Original Article

Characterization of Immune Responses to *Mycobacterium tuberculosis* Rv2041c Protein

Su-Young Kim[1], A-Rum Shin[2,4], Byung-Soo Lee[2,4], Hwa-Jung Kim[2], Bo Young Jeon[5],
Sang-Nae Cho[5], Jeong-Kyu Park[2,3*] and Sung Jae Shin[2,4†]

[1]Division of Pulmonary and Critical Care Medicine, Department of Medicine, Samsung Medical Center,
Sungkyunkwan University School of Medicine, Seoul, Korea; [2]Department of Microbiology,
[3]Cancer Research Institute, [4]Infectious Signaling Network Research Center, College of Medicine,
Chungnam National University, Daejeon, Korea; [5]Department of Microbiology and Brain
Korea 21 Project for Medical Science, Yonsei Univeristy College of Medicine, Seoul, Korea

Tuberculosis, which is caused by *Mycobacterium tuberculosis* (*M. tb*), is one of the most important infectious diseases in the world. Although many functional studies have been conducted on *M. tb* proteins in the post-genomic era, little is known about the function of many proteins expressed specifically during latency. Previously, we reported that *Rv2041c* from *M. tb* H37Rv is highly expressed under conditions of low pH and hypoxia, which represent the *in vitro* mimicry of latent tuberculosis. In the present study, increased expression levels of *Rv2041c* under hypoxia and low pH *in vitro* culture was confirmed by RT-PCR. Interestingly, *Rv2041c* showed significantly increased expression among genes of the same operon and genes belonging to the same functional group. Finally, the immune responses elicited by the recombinant (r) Rv2041c protein were investigated using *ex vivo* and *in vivo* models of *M. tb* infection. A significantly high level of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-12p40 was detected in a dose-dependent manner by treatment of murine bone marrow-derived macrophages with rRv2041c protein. In addition, IFN-γ and TNF-α secretion increased after stimulation with purified Rv2041c protein to lymphocytes from latent and active TB mice in a modified Cornell model. In conclusion, our findings suggest that Rv2041c is a new T-cell antigen and could be a potential vaccine candidate against *M. tb* infection by inducing a strong cellular immune response.

Key Words: Rv2041c, *M. tuberculosis*, T-cell antigen, Immune response, Vaccine candidate

INTRODUCTION

Tuberculosis (TB) causes more than 2 million deaths

every year (1). The ability of *Mycobacterium tuberculosis* (*M. tb*) to persist in the host for long periods of time causes long-term disease (2). The chronic nature of the disease is likely due to the bacterium needing to adapt to a continually changing host environment, severely complicating any attempts to identify better therapeutics or diagnostics for this disease (3).

Although drug-susceptible TB can be treated successfully with a 6-month regimen involving three of four drugs (WHO DOTS strategy, >95% cure rate), the combined challenges of timely diagnosis, socioeconomic factors in TB endemic areas, and the fact that bacterial clearance requires many months of treatment have prevented the successful global control of TB (4). The only current Bacillus

Calmette-Guérin (BCG) vaccine, the most widely used in the world, is not completely protective. BCG is ineffective in individuals pre-sensitized by exposure to environmental mycobacteria prior to vaccination or *M. tb* infection (5, 6). In addition, BCG is a live vaccine and the development of protective immunity after vaccination appears to require BCG replication in the host, which can be prevented by a pre-existing immune response that can cross-react with BCG (7). Therefore, a novel and efficacious vaccine to replace BCG is needed urgently to control and eliminate TB.

Progress in mycobacterial proteomics, functional genomics, and recombinant biotechnology to produce and purify mycobacterial proteins might lead to the development of vaccines from putative candidates (8). However, little is known about the actual functions of many proteins from *M. tb* or the proteins induced in the host post-infection. For this reason, many studies have been conducted to find the proteins expressed specifically during *M. tb* infection using *in vitro* dormancy, *ex vivo* macrophage, and *in vivo* animal models. In the screening of antigens for the development of a TB vaccine or diagnosis, IFN-γ, and TNF-α inducing proteins are also very considerable candidates.

