment of four independent experiments is shown. Data are shown as the mean ± SD of each group. $*p < 0.05$, $**p < 0.01$.

secretion of pro-inflammatory cytokines through the TLR2 pathway.

3.5. rRv1737c induced IDO1-expressed macrophages

IDO1, an enzyme that regulates tryptophan metabolism, is well-known for its immunosuppressive effects in tumors, chronic infections, and autophagy diseases [14–17]. Increased activity of IDO has been reported in TB patients [18]. DosR antigens are detected from latent infection of *Mtb*, and recent reports have indicated that the influence of IDO is associated with *Mtb* immune escape [19]. Previous studies have supported a role for inducible nitric-oxide synthase (iNOS) in the pathogenesis of *Mtb* infection. Macrophages are the main cellular source for iNOS production.

Therefore, to identify the functional changes of IDO1 and iNOS in rRv1737c-treated macrophages, the mRNA levels of IDO1 and iNOS in PMA-differentiated THP-1 cells was measured. The results showed that the levels of IDO1 mRNA increased in a dose-dependent pattern. However, the levels of iNOS changes had no statistical significance (Fig. 5). IDO1 upregulation may be one of the mechanisms leading to latent infection in the host.

3.6. rRv1737c-activated macrophages induced Th2-type immune response and Treg production

The expression of co-stimulatory molecules on the surface of macrophages, and the secretion of proinflammatory cytokines enhances its interaction with T cells. To further investigate the functions of Rv1737c-activated macrophages, we performed co-culture of rRv1737c-treated macrophages with rRv1737c-specific T cells. As shown in Fig. 6, the frequency of CD4 T cells expressing Foxp3, IL-10, and IL-4 was significantly increased compared to control cells. These results suggested that the rRv1737c-treated macrophages directed naive T cell proliferation toward Th2, IL-10^+Treg, and $Foxp3^+$ Treg phenotypes.

4. Discussion

Tuberculosis manifests several clinical stages. Latent infection is usually found in the initial infected patients without any clinical symptoms. Active *Mtb* infection is believed to develop from the re-activation of undiagnosed latent infection when host immune response is compromised [20]. Therefore, it is crucial to understand the role of factors released during LTBI by *Mtb* and their roles in impairing host immunity. Recently, proteins encoded by the DosR genes have become

