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# *Mycobacterium tuberculosis* conserved hypothetical protein rRv2626c modulates macrophage effector functions

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## Summary

**Secretory proteins of *Mycobacterium tuberculosis* are the major immunomodulators of the host immune response. Open reading frame (ORF) *Rv2626c*, encoding a conserved hypothetical protein eliciting a strong humoral immune response in patients with tuberculosis (TB), was shown to be up-regulated upon infection in mice under hypoxic conditions. We now show that recombinant Rv2626c protein (rRv2626c) can bind to the surface of murine macrophages and elicit the type-1 immune response, as manifested by nitric oxide (NO) secretion and expression of inducible nitric oxide synthase (iNOS). Significant induction of pro-inflammatory cytokines [interleukin (IL)-12 and tumour necrosis factor (TNF)-α] was evident upon stimulation of murine macrophages, as well as peripheral blood mononuclear cells (PBMCs) isolated from patients with active TB disease, with rRv2626c. Stimulation with rRv2626c also enhanced the expression of costimulatory molecules such as B7-1, B7-2 and CD40 on murine macrophages. We further show that the production of NO and pro-inflammatory cytokines in response to rRv2626c is mediated by the transcription factor nuclear factor (NF)-κB, and this was further confirmed using pyrrolidine dithiocarbamate (PDTC), a specific pharmacological inhibitor of NF-κB. Rv2626c therefore appears to modulate macrophage effector functions by eliciting both innate and adaptive immune responses, suggesting its possible use as a vaccine candidate.**

**Keywords:** *M. tuberculosis*; innate and adaptive responses; Rv2626c; vaccine

## Introduction

Tuberculosis (TB), a progressive disease that takes one human life approximately every 15 seconds globally, is caused by invasion of macrophages by *Mycobacterium tuberculosis*. The use of Bacillus Calmette-Guerin (BCG) as a protective vaccine for TB is questionable as it provides only 50% protection in pulmonary TB and is not effective in adults.[1] In addition to the problem of its limited protective value, use of BCG in immunocompromised individuals with human immunodeficiency virus (HIV) infection or acquired immune deficiency syndrome (AIDS) can cause disseminated disease.[2–5] Secretory proteins (culture filtrate proteins) of the bacterium are recognized directly by the host immune system, and some of these, such as Ag-85, MPT-64, MPB-70, culture filtrate protein (CFP)-10 and early secreted antigenic target-6 (ESAT-6), are promising subunit vaccine candidates for vaccination against TB.[6–8] Although several vaccine candidates are under development, a better vaccine which could provide long-term protection against TB is unlikely to be developed in the near future.[9]

Protection against *M. tuberculosis* infection requires activation of both innate and adaptive immunity.[10] Activated T cells mainly restrict progression of TB in the host.[2] Effective activation of T cells depends on the interaction of various T-cell receptors (TCRs) (e.g. CD28 and CD40L) with their counterparts [major histocompatibility complex (MHC)–peptide complex, B7 molecules and CD40] on antigen-presenting cells (APCs).[11,12] Host resistance to *M. tuberculosis* infection is governed by the secretion of pro-inflammatory cytokines against *M. tuberculosis* invasion and the balance with inhibitory or suppressive cytokines such as interleukin (IL)-10 and transforming growth factor (TGF)-β. Host pro-inflammatory cytokines such as interferon (IFN)-γ, tumour necrosis factor

  

(TNF)-α and IL-12 are important resistance factors against TB.[13–17] Pro-inflammatory gene knockout mice were found to be susceptible to TB infection, indicating a direct role of these cytokines in immunity to TB.[18,19]

In addition to the pro-inflammatory cytokines, production of nitric oxide (NO) by macrophages is an effective host defence mechanism against *M. tuberculosis*. Up-regulation of the expression of inducible nitric oxide synthase (iNOS) was found to be an important component of host defence against *M. tuberculosis*.[20] NO exhibits efficient microbicidal activity even at concentrations < 100 ppm, killing 99% of *M. tuberculosis* in culture.[21] The importance of NO in providing protection against TB is clear from experiments in iNOS knockout mice, which showed higher mortality and increased dissemination.[20] A wide variety of cytokines and inflammatory mediators such as TNF-α, IFN-γ, lipopolysaccharide (LPS) and IL-1β are known to induce iNOS expression.[22] Several *M. tuberculosis* components, such as lipoarabinomannan (LAM),[23] ESAT-6 antigen,[24] and *M. tuberculosis*-specific antigen (MTSA) or CFP-10,[25] can also stimulate macrophages to release NO. Many mycobacterial products, such as lipomannan (LM), dimannosylated phosphatidylinositides (e.g. PIM2), mycolyl arabinogalactan–peptidoglycan complex, phospholipase C and lipoproteins, also have the potential to induce iNOS expression.[23,26]

The hypothetical protein coded by *M. tuberculosis* open reading frame (ORF) *Rv2626c* has been shown to elicit a high serum antibody response in patients with active TB, suggesting that this antigen is important in immunoprofiling of disease states.[27] *Rv2626c* expression was up-regulated in hypoxic conditions[28] and found in culture filtrates as well as in lysates in peptide mass fingerprinting and immune detection studies using an *in vitro* latency model. [29] Further studies in mice showed increased expression of *Rv2626c* at the terminal stages of infection in the lungs. *Rv2626c* and other *M. tuberculosis* ORFs encoding α-crystallin (acr), Rv2623, sodC, sodA and fbpB were found to be differentially expressed in IFN-γ deleted mice. An increase in T helper type 1 (Th-1)-mediated immune responses (IFN-γ/iNOS induction) correlated well with increased mRNA synthesis of *Rv2626c* in *M. tuberculosis*, suggesting its up-regulation under stress conditions.[30] Studies using real-time reverse transcription–polymerase chain reaction (RT-PCR) to monitor *Rv2626c* mRNA synthesis just prior to stress-induced reduction of bacterial multiplication have suggested a role of Rv2626c as a transcription signature for non-replicating persistence.[30] In another study where the eight DosR regulon-encoded antigens (Rv1733c, Rv1738, Rv2029c, Rv2031c, Rv2032, Rv2627c, Rv2628 and Rv2626c) were analysed for their immunogenicity in BALB/c and C57BL/6 mice following vaccination with DNA constructs, it appeared that Rv2626c and Rv2031 could provide strong humoral and/or cellular Th-1 responses.[31] Furthermore, peripheral blood mononuclear cells (PBMCs) from *M. tuberculosis*-infected patients recognize Rv2626c and induce major Th-1 cytokines such as IFN-γ.[32] A correlation between increased expression of *Rv2626c* (and the other *M. tuberculosis* ORFs *Rv3286c*, *Rv2031* and *Rv3133c*) and phenotypical tolerance of *Mycobacterium bovis* BCG to rifampicin and metronidazole under anaerobic growth conditions has been found.[33] In the present study we describe the immunostimulatory role of the secretory 16-kDa conserved hypothetical protein coded by the *M. tuberculosis* ORF *Rv2626c*. Our study shows that recombinant Rv2626c (rRv2626c) binds to the surface of murine macrophages and up-regulates NO production and iNOS expression. In addition, we report that rRv2626c induces the expression and secretion of pro-inflammatory as well as Th-1 type cytokines such as TNF-α, IL-12 and IFN-γ as well as the up-regulation of various costimulatory molecules such as B7-1, B7-2 and CD40. We further show that the induction of iNOS expression and NO production by rRv2626c is mediated through the nuclear factor (NF)-κB-dependent pathway.