Several studies have reported on the genes and proteins that are highly expressed in macrophages and in the *in vitro* dormancy state to discover vaccine candidates (9~11). However, the contribution of these proteins to host immune responses must be evaluated following *ex vivo* and *in vivo* *M. tb* infection models.

In a previous study, the *Rv2041c* of *M. tb* H37Rv was found (by differential expression using a customized amplification library [DECAL]) to be specifically induced under acidic and hypoxic conditions similar to those in a phagocytosed environment (12), and may be important for survival in the host. Currently, the host immune response to Rv2041c of *M. tb* H37Rv is not completely elucidated.

Rv2041c is thought to be a sugar-binding lipoprotein component of the ATP-binding cassette (ABC) sugar transport system. In total, 38 ABC transporter proteins have been identified in *M. tb*, only four of which are assigned for a role in carbohydrate import (13). The four identified sugar importers are encoded by genes clustered in the

following operons: *Rv2041c-Rv2040c-Rv2039c-Rv2038c*, *ugpA-ugpE-ugpB-ugpC*, *sugC-sugB-sugA-lpqY*, and *uspA-uspE-uspC*. These consist of membrane spanning domains (MSDs), cytoplasmic nucleotide binding domains (NBDs), and extra-cytoplasmic substrate binding protein (SBP). Each of these four transporters is composed of one SBP (Rv2041c, UgpB, LpqY, or UspC).

This SBP is a membrane-bound lipoprotein and may act as an antigenic membrane-surface component in interactions with the host cell. Nearly half of the annotated *M. tb* lipoproteins do not share conserved domains with proteins outside of the genus *Mycobacterium* and thus represent proteins unique to mycobacteria (14). Experimental data concerning mycobacterial lipoprotein function are rare.

Key immune factors controlling tuberculosis and the reactivation of infection include T cells and macrophages. After phagocytosis of *M. tb* by macrophages, activated macrophages induce IL-12 and reactive nitrogen intermediates production and expression of costimulatory molecules. This, in turn, leads to the activation of T and NK cells and IFN-γ and TNF-α production, thus linking the innate and adaptive immune responses, augmenting the microbicidal activity of the phagocytes. TNF-α and IFN-γ play critical roles in protective immunity against mycobacterial infection and immunopathology (15). Therefore, the identification of mycobacterial antigens that preferentially induce these cytokines is critical to the development of new anti-TB vaccines.

The goal of this study was to investigate the significant induction of Rv2041c transcripts in *M. tb* growing *in vitro* and to assess the immune responses by cytokine production in macrophage infection as well as IFN-γ and TNF-α increase in primed lymphocytes from *M. tb*-infected mouse. The improved knowledge of immunoreactive Rv2041c properties may be conducive to the development of an efficacious anti-tuberculosis vaccine.

MATERIALS AND METHODS

Plasmid and vector construction

The cloning vector plasmid pGEM®-T Easy and

expression vector pET-28a were purchased from Promega (Madison, WI, USA) and Novagen (Madison, WI, USA), respectively. The plasmid pGEM®-T Easy and pET-28a contained an ampicillin and a kanamycin resistance marker, respectively. The expression vector contained a T7 promoter and a N-terminal 6× His-tag coding sequence.

Expression and purification of the recombinant Rv2041c protein

The *Rv2041c* was amplified by PCR from *M. tb* H37Rv genomic DNA and then cloned into the pET-28a. The correct construct was transformed into competent *Escherichia coli* BL21 cells. For the expression of the Rv2041c, transformed *E. coli* BL21 cells were grown overnight at 37℃ in Luria-Bertani (LB) medium containing 30 μg/ml kanamycin (Sigma, St. Louis, MO, USA). The culture was then inoculated into LB medium containing the same concentration of kanamycin and grown at 37℃ until the optical density at 600 nm (OD_{600}) reached to 0.6. Expression of the protein was induced by adding 1 mM isopropyl β-D-thiogalactoside (IPTG; Bioneer, Daejeon, Korea), and the culture was grown at 25℃ for an additional 4 h. For preparative purification, induced *E. coli* BL21 cells from a 1-L culture grown under optimum conditions were harvested by centrifugation at 4000 g for 20 min at 4℃. The cell pellet was stored at -20℃.