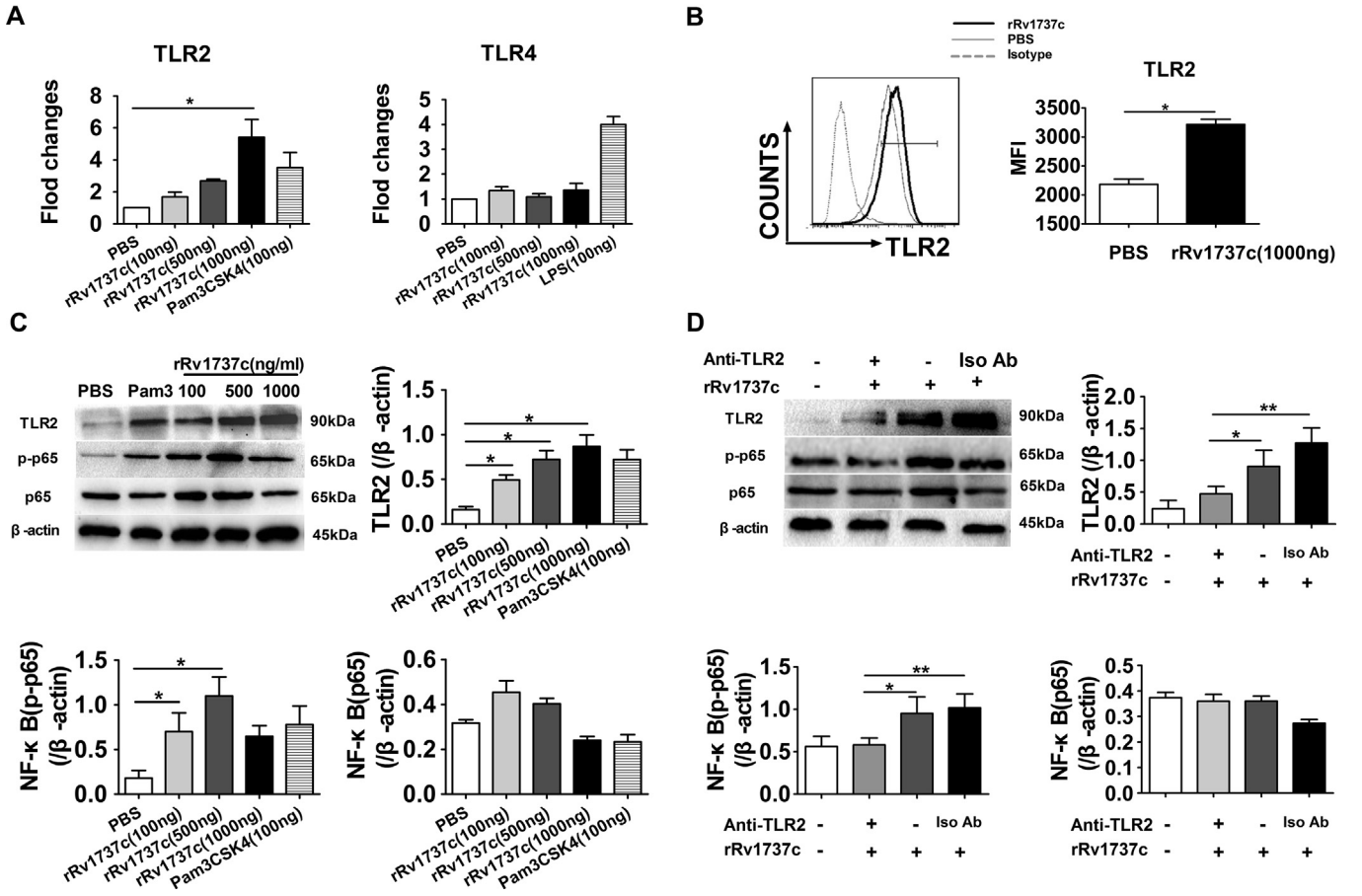

Fig. 2. rRv1737c induced the expression of TLR2 and NF-κB (p-p65) in THP-1 cells. THP-1 cells were cultured in 6-well plates (2×10^6/well) and stimulated with PMA for 24 h in complete RPMI-1640 medium at 37 °C. rRv1737c (100 g, 500 ng, 1000 ng/ml) was added and incubated for 24 h. LPS/Pam3CSK4 (100 ng/ml) was used as a positive control. A, The TLR2 and TLR4 mRNA levels in PMA-differentiated THP-1 cells treated with rRv1737c protein were detected by RT-PCR. mRNA levels normalized to β-actin were then compared to the PBS group and calculated by $2^{-\triangle\triangle Ct}$. B, Surface expression of TLR2 by flow cytometry and mean fluorescence intensity (MFI) of TLR2 was calculated and shown. C, The expression of TLR2 and NF-κB (p-p65) in PMA-differentiated THP-1 cells stimulated with different concentrations of rRv1737c. β-Actin was used as a control. The fold changes of TLR2, NF-κB, and phosphonated NF-κB was analyzed and graphed. D, PMA-differentiated THP-1 cells were incubated with anti-TLR2 and isotype control Ab (iso Ab) before stimulation with rRv1737c. The expression of TLR2 and NF-κB (p-p65) was measured by Western blot. One representative experiment of three independent experiments with similar results is shown. *$p < 0.05$, **$p < 0.01$.

increasingly well known. Rv1737c, one of the DosR genes, encodes nitrate/nitrite-transport integral membrane protein [21]. However, the molecular mechanism of Rv1737c in maintaining latent infection is not fully understood.