## Materials and methods

### *Cloning, expression and purification of* M. tuberculosis *Rv2626c*

The ORF encoding the hypothetical protein Rv2626c of *M. tuberculosis* was PCR-amplified from the *M. tuberculosis* H37Rv cosmid library (kindly provided by Dr Stewart Cole; Institut Pasteur, Paris, France) using a forward primer (5′-GGC ATA TGA CCA CCG CAC GCG ACA TCA TG-3′) and a reverse primer (5CCG CTC GAG GCT GGC GAG GGC CAT GGG C-3′) harbouring *Nde*I and *Xho*I restriction sites (underlined), respectively. The *Nde*I/*Xho*I-digested 432-bp PCR product was cloned in the expression vector pET23a (Novagen, Merck Chemicals Ltd, Nottingham, UK). The clones were confirmed by sequencing with the T7 promoter primer on an Applied Biosystems Prism 377 DNA sequencer (Biosystems, Foster City, CA). The *Escherichia coli* BL21pLys (DE3) strain was transformed with the pET23a-2626c construct and the recombinant protein was expressed and affinity-purified on a Talon Column (Takara Bio, Madison, WI) as described previously.[34] The protein was eluted with 250 mM imidazole in lysis buffer. The elution fractions were 95% homogenous as analysed on a 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) gel followed by Coomassie blue staining. The purified rRv2626c protein was dialysed against 10 mM Tris/100 mM NaCl to remove the imidazole and quantified using the bicinchoninic acid test (Micro BCA Protein Assay kit; Pierce, Rockford, IL). The purified recombinant protein was incubated overnight at 4° with 10% volume/volume (v/v) polymyxin B-agarose beads

(Sigma-Aldrich St Louis, MO) to remove any endotoxin contamination. Further evaluation of bacterial endotoxin was carried out with the amebocyte lysate assay (E-toxate Kit; Sigma-Aldrich). The purified rRv2626c protein was stored in small aliquots at −20° and used in further experiments.

### *Macrophage binding assay*

In order to study cell surface binding of rRv2626c, antibody against rRv2626c was generated in BALB/c mice in the animal facility of Indian Immunological Limited (Hyderabad, India). For binding assays, approximately $1 \times 10^6$ RAW 264·7 macrophages were washed with wash buffer [phosphate-buffered saline (PBS) with 1% bovine serum albumin and 0·01% sodium azide] twice and then incubated with rRv2626c (10 μg) for various times on ice. After washing, RAW 264·7 macrophages were incubated with the anti-Rv2626c antibody at 1 : 2500 dilution for 1 hr at 4° followed by incubation with anti-mouse fluorescein isothiocyanate (FITC) conjugate for 40 min at 4°. After a final washing, RAW 264·7 macrophages were suspended in sheath fluid and analysed on a fluorescence-activated cell sorter (FACS) machine (FACS Vantage SE; Becton Dickinson, San Jose, CA). For control experiments, cells were treated with (i) medium plus anti-Rv2626c antibody, (ii) 10 μg of rRv2626c protein plus normal mouse serum (NMS), or (iii) 10 μg of rRv2626c plus anti-Rv2626c antibody preincubated with recombinant Rv2626c proteins.

### *Nitrite and cytokine assay*

The mouse macrophage cell lines RAW 264·7 and J774·1 were obtained from the National Centre for Cell Science, Pune, India and were maintained in Dulbecco's modified Eagle's minimal essential medium (DMEM; Invitrogen, Grand Island, NY) supplemented with sodium bicarbonate, 10% fetal calf serum (FCS) and 1% antibiotics. For the NO and cytokine assay, approximately $3 \times 10^5$ RAW 264·7 or J774·1 macrophages were seeded in a 96-well culture plate (BD, Falcon, San Jose, CA). Cells were stimulated with different concentrations of rRv2626c and incubated at 37° for 48 hr. The positive control group received LPS (1 μg/ml) and IFN-γ (1 ng/ml). As and when required, cells were pretreated by adding 10 μM pyrrolidine dithiocarbamate (PDTC; Sigma) and incubating for 1 hr, followed by stimulation with various concentrations of rRv2626c. For NO estimation by the Griess assay, equal aliquots of the culture supernatants were dispensed in duplicate into a 96-well culture plate and mixed with an equal volume of Griess reagent,[35] composed of 1% [weight/volume (w/v)] sulphanilamide, 0·1% (w/v) napthyl-ethylenediamine hydrochloride and 2·5% (v/v) $H_3PO_4$. After incubation at room temperature for 5 min, the absorbance was measured at 540 nm in an Ultra Microplate Reader (Bio-Tek, Winooski, VT). The concentration of nitrate was interpolated from the $NaNO_2$ standard curve.

TNF-α and IL-12 p40 levels in the culture supernatants were measured by enzyme immunoassay (EIA) (BD Biosciences Pharmingen, San Diego, CA) as described previously.[36] Standard curves for each cytokine were obtained using the recombinant standard protein provided by the manufacturer.