The recombinant (r) Rv2041c protein was extracted after cell disruption by sonication. The rRv2041c containing N-terminal histidine tag was purified using Ni-nitrilotriacetic acid (NTA) resin (Qiagen, Chatsworth, CA, USA). The rRv2041c was dialyzed five times in 10 mM phosphate buffered saline (PBS, pH 7.2) using a Slide-A-Lyzer Dialysis Cassette with a 3 kDa molecular weight cut-off (Pierce, Rockford, IL, USA). After dialysis, endotoxin contamination was removed using Detoxi-Gel Affinity Pak Columns (Pierce). The rRv2041c protein was incubated with endotoxin removal resin for overnight to remove LPS and concentrated by Centricon (2,000 MW cut-off; Millipore, Billerica, MA, USA). In addition, endotoxin was assayed under endotoxin-free experimental conditions using a *Limulus* amebocyte lysate pyrogen kit (Biowhittaker,

Walkersville, MD, USA). The quantity of endotoxin in the rRv2041c was detected ≤0.01 ng/mg. The final concentration of purified rRv2041c protein was determined using a BCA protein assay kit (Pierce). The dialyzed protein was analyzed by SDS-PAGE with Coomassie brilliant blue staining.

Cultivation of *M. tuberculosis* H37Rv under limited culture conditions

M. tb H37Rv was grown in minimal Sauton's medium as surface pellicles as previously described (16). The medium was then removed by filtration, and the bacterial cells were transferred to normal (pH 7.2) or mildly acidic (pH 6.0) 7H9 medium and cultured static for 8 days at 37℃ in a CO_2 incubator set at 21% or 13% O_2, or with 0% O_2 in an anaerobic jar (Oxoid, Cambridge, UK) containing anaerogen (Oxoid) and an anaerobic indicator (Oxoid); normal condition (pH 7.2 and 21% O_2), normal pH with hypoxia (pH 7.2 and 13% O_2), normal pH with anoxia (pH 7.2 and 0% O_2), mildly acidic pH with hypoxia (pH 6.0 and 13% O_2), and mildly acidic pH with anoxia (pH 6.0 and 0% O_2). The acidic media were buffered with 100 mM morpholine-propanesulfonic acid (MOPS; Sigma) and adjusted to pH 6.0.

Extraction of RNA from *M. tuberculosis* H37Rv

Total RNA from *M. tb* H37Rv was isolated using a catrimox-14 RNA isolation kit (Takara, Shiga, Japan) according to the manufacturer's instructions, with a protocol as described previously (17). Bacterial cells were pelleted, resuspended in catrimox-14, and sonicated on ice. The cell lysate was vortexed for 1 min and left to allow micelle formation. The lysate was then centrifuged. The pellet was resuspended in guanidium solution (4 M guanidium isothiocyanate, 0.2 M sodium acetate, pH 4.0), and subjected to phenol/chloroform/isoamyl alcohol extraction. The aqueous layer was precipitated in isopropanol, washed with 70% ethanol, and air dried. The RNA pellet was resuspended in DEPC-treated water, and assessed by gel electrophoresis.