After infection with *Mtb*, alveolar macrophages are the first line of immune defense to efficiently phagocytose bacteria. However, the bacterium methodically antagonizes the activity of these cells to grow or multiply in these cells. The fighting between macrophages and bacterium results in the development of functional distinct subsets of macrophages, which is critical for the infection outcome. Macrophages recognize the pathogen, relying primarily on its membrane pattern recognition receptors, such as Toll-like receptors, scavenger receptors, complement receptors, mannose receptors, or c-type lectin receptors [22,23]. Among them, Toll-like receptors play a key role in connecting innate and acquired immunity [24]. Particularly, TLR2, TLR9, and TLR4 are responsible for the *Mtb* recognition and are involved in the activation of innate and adaptive immune responses [24,25]. In addition to immune activation, the involvement of certain TLRs by *Mtb* is also associated with immune evasion [26,27]. TLR2 is essential in anti-tuberculosis infection [28]. TLR-deficient mice showed increased susceptibility to tuberculosis compared with wild-type mice in early infection, particularly in TLR2 −/− mice, followed by TLR4 −/− mice [29].

The formation of granulomatous tissue induced by TLR2-mediated immune response prolongs the survival of *Mtb* in macrophages [30]. At present, it has been confirmed that three different lipoproteins (LpqH, LprG, LprA) on the surface of *Mtb* are TLR2 ligands, which inhibit MHC-II expression that retard the activation of the cell-mediated immune responses [31–33]. Some studies have shown that DosR antigens Rv0079 and Rv3131 induced cytokine secretion by interaction with macrophages or peripheral blood mononuclear cells (PBMCs) through TLR2, which may be one of the reasons for tuberculosis phasing in latent infection [12,34]. In this study, we observed that rRv1737c induced TLR2 upregulation on activated macrophages after promoting mouse peritoneal macrophage aggregation. TLRs are mainly transduced through Toll/IL-1R in the cytoplasm and activate the NF-κB through the MyD88-dependent or MyD88-independent pathways [35]. Our study confirmed the NF-κB phosphorylation through TLR2 activation by rRv1737c. The data indicated that the expression of TLR2 and phosphorylated NF-κB in the TLR2-blocking group was much lower than those in the mock group. Expression of costimulatory molecules is important for macrophage function. Our results showed that the CD40, CD80, and CD86 expression on rRv1737c-induced macrophages were significantly increased, but their sensitivity to rRv1737c was not equal. In addition, The CD40 and CD80 expression on macrophages decreased when the TLR2 signal pathway was blocked. Our study indicated that TLR2 was one of the pathways by which rRv1737c activated macrophages.