### *Flow cytometric analysis of B7-1, B7-2 and CD40 expression*

RAW 264·7 macrophages ($2 \times 10^6$ cells/well in a six-well culture plate) were left untreated or treated with 3 μg/ml of rRv2626c in the absence or presence of LPS and IFN-γ. After 24 hr of incubation, cells were harvested and washed three times with ice-cold FACS buffer [PBS containing 1% bovine serum albumin (BSA) and 0·01% sodium azide] and then re-suspended in FACS buffer and incubated with anti-mouse B7-1 (clone 1G10; BD Biosciences Pharmingen), anti-mouse B7-2 (clone GL1; BD Biosciences Pharmingen) and anti-mouse CD40 (clone 3/23; BD Biosciences Pharmingen). The control group received isotype control antibody. Cells were washed again with FACS buffer and incubated with anti-mouse FITC (Sigma-Aldrich). Flow cytometric analysis was performed (Becton Dickinson, San Jose, CA) and the FACS data were recorded for 20 000 events for each labelled cell population. Flow cytometry data analyses were carried out using CELL QUEST data analysis software (BD Biosciences, San Jose, CA).

### *Preparation of whole-cell extract*

The RAW 264·7 macrophages were seeded at a density of $5 \times 10^6$ per well in a six-well culture plate and were either left untreated or pretreated with PDTC for 1 hr followed by stimulation with either 5 μg of rRv2626c or a combination of LPS and IFN-γ. Cells were harvested and the whole-cell extract was prepared as described previously.[37] Equal amounts of the protein (30 μg), after protein estimation using the Micro BCA Protein Assay kit from each experimental set (namely, stimulated with rRv2626c, or rRv2626c + LPS or rRv2626c + IFN), were fractionated on a 10% SDS-PAGE gel under reducing conditions and transferred onto a nitrocellulose membrane (GE Healthcare, Piscataway, NJ). The membrane was then incubated with rabbit polyclonal iNOS antibody (Sigma) followed by anti-rabbit immunoglobulin-horse radish peroxidase (Ig-HRP) conjugate (Sigma-Aldrich). Bound enzyme was detected by chemiluminescence following the manufacturer's protocol (GE Healthcare, Piscataway, NJ).

  

*Nuclear extract*

RAW 264·7 macrophages were seeded at a density of $5 \times 10^6$ per well in a six-well culture plate and either left untreated or pretreated with PDTC for 1 hr, followed by stimulation with 5 μg of rRv2626c alone or with a combination of LPS and IFN-$\gamma$. Cells were harvested and nuclear extract was prepared from NP-40 lysed cells.[36] Equal amounts of the protein extracts (50 μg) were fractionated on a 10% SDS-PAGE gel. The nuclear proteins were transferred onto a nitrocellulose membrane and incubated with polyclonal rabbit antibody to NF-$\kappa$B p50 or NF-$\kappa$B p65 (Santa Cruz Biotech, Santa Cruz, CA) followed by incubation with anti-rabbit Ig-HRP conjugate. Bound enzyme was detected by chemiluminescence (ECL).

*Electrophoretic mobility shift assay (EMSA)*

An equal amount of the nuclear extract (10 μg) from each set (cells stimulated with rRv2626c, or rRv2626c + LPS or rRv2626c + IFN) was incubated at 37° for 30 min with 1 ng of $\gamma$-$P^{32}$-radiolabelled consensus oligodeoxyribonucleotides containing the binding site for NF-$\kappa$B (5′-ttgttacaagggactttccgctggggactttccagggaggcgtgg-3′; Santa Cruz Biotech) in a binding buffer [10 mM Tris, pH 7·5, 50 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 1 μg of poly dIdC, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF) and 50 mM $MgCl_2$]. For competition experiments, 100-fold molar excess of unlabelled consensus NF-$\kappa$B or mutant NF-$\kappa$B oligos was used to check the specificity of the DNA–protein complex. The DNA–protein complexes were resolved by electrophoresis on a 7% native PAGE gel at 4° in 1× Tris-borate-EDTA (TBE). After electrophoresis, the gel was dried and exposed to Phosphor Imager screen (Fuji Film, Tokyo, Japan) at room temperature for 12 hr and the screen was scanned using the Typhoon system (GE Healthcare, Piscataway, NJ).

### Study using PBMCs derived from patients with active TB

Patients with TB who participated in this study were diagnosed at the Mahaveer Hospital and Research Centre, Hyderabad, India; their TB was confirmed by a tuberculin skin test, radiographic examination, and observation of acid-fast bacilli in sputum. Healthy controls were volunteers at the Centre for DNA Fingerprinting and Diagnostics who had no clinical symptoms of TB disease. Blood samples (2–3 ml) were collected from patients with TB ($n = 48$) as well as from healthy controls ($n = 9$), followed by separation of PBMCs on Ficoll-Histopaque (Sigma-Aldrich) as described previously.[38] PBMCs were plated at a density of $2 \times 10^5$ per well in a 96-well culture plate and treated with rRv2626c (5 μg/ml) for 72 hr. The culture supernatants were harvested and assayed for cytokines (TNF-$\alpha$, IFN-$\gamma$ and IL-12) by EIA as described previously.[39] The institutional ethical committee approved this study.

*Statistical analysis*

The statistical significance of the results was determined using Student's *t*-test. The results are presented as mean ± standard deviation (SD).

## Results

### Expression and purification of recombinant Rv2626c

The 16-kDa recombinant protein coded by *Rv2626c* was expressed in the *E. coli* BL21plys (DE3) strain and purified using metal affinity chromatography, giving a yield of 10 mg/l culture. The purified rRv2626c when analysed by SDS–PAGE (Fig. 1) or even after silver staining (data not shown) did not reveal any major contaminating protein band. The endotoxin content in the purified recombinant protein was checked using the amoebocyte lysate assay and was found to be extremely low (0·05 pg/μg of protein).

### rRv2626c binds to the surface of RAW 264·7 macrophages

Previous studies have revealed that Rv2626c is a secretory protein, indicating that Rv2626c could influence the host immune response by interacting with macrophage surface receptors. In order to assess the ability of rRv2626c to

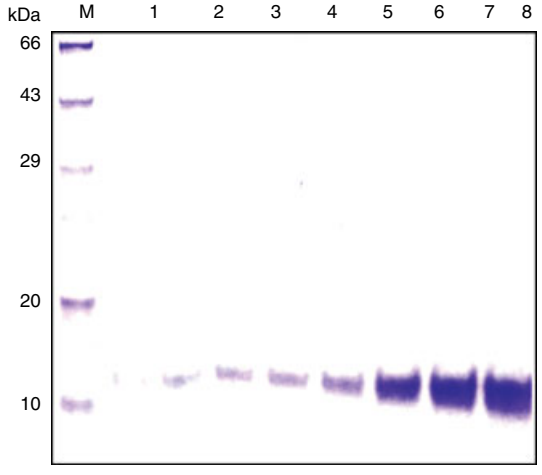


**Figure 1.** Purification of recombinant Rv2626c. His-tagged Rv2626c was over-expressed in the *Escherichia coli* pLys strain and purified by $Co^{++}$-affinity chromatography. Lanes 1–8 show the successive elution fractions of the recombinant protein. M is the protein molecular size marker. Tris-Tricine sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) fractionation and subsequent Coomassie blue staining revealed 99% homogeneity of the purified recombinant protein.