Table 1. Primers used in RT-PCR

Gene		Primer sequence	Product size
rpoB	sense	5'-CGAGTGCAAAGACAAGGACATG-3'	486 bp
	anti-sense	5'-CGCAGCTTGCGGTAGATGTC-3'	
lpqY	sense	5'-GATTTGGTAAATAGCCCGCC-3'	255 bp
	anti-sense	5'-AGCCACCGATTTGAGGATCT-3'	
uspC	sense	5'-AGTGGCCGGCTAATGAAGAT-3'	191 bp
	anti-sense	5'-CTCCATCGCAAGTTGTCCAC-3'	
ugpB	sense	5'-GTCAACGCGCTCAAGTTCAT-3'	190 bp
	anti-sense	5'-CGTAGTCTTGTGTCCGGGTG-3'	
Rv2038c	sense	5'-ACGGTGTCACTGGGTGATTG-3'	206 bp
	anti-sense	5'-ATTGACTGGTCATTTCGCA-3'	
Rv2039c	sense	5'-CGCGATACGTTGTTCGTCTT-3'	255 bp
	anti-sense	5'-CAGCAGAATCCGCCAGTAGA-3'	
Rv2040c	sense	5'-TTATCGTCAGCCTCGTCGTC-3'	205 bp
	anti-sense	5'-GGTTCGATTAGCCATGGGAT-3'	
Rv2041c	sense	5'-TATCCCGATATCAAGGTACG-3'	233 bp
	anti-sense	5'-GTGAAAGTCTCATACAGGGC-3'	

Semi-quantitative RT-PCR

The first-strand cDNA was obtained from 1 μg total RNA with reverse transcriptase and random primer at 42℃ for 60 min. Reactions were stopped by heat inactivation for 5 min at 95℃ and chilled on ice. Initially RT-PCR was carried out using primers for amplifying *rpoB* to optimize the cDNA concentration. The PCR was carried out using 1 μl of single-stranded cDNA for 30 cycles of 1 min denaturing at 95℃, 1 min annealing at 51℃, and 1 min extension at 72℃. Sequences of the specific oligonucleotide primers used for PCR amplification and the size of the predicted PCR products are shown in Table 1. The PCR products were analyzed with 1% agarose gel electrophoresis and the intensity of each band was calculated using Quantity One software (Version 4.1.0; Bio-Rad, Hercules, CA, USA). All samples were compared to normal culture (oxygen pressure of 21%, pH 7.2; condition 1) and the gene expression level of this condition corresponds to 1. The results were expressed as the fold induction relative to the control value of normal condition.

Cytokine production in murine BMDMs by stimulation with Rv2041c protein

To determine the responses in macrophages by the stimulation with rRv2041c, murine bone marrow-derived macrophages (BMDMs) were used for *ex vivo* macrophage *M. tuberculosis* infection. Specific pathogen-free female C57BL/6 mice (Japan SLC, Shijuoka, Japan) at 5~6 weeks of age were used for the isolation of BMDMs. BMDMs were differentiated for 6 days in M-CSF-containing media, as described previously (18, 19). The culture medium consisted of DMEM that was supplemented with 20% L929 cell-conditioned medium (as a source of M-CSF), 10% heat-inactivated FBS, 1 mM sodium pyruvate, 50 U/ml penicillin, 50 μg/ml streptomycin, and 5×10^{-5} M 2-mercaptoethanol. The prepared BMDMs were cultured in DMEM medium in the presence of diverse concentration (0.5, 1, 5 μg/ml) of rRv2041c and 1 μg/ml of LPS (as a positive control; Sigma) for 18 h. Non-treated cells were used as a negative control. This experiment was conducted in triplicate and repeated 3 times. Culture supernatants were
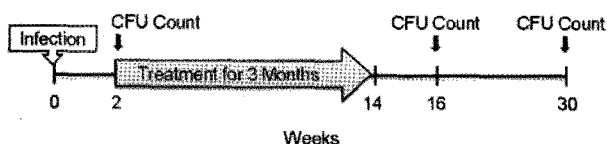
Figure 1. Experimental schedules for the animal models of latent and active TB. Mice were exposed to *M. tb* H37Rv in the inhalation chamber of an airborne infection apparatus. Bacteria were counted 2 weeks after exposure. Mice were then treated with INH and PZA for 3 months, starting 2 weeks after aerosol infection. Bacterial counts were obtained from lung and spleen of mice at 2 and 16 weeks after the completion of 3 months of chemotherapy. The active TB mice in the Cornell model were obtained by natural reactivation.

then collected and frozen at -80℃ until ELISA analysis.