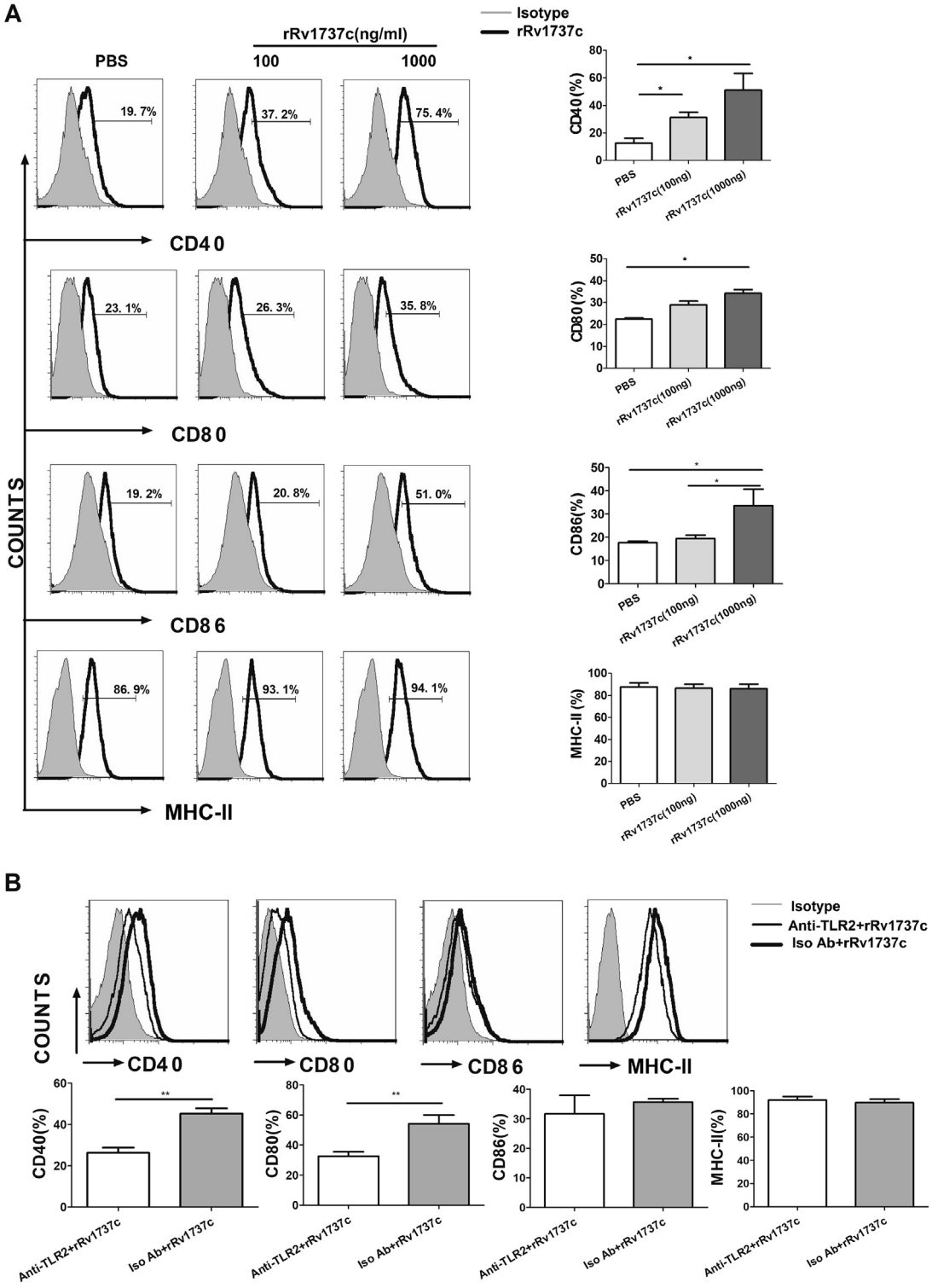

Fig. 3. The surface marker expression on macrophages partially depends on TLR2 signaling after rRv1737c treatment. PMA-differentiated THP-1 cells were stimulated with rRv1737c (100 ng, 1000 ng/ml) for 24 h. The cells were co-stained with surface molecules CD40-APC, CD86-Percp-Cy5.5, CD80-PE, and MHC-II-FITC for analysis of costimulatory molecule expression using flow cytometry. A, The surface marker expression of CD40, CD80, CD86, and MHC-II was analyzed by flow cytometry. Gray shadow represents the isotype. The summarized data are presented. B, PMA-differentiated THP-1 cells were incubated with anti-TLR2 and isotype control Ab (Iso Ab) before stimulation with rRv1737c. The macrophages were analyzed using flow cytometry. The representative FACs data are shown. The data were analyzed as mean ± SD. One representative experiment of three independent experiments with similar results is shown. $*p < 0.05$, $**p < 0.01$.