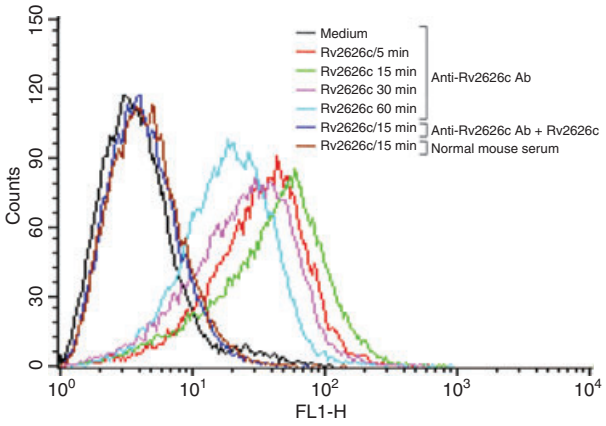


**Figure 2.** Recombinant Rv2626c (rRv2626c) binds stably to the murine macrophage surface with high affinity. RAW 264.7 macrophages were incubated with rRv2626c (3 μg) at 4° for 60 min and with normal mouse serum or anti-Rv2626c antibody (raised in a mouse), followed by incubation with anti-mouse fluorescein isothiocyanate (FITC) conjugate. The fluorescence was monitored by fluorescence-activated cell sorter (FACS) analysis.

bind to the surface of RAW 264·7 macrophages, cells were incubated with 10 μg of rRv2626c for various times and the bound rRv2626c was investigated using anti-rRv2626c antibody in a FACS analysis. The binding of rRv2626c with macrophages could be seen as early as 5–10 min after the start of incubation, and remained noticeably high until 60 min (Fig. 2). It could be seen (Fig. 2, brown curve) that the binding of rRv2626c to macrophages was inhibited when the cells were incubated with anti-Rv2626c antibody preincubated with rRv2626c. This clearly indicates that rRv2626c binds with high affinity and specificity to the surface of RAW 264·7 macrophages. Similar observations were obtained for phorbol 12-myristate 13-acetate (PMA)-differentiated THP-1 macrophages (data not shown).

### rRv2626c activates NO production by macrophages

Having demonstrated binding of Rv2626c to the surface of murine macrophage cells cultured *in vitro*, the ability of rRv2626c to induce NO production via *de novo* expression of iNOS in the macrophages was assessed. RAW 264·7 macrophages were stimulated with different concentrations of rRv2626c protein (Fig. 3a; bars 3, 4 and 5). Stimulants such as LPS and IFN-$\gamma$ were used as positive controls (Fig. 3a; bar 2) for NO production and iNOS expression (Fig. 3b; lane 2) in RAW 264·7 macrophages. NO production increased in RAW 264·7 macrophages with the addition of rRv2626c in a dose-dependent manner (Fig. 3a; bars 3, 4 and 5). Similar observations were obtained in J774·1 macrophages (data not shown). NO production by the cells was not observed when cells were stimulated with proteinase K-treated rRv2626c protein

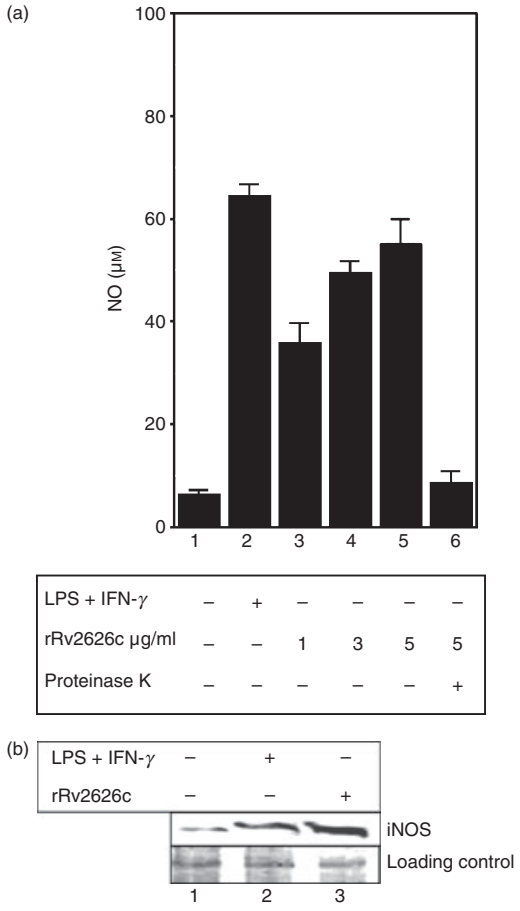


**Figure 3.** Recombinant Rv2626c (rRv2626c) induces nitric oxide (NO)/inducible nitric oxide synthase (iNOS) in murine macrophages. (a) The dose-dependent effect of rRv2626c on NO production in RAW 264.7 macrophages. RAW 264.7 macrophages ($3 \times 10^5$) were treated with either rRv2626c or proteinase K (200 ng/ml) treated rRv2626c for 48 hr and the supernatants were assayed for NO by the Griess reagent assay. A combination of lipopolysaccharide (LPS) (1 μg/ml) and interferon (IFN)-$\gamma$ (1 ng/ml) was used as a positive control. Data represent the mean ± standard deviation (SD) of at least three triplicate experiments. Statistical significance was determined using Student's *t*-test and the effect was found to be significant at $P < 0{\cdot}001$. (b) RAW 264·7 macrophages were stimulated with rRv2626c. The positive control group received LPS plus IFN-$\gamma$. The whole-cell extracts were prepared from these cells to measure iNOS expression by immunoblotting using polyclonal iNOS antibody. Equal loading of protein was confirmed by Ponceau S stain. The blot represents one of the three immunoblots showing similar results.