Animal model in latent and active TB

Specific pathogen-free female C57BL/6 mice at 5~6 weeks of age were purchased from Japan SLC, Inc. and maintained under barrier conditions in a BL-3 biohazard animal room at Yonsei University Medical Research Center. The animals were fed on a sterile commercial mouse diet and provided with water *ad libitum*. All animal experiments were done according to the regulation of Institutional Animal Care and Use Committee, Yonsei University Health System.

The Cornell model described by McCune *et al.* was employed with minor modification (20, 21) (Fig. 1). Briefly, mice were exposed to a predetermined dose (low-dose; 50 CFU) of *M. tb* H37Rv for 60 min in the inhalation chamber of an airborne infection apparatus (Glas-Col, Terre Haute, IN, USA). Bacteria were counted 2 weeks after exposure. Mice were then treated with INH at 25 mg/kg/day and PZA at 1000 mg/kg/day, in diet for 3 months, starting 2 weeks after aerosol infection. Bacterial counts were taken from the lungs and spleens of mice at 2 weeks after the completion of 3-month chemotherapy to confirm the clearance of cultivable organisms. At 18 weeks post-infection (2 weeks after completion of the 3-month treatment with INH and PZA), viable *M. tb* in the lungs and spleens of mice was not found by culture on laboratory media. Bacteria were counted 30 weeks after infection, and approximately 75 and 50 viable *M. tb* were found to have been delivered to the lungs and spleens, respectively. At this point of time,

this established infection model represents latent infection. The active TB mice in the modified Cornell model were obtained by naturally reactivation.

Preparation of lymphocytes from spleens in latent and active TB mice and stimulation with Rv2041c protein

At 30 weeks post-infection and naturally reactivation after the completion of 3-month chemotherapy, mice were euthanized with CO_2, and spleens were removed aseptically. Spleens were homogenized in 0.04% Tween 80 saline and lymphocytes were prepared from tissue suspension. Erythrocytes were lysed with a lysis buffer (155 mM ammonium chloride and 10 mM potassium bicarbonate), and cells were washed. Splenocytes were plated at 2×10^5 cells per well and cultured in RPMI medium in the presence of the purified Rv2041c and concanavalin A (Con A; Sigma) at a concentration of 1 μg/ml for 1 or 4 days. The non-treated and Con A-treated cells were served as a negative and a positive control, respectively. Culture supernatants were then collected and frozen at -80℃ until ELISA analysis.

Determination of cytokine concentration by ELISA

Cytokine production (IFN-γ, TNF-α, IL-6 and IL-12p40) was measured with commercial mouse enzyme-linked immunosorbent assay (ELISA) kits (BD Pharmingen, San Diego, CA, USA) following the procedure provided by the manufacturer. Cytokine concentrations in the samples were calculated using standard curves generated from recombinant cytokines, and the results were expressed in picograms per milliliter.