Some studies have confirmed that *Mtb*-specific antigens induced the secretion of inflammatory factors through TLR2 signaling [12,34,36]. Our study also confirmed the increased levels of inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-8 expression in THP-1 cells with the treatment of rRv1737c, which are dependent on TLR2. rRv1737c-induced secretion of pro-inflammatory cytokines from macrophages may promote the formation of macrophage granulomas. The local microenvironmental changes limit *Mtb* growth in granulomatous tissue and

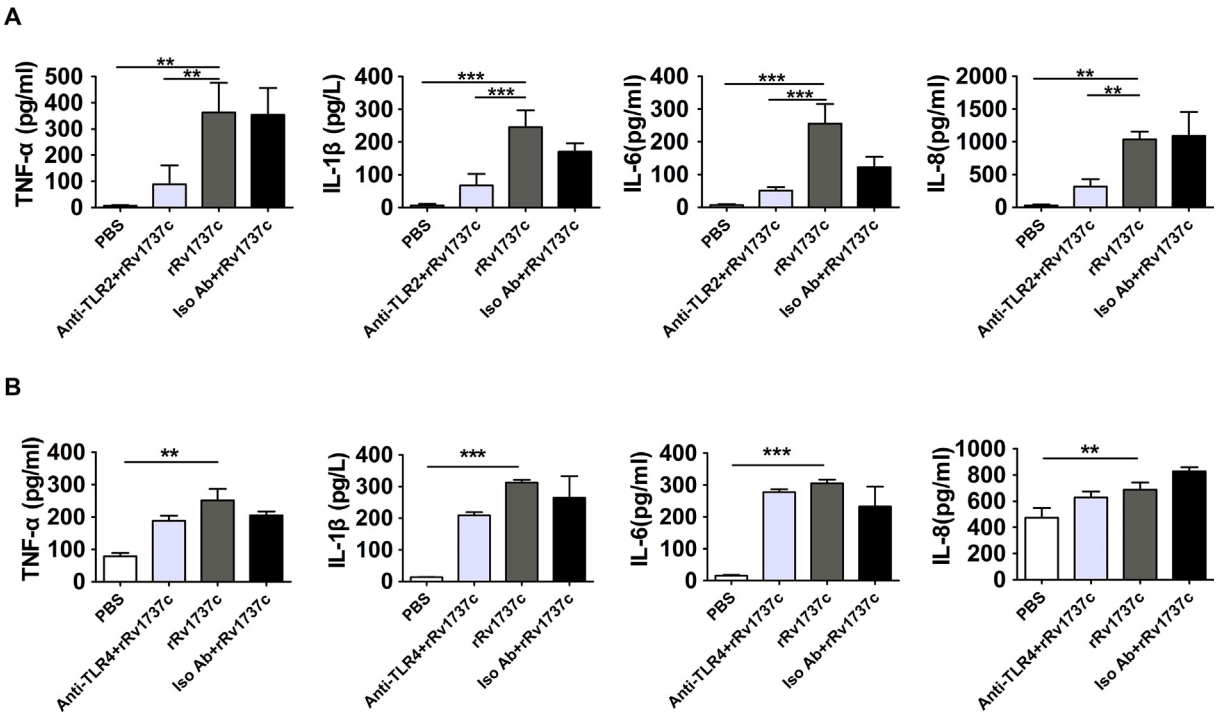

Fig. 4. rRv1737c stimulates the release of inflammatory cytokines in THP-1 cells. The level of cytokines released by PMA-differentiated THP-1 cells after rRv1737c (l000 ng/ml) treatment for 24 h were detected by ELISA. A, the secretion of the cytokines by PMA-differentiated THP-1 cells infected with rRv1737c with or without anti-TLR2 or isotype control Ab (Iso Ab). B, PMA-differentiated THP-1 cells were incubated with anti-TLR4 and isotype control Ab (Iso Ab) before stimulation with rRv1737c. Data represent the mean ± SD of the results of three replicates. $^{**}p < 0.01$, $^{***}p < 0.001$.