(Fig. 3a; bar 6), indicating that the NO production was specifically attributable to the presence of rRv2626c and was not a result of endotoxin contamination in the protein preparation. This increased NO production correlated well with the increase in iNOS expression in cells stimulated with rRv2626c (Fig. 3b; lane 3) as compared with the unstimulated group (Fig. 3b; lane 1). This clearly indicates that Rv2626c can stimulate macrophages to produce NO and that this is mediated by iNOS.

  

### rRv2626c-induced NO production is mediated by NF-κB

iNOS expression and NO production are known to be dominantly regulated by the transcription factor NF-κB.[23,40] Therefore, we first checked whether rRv2626c activates the NF-κB transcription factor in macrophages. RAW 264·7 macrophages were either left untreated or treated with rRv2626c (5 μg). The positive control group received LPS plus IFN-γ. Nuclear extracts were prepared from these cells and the expression of NF-κB was mounted using an electrophoretic mobility shift assay. It was observed that stimulation with rRv2626c caused an increase in the intensity of the NF-κB complex *in vitro* compared with the untreated group (Fig. 4a; compare lane 4 with lane 2) suggesting induced expression of NF-κB. A similar increase was apparent in cells stimulated with LPS plus IFN-γ (lane 3) as compared with the control (lane 2). The specificity of the DNA–protein interaction was confirmed by homologous and heterologous competition during the binding reaction. In the presence of a 100-fold molar excess of unlabelled wild-type consensus NF-κB oligonucleotides, the complex completely disappeared (lane 6) but was unaffected even in the presence of a 100-fold molar excess of unlabelled NF-κB mutant oligonucleotides (lane 7) that carried a mutation in the bases critical for NF-κB binding. To conclusively demonstrate the specific involvement of NF-κB, a nuclear extract prepared from RAW 264·7 cells treated with PDTC, a specific inhibitor of this transcription factor,[41–43] was used in the electrophoretic mobility shift assay. PDTC treatment was found to inhibit rRv2626c-induced NF-κB activity (compare lane 5 with lane 4). The levels of nuclear p50 and p65 subunits of NF-κB present in rRv2626c-stimulated macrophages were further confirmed using NF-κB-specific antibody. The immunoblotting results again showed increased nuclear translocation of p50 and p65, indicating that rRv2626c induces NF-κB activity (Fig. 4b; compare lane 3 with lane 1) in macrophages, and this was almost comparable to that induced by LPS plus IFN-γ (lane 2). Treatment with PDTC, as expected, caused a reduction in nuclear translocation of both p50 and p65 subunits of NF-κB (lane 4).

Having shown the direct involvement of NF-κB, we once again assayed for activation of iNOS by western blotting as well as NO production in the presence or absence of PDTC followed by stimulation with rRv2626c. While rRv2626c induced iNOS expression (Fig. 4c; lane 3) comparable to that induced by LPS plus IFN-γ (Fig. 4c; lane 2), treatment with PDTC inhibited rRv2626c-induced iNOS expression (Fig. 4c; compare lane 4 with lane 3). The subsequent production of NO in these experimental groups was measured. Again, it was observed that rRv2626c increased NO production as a function of concentration (Fig. 4d; bars 2, 3 and 4), and NO production was inhibited by PDTC treatment (Fig. 4d; bars 5, 6 and 7) in a concentration-dependent manner. These observations provide direct evidence that rRv2626c induces iNOS expression via signalling pathways associated with NF-κB activation.

### rRv2626c activates the pro-inflammatory response in RAW 264·7 cells

A recent study has shown that DNA vaccination with *Rv2626c* in infected mice increases levels of Th-1 type cytokines such as IFN-γ and IL-2 and cytotoxic activity *in vivo*.[31] Th-1 responses are regulated at the level of IL-12,[44,45] and both IL-12 and TNF-α are protective against TB.[46] We therefore checked whether rRv2626c actually activates macrophages to induce a Th-1 response. TNF-α as well as IL-12 production was measured in macrophages after treatment with different concentrations of rRv2626c protein. The culture supernatants were harvested after 48 hr and TNF-α and IL-12 production was measured by EIA as described previously.[36,39] It was observed that treatment with rRv2626c increased production of TNF-α (Fig. 5a) and IL-12 (Fig. 5b) as a function of protein concentration (Fig. 5a,b; compare bars 3, 4 and 5 with bar 1 in both cases). Treatment with LPS plus IFN-γ (bar 2) was used as a positive control. These results demonstrate that rRv2626c can act as an immunomodulator by activating the pro-inflammatory cytokines.

### rRv2626c promotes Th-1 response in PBMCs derived from patients with active TB disease

Having shown the ability of rRv2626c to act as an immunomodulator using *in vitro* cultured macrophage cell lines (RAW 264·7), we further investigated the immunomodulatory effect of Rv2626c on PBMCs isolated from patients with active TB. This investigation was carried out by quantifying the levels of various Th-1 type cytokines such as IFN-γ (Fig. 6a), TNF-α (Fig. 6b) and IL-12 (Fig. 6c) in an EIA using culture supernatants of PBMCs treated with rRv2626c (5 μg/ml) for 72 hr. It was observed that rRv2626c was able to increase IL-12, TNF-α and IFN-γ secretion in PBMC cultures from TB patients as compared with those from healthy controls (Fig. 6a,b,c). These results clearly demonstrate the involvement of rRv2626c as an immunomodulator when assayed using PBMCs from patients with active TB.