RESULTS

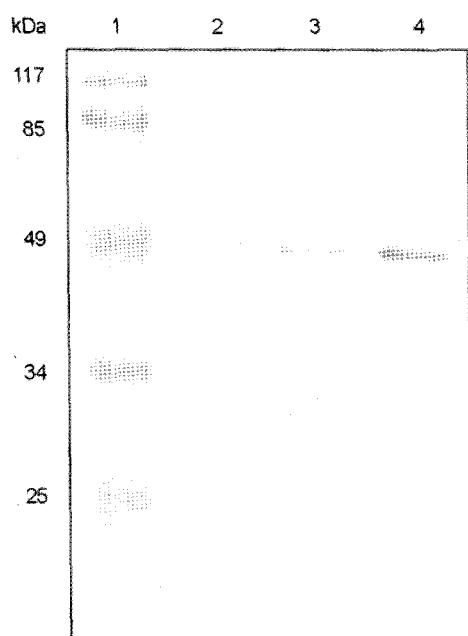### Purification of the rRv2041c protein

The rRv2041c protein containing a C-terminal histidine tag was purified using Ni-NTA resin. The purified protein displayed the expected molecular mass by SDS-PAGE analysis (Kim *et al.*, submitted). The purified protein was dialyzed against PBS to remove the salts, followed by SDS-PAGE analysis to confirm that the dialyzed protein remained intact (Fig. 2). The small sized peptide bands are

most probably derived from the degradation of recombinant protein. For subsequent experiments using rRv2041c protein, macrophage viability was tested by post-treatment with rRv2041c protein. There were no statistically significant differences in the percentage of dead cells in murine BMDM cultures exposed up to 500 μg/ml rRv2041c protein when cell viability was assessed using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). This indicates that our rRv204c protein is not cytotoxic to BMDMs and does not contain significant amounts of endotoxin that would interfere at concentrations below 500 μg/ml. Finally, the protein concentration was determined and the purified rRv2041c protein was used in the subsequent experiments.
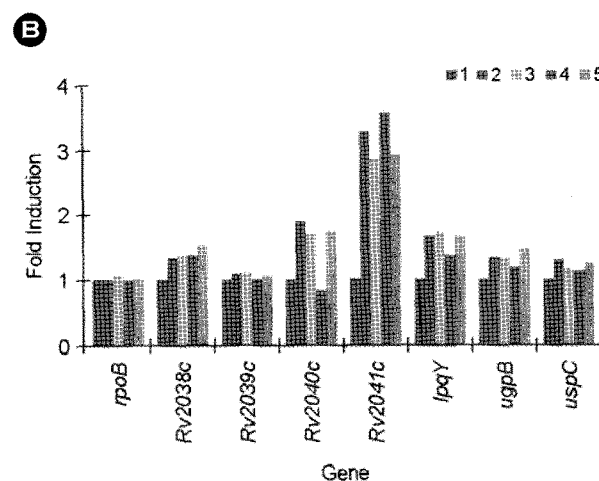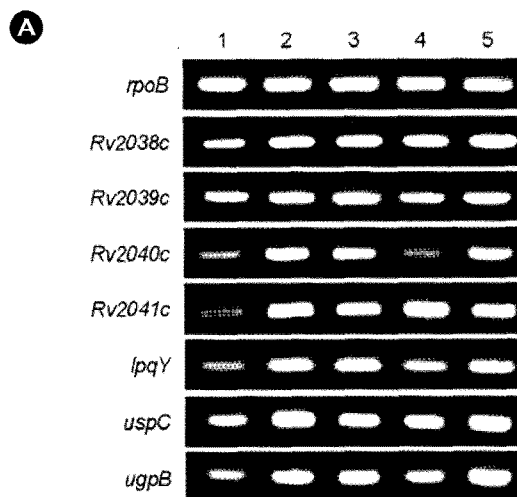
Confirmation of the expression of Rv2041c at the transcriptional level in vitro by semi quantitative RT-PCR