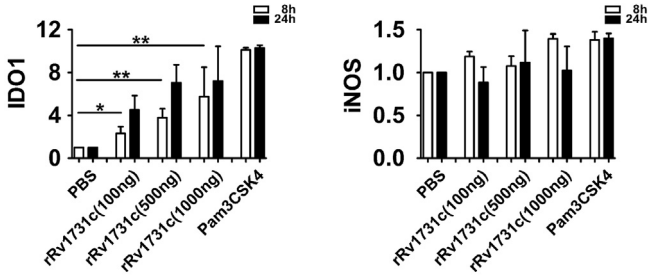

Fig. 5. The levels of IDO1 and iNOS mRNA in THP-1 cells after rRv1737c exposure. PMA-differentiated THP-1 cells were cultured with rRv1737c (100 ng, 500 ng, 1000 ng/ml) for 24 h at 37 °C. IDO1 and iNOS levels were detected by real-time PCR. β-Actin was used as a control. The relative expression of target gene mRNA was calculated by $2^{-\triangle\triangle Ct}$. Fold changes were graphed. One representative experiment of three independent experiments with similar results is shown. $^{*}p < 0.05$, $^{**}p < 0.01$.

result in latent *Mtb* infection. It is well known that TNF-α plays an important role in the formation of granulomatous tissue [37,38]. TNF-α can mediate the activation of the NF-κB signaling pathway, leading to IL-1β, IL-6, and IL-8 cytokine release [39]. IL-1β and IL-6 initiate early pro-inflammatory responses and promote the recruitment of other immune cells. Further studies are needed to understand the roles of cytokines, as a recent study suggested that these cytokines can be protective or detrimental depending on the stage of *Mtb* infection.

Surprisingly, this study also found that IDO1 expression increased in activated macrophages with high expression of CD40/CD80. IDO1 is always reported with an immunosuppressive effect in tumor-associated immune escape [40]. There is evidence that it plays a similar role in TB latent infection [19]. This may be one of the strategies that *Mtb* manipulated to persist in latent infection. *Mtb* employed its latent-associated antigen, Rv1737c, to break the code of costimulatory molecules to enhance the suppressive subset differentiation. Most existing studies have reported that Th2 immune response plays a negative role in the body's fight against tuberculosis infection [41,42]. During the initial immune response in tuberculosis infection, *Mtb* induces the expansion of Treg cells that delays the adaptive immune activation to benefit its persistence in lung tissue [43]. In this study, we observed that rRv1737c-activated macrophages induced a Th2-type immune response and increased the proportion of Treg in vitro, which is different from some DosR antigens that induced Th1 immune response [44]. Our previous study confirmed that rRv1737c induces Th2-type immune responses in vivo, which may be one of the mechanisms leading to the retention of *Mtb* [45].

In essence, these findings suggest that Rv1737c plays a key role in the activation of the IDO^{+} macrophage subset and the Th2/Treg response. Rv1737c interaction with macrophages partly depended on the TLR2 signaling pathway. Proinflammatory factor secretion may lead to the formation of granulomatous tissue, supporting *Mtb* long-term survival in a latent phase. This study showed that Rv1737c may not be a good vaccine candidate compared to other DosR antigens. Our study contributes to understanding the roles of DosR antigens in LTBI.

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Declaration of Competing Interest

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

Footnotes

Author Contributions: C.W. performed tests, analyzed the data and drafted the manuscript. Y.W. and W.H. helped and perform partial