### rRv2626c increases expression of costimulatory molecules

We next examined whether rRv2626c has any role in the modulation of macrophage costimulatory molecules, which are important for the activation of the adaptive immune response. Therefore, RAW 264·7 macrophages

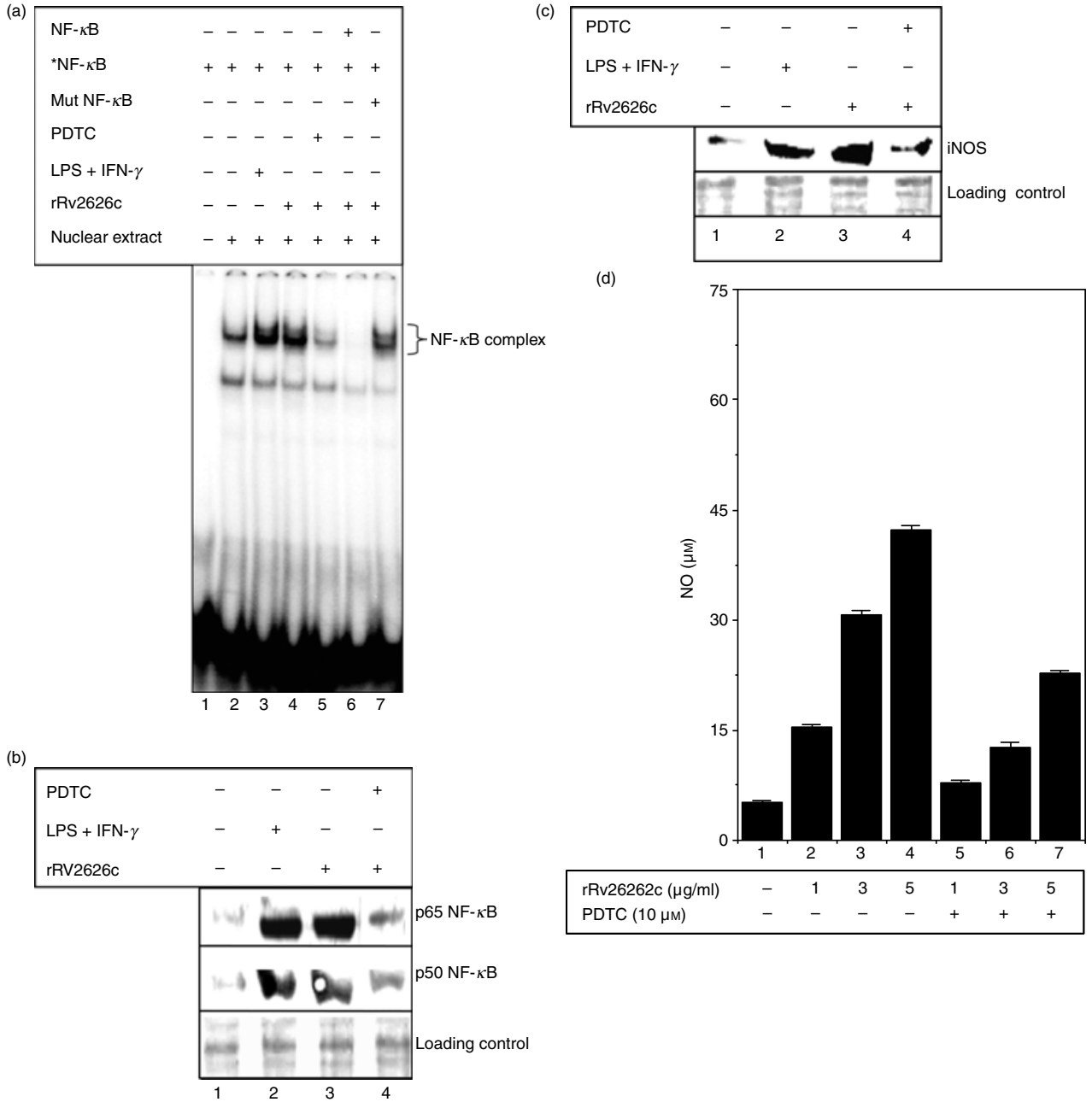


**Figure 4.** Recombinant Rv2626c (rRv2626c)-induced nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) expression is mediated by nuclear factor (NF)-$\kappa$B. (a) RAW 264·7 macrophages were treated with rRv2626c in the absence or presence of pyrrolidine dithiocarbamate (PDTC) (10 μM). The positive control group received lipopolysaccharide (LPS) plus interferon (IFN)-$\gamma$. Nuclear extracts prepared from these cells were used to measure NF-$\kappa$B activation by electrophoretic mobility shift assay (EMSA). (b) In a different experiment, RAW 264.7 macrophages were treated with rRv2626c in the presence or absence of PDTC (10 μM). The positive control group received LPS plus IFN-$\gamma$. Nuclear extracts prepared from these cells were used to examine the nuclear translocation of the p50 and p65 subunits of NF-$\kappa$B by immunoblotting using specific antibody. Ponceau S staining of the membrane confirmed equal loading of the nuclear extract. The blot represents one of three immunoblots showing similar results. (c) In another experiment, RAW 264.7 macrophages were treated with rRv2626c in the presence or absence of PDTC (10 μM). The positive control group received LPS plus IFN-$\gamma$. The whole-cell extracts were prepared from these cells to measure iNOS expression by immunoblotting. Ponceau S staining of the membrane was used as a protein loading control. The blot represents one of three immunoblots showing similar results. (d) RAW 264·7 macrophages were treated with different concentrations of rRv2626c in the presence or absence of PDTC (10 μM). The LPS plus IFN-$\gamma$ treatment served as a positive control. After 48 hr, culture supernatants were harvested to measure NO production by the Griess assay. Data represent the mean ± standard deviation (SD) of three experiments. Statistical significance was determined using Student's *t*-test and the effect was found to be significant at $P < 0{\cdot}001$.

  

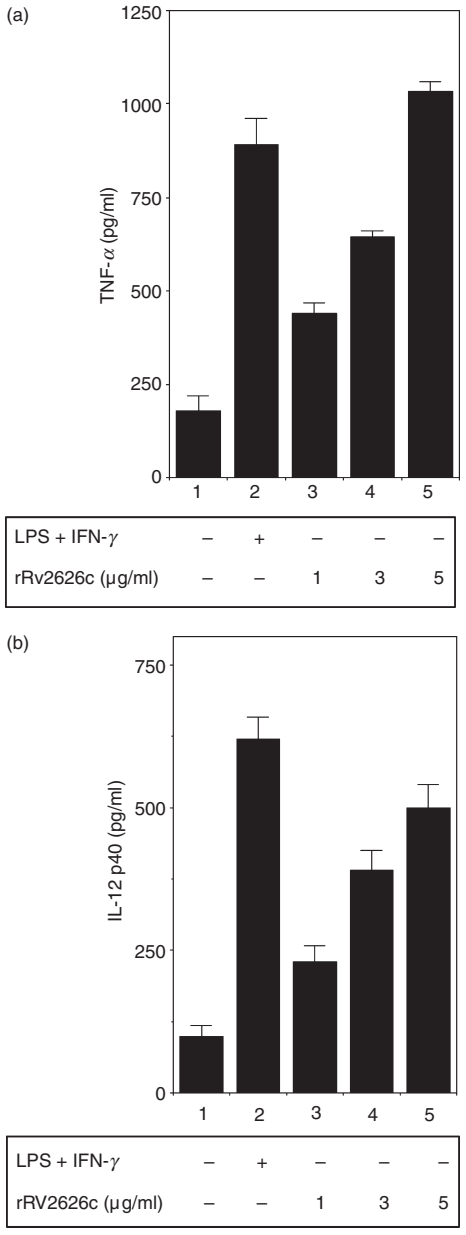


**Figure 5.** Recombinant Rv2626c (rRv2626c) promotes pro-inflammatory cytokine response in RAW 264·7 murine macrophage cells in a dose-dependent manner. RAW 264·7 ($3 \times 10^5$) macrophages were treated with various concentrations of rRv2626c. The positive control group received a combination of lipopolysaccharide (LPS) and interferon (IFN)-$\gamma$. After 48 hr the culture supernatants were harvested and assayed for tumour necrosis factor (TNF)-$\alpha$ (a) and interleukin (IL)-12 (b) levels by enzyme immunoassay (EIA). Data represent the mean ± standard deviation (SD) of three triplicate experiments. Statistical significance was determined using Student's *t*-test and the effect was found to be significant at $P < 0{\cdot}001$.

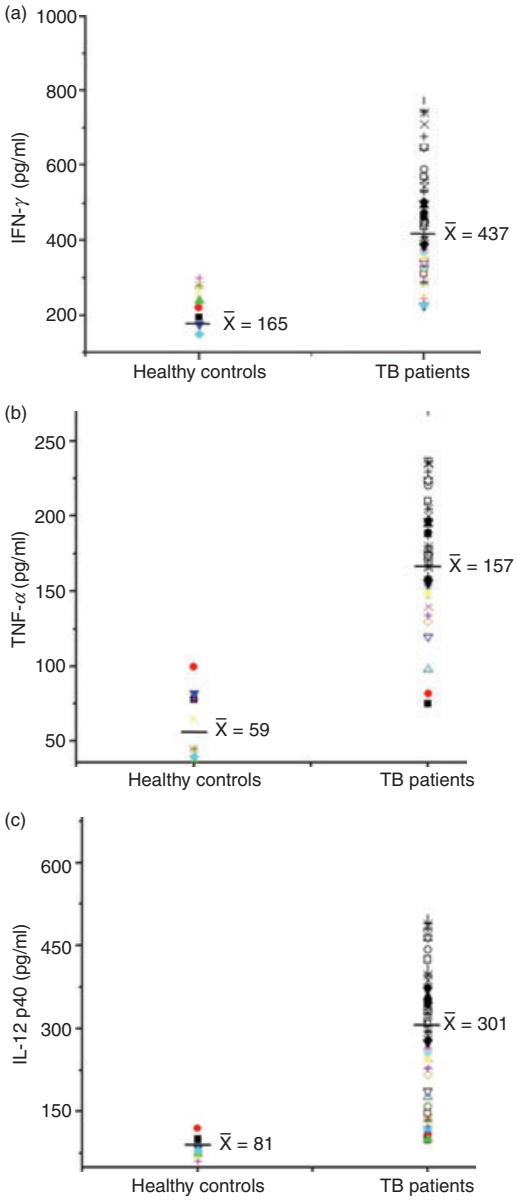


**Figure 6.** Recombinant Rv2626c (rRv2626c) promotes the expression of T helper type 1 (Th-1) cytokines in peripheral blood mononuclear cells (PBMCs) isolated from patients with active tuberculosis (TB) disease. PBMCs ($2 \times 10^5$) from healthy controls ($n = 9$) and patients with TB ($n = 48$) were stimulated with rRv2626c at a concentration of 5 μg/ml for 72 hr. Culture supernatants were collected and the expression of interferon (IFN)-$\gamma$ (a), tumour necrosis factor (TNF)-$\alpha$ (b) and interleukin (IL)-12 (c) was quantified by enzyme immunoassay (EIA). Statistical significance was determined using Student's *t*-test and the effect was found to be significant at $P < 0{\cdot}001$.

were treated with 3 μg/ml rRv2626c protein in the presence or absence of LPS plus IFN-$\gamma$ and the surface expression profiles of various costimulatory molecules were examined after 24 hr by FACS analysis. It was seen that stimulation with rRv2626c alone was able to up-regulate the expression of costimulatory molecules such as B7-1, B7-2 and CD40 (Fig. 7a, b and c) at levels comparable to those induced by LPS plus IFN-$\gamma$. Thus, rRv2626c can influence the antigen-presenting activity of macrophages