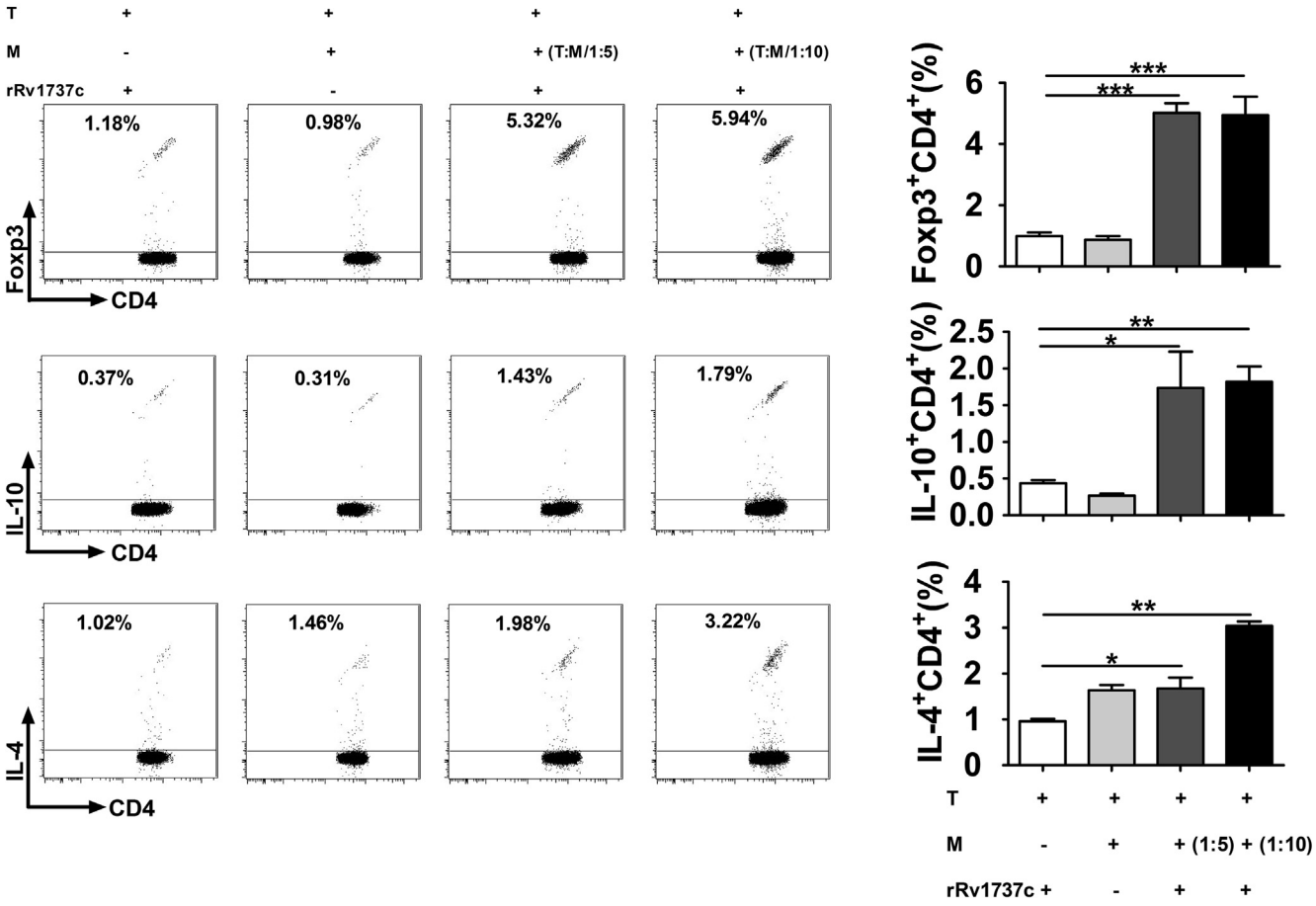

Fig. 6. rRv1737c-treated macrophages induce Treg and Th2 immune responses. rRv1737c-activated CD4 T cells were obtained from splenocytes of C57BL/6 mice that were immunized with rRv1737c. Mouse peritoneal macrophages were isolated from C57BL/6 mice. CD4 T cells and macrophages were co-cultured at a ratio of 1:5/1:10 (T:M) for 48 h at 37 °C. One representative experiment of three independent experiments with similar results is shown. $*p < 0.05$, $**p < 0.01$.

experiments. R.Y., X.X., Y.L., Y.J and H.L. provided help and advice. X.G., B.Z. designed the research, directed the experiments and wrote the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2019.103947.

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