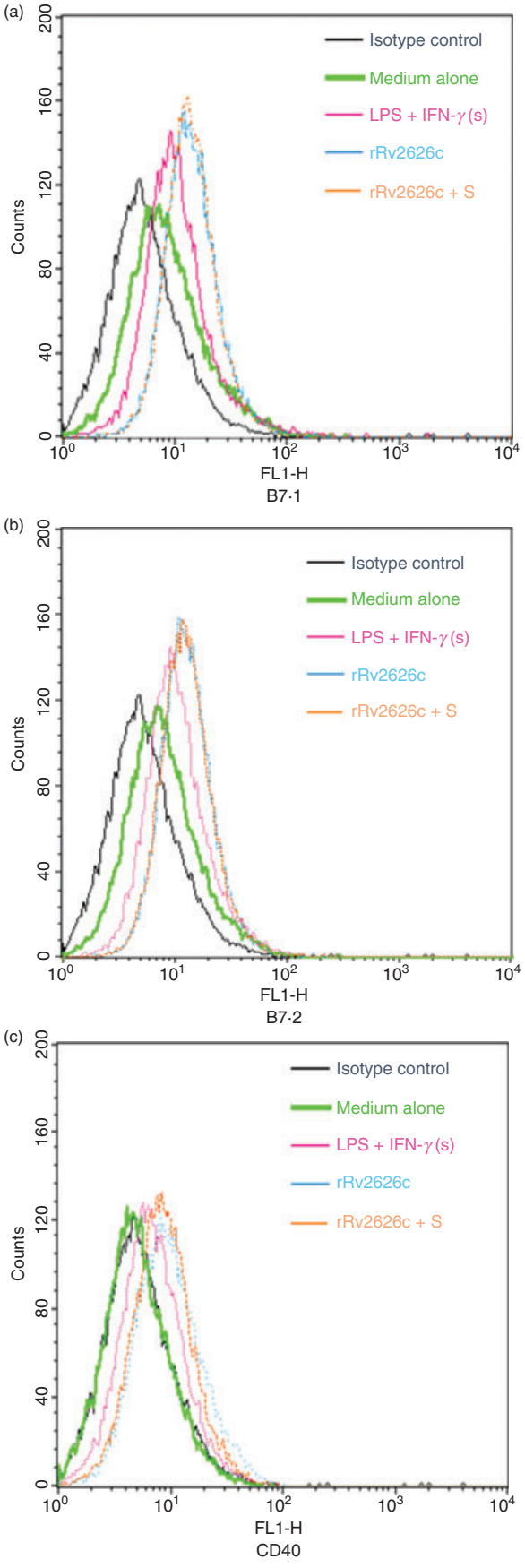


**Figure 7.** Recombinant Rv2626c (rRv2626c) increases the expression of costimulatory molecules such as B7-1, B7-2 and CD40 in RAW 264.7 macrophages. RAW 264·7 macrophages were treated with rRv2626c at 3 μg/ml for 24 hr in the presence or absence of interferon (IFN)-γ and lipopolysaccharide (LPS). Cells were washed, incubated with mouse anti-B7-1 (a), mouse anti-B7-2 (b) and mouse anti-CD40 (c) and then incubated with anti-mouse fluorescein isothiocyanate (FITC) conjugate. The control group received isotype immunoglobulin G2a (IgG2a). This experiment is representative of three experiments showing similar results.

to prime T cells by directly activating the expression of these costimulatory molecules.

## Discussion

Manipulations of the immune systems of mice with neutralizing antibodies or gene knockouts have provided strong evidence that anti-mycobacterial immunity correlates with the Th1 immune response. In TB, macrophages play an important role in controlling the infection by regulating both the innate signalling and the T cell-mediated Th1/Th2 immune response.[47–49] The interaction between T cells and macrophages is known to be critical for prevention of bacterial growth.[50–53] However, it is not clear how various *M. tuberculosis* proteins can trigger the Th1 response. Several factors, such as the affinity between the T-cell receptor (TCR) and peptide–MHC ligand, peptide ligand density and costimulatory signalling during T-cell activation, can play important roles in the regulation of the Th1/Th2 T-cell response.[11,12,54–57] Cytokines induced during innate activation of macrophages have also been shown to be extremely important in controlling the Th1/Th2 balance. For example, induction of IL-12 or TNF-α can trigger a Th1 response;[58,59] however, if more IL-10 is produced, the response is likely to be biased towards the Th2 type response.[60,61] It has been shown that various *M. tuberculosis* secretory proteins bind to a specific receptor on macrophages and influence the downstream signalling cascades and the induction of pro-inflammatory cytokines.[62]

Although up-regulation of iNOS expression and NO production during infection with *M. tuberculosis* is well known, very few studies have actually identified the *M. tuberculosis* proteins directly involved in the up-regulation of the iNOS gene. Our study indicates that rRv2626c affects the macrophage-signalling cascades and up-regulates iNOS induction and NO production mainly by increasing NF-κB activity. Interestingly, flow cytometry data indicate that Rv2626c binds to the macrophage surface with high affinity and specificity. It is possible that the specific binding of Rv2626c on the macrophage surface causes modulation of the downstream signalling pathways triggering NF-κB signalling, which results in increased induction of iNOS[23] as well as the cytokines TNF-α[63] and IL-12.[64] Although the exact beneficial role

  

of iNOS/NO in anti-mycobacterial killing has not been uniformly elucidated,[65] studies have confirmed that iNOS/NO is crucial in limiting bacterial growth.[66,67] Similarly, the role of TNF-α in TB is paradoxical because, although there is evidence of its protective role,[68] it can play a part in the tissue damage that characterizes human disease.[68] A recent study also indicates that *M. tuberculosis* activates TNF-α production to induce apoptosis of macrophages.[62]

Our study clearly demonstrates that the secretory *M. tuberculosis* Rv2626c protein induces pro-inflammatory responses by modulating the expression of iNOS and increasing the secretion of IL-12 and TNF-α, which may play an important role in the initiation of the adaptive immune response in the host. *Mycobacterium tuberculosis* proteins that induce the Th1 response have been used as targets for subunit vaccines. For example, use of the mycobacterial 30-kDa major secretory protein (antigen 85B, Ag85B) was found to protect animals from *M. tuberculosis* infection by inducing a Th1-dominant response.[69] Further, the recombinant BCG vaccine over-expressing Ag85B also induced better protection against TB as compared with the parental BCG strain;[70] however, this was not very successful in a phase 1 clinical trial. The hybrid protein consisting of Mtb39 and Mtb32 (Mtb72F) was also found to be immunogenic and produced an enhanced Th1 response to BCG in mice but failed to reduce the bacterial load in the lungs after an aerosol challenge.[71] Interestingly, the co-administration or boosting of BCG vaccination with Mtb72F conferred protection in both mouse and guinea pig models.[71] Similar to Rv2626c, the Rv1860 of *M. tuberculosis* also elicited both a lymphoproliferative response and IFN-γ production from PBMCs, and the response was found to be different in PPD-positive healthy controls and patients with pulmonary TB;[72] the protein also offered protection in guinea pigs after *M. tuberculosis* challenge.

Rv2626c could also influence macrophage signalling for induction of higher levels of B7·1 and B7·2 and CD-40 costimulatory molecules on the macrophage surface, which may contribute to increased T-cell proliferation, as observed in the *in vitro* T-cell proliferation assay. Priming of T cells by expression of costimulatory molecules, MHC molecules and the necessary cytokines is important for T-cell polarization. Although some secretory proteins of *M. tuberculosis* have been found to increase IL-12 production and induce a pronounced Th1 response,[73,74] to the best of our knowledge, this is the first report showing that Rv2626c can both activate costimulatory signalling and trigger induction of the cytokines IL-12 and IFN-γ. Thus, Rv2626c may be a promising T-cell vaccine candidate. The protective role of the Rv2626c protein was evident from earlier studies showing that immunization of mice with *Rv2626c* gave better protection against the bacilli relative to the control.[31] A detailed understanding of the signalling pathway exploited by this protein will therefore be helpful in designing better therapeutics against *M. tuberculosis.*

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## Disclosures

The authors declare that there is no conflict of interest.

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