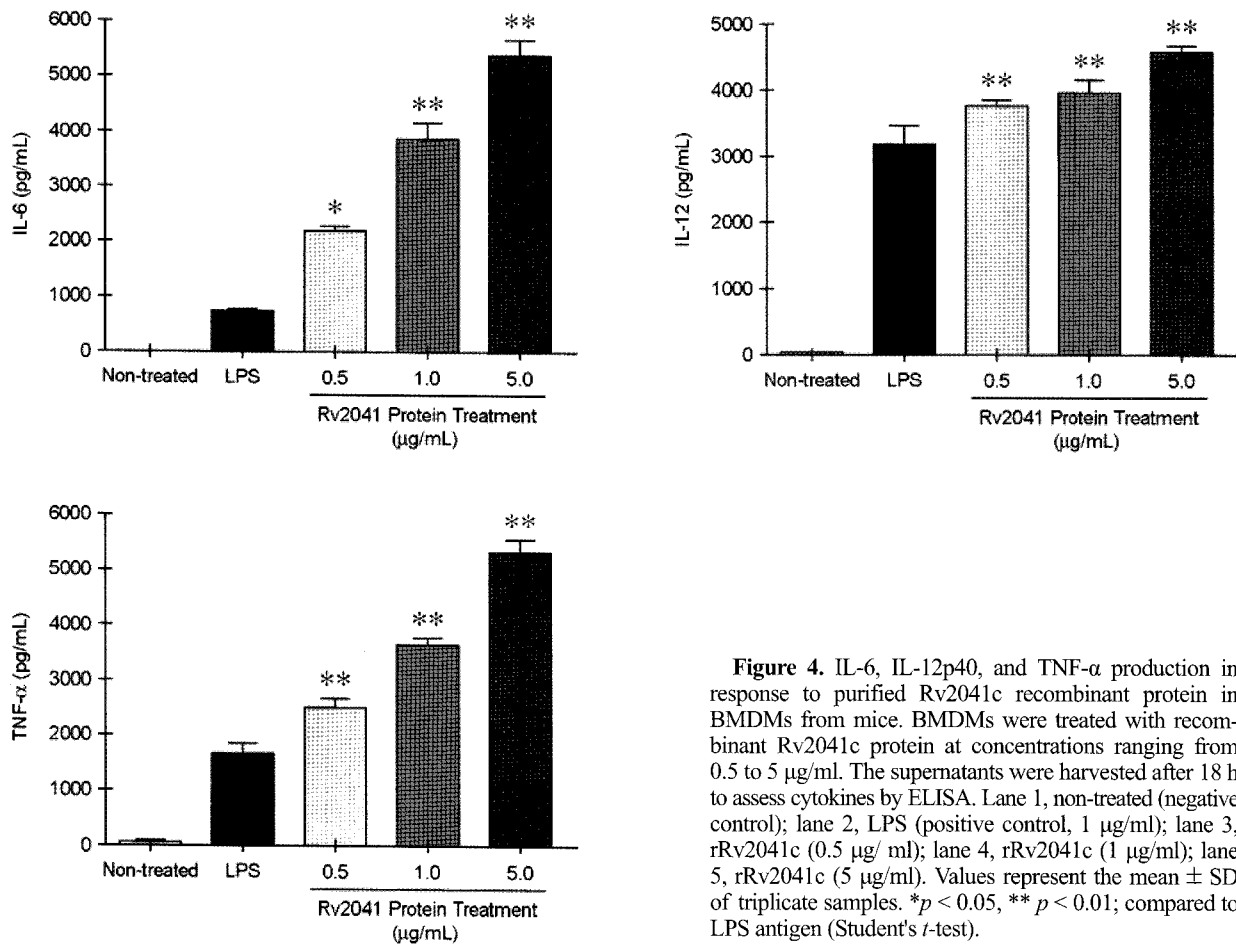The Rv2041c of M. tb H37Rv was found (using differential expression and a customized amplification library [DECAL]) to be specifically induced under acidic and hypoxic conditions. The transcriptional expression level of Rv2041c was compared to that of genes in the same operon and to genes belonging to the same functional group under the culture conditions described in MATERIALS AND METHODS. To determine Rv2041c expression at the transcriptional level at different conditions, mRNA of M. tb H37Rv cultured in normal pH (7.2) or mildly acidic (6.0)

Figure 3. RT-PCR analysis of Rv2038c, Rv2039c, Rv2040c, Rv2041c, lpqY, ugpB, and uspC in M. tb H37Rv cultured under various conditions. The transcription level of each gene from M. tb cultured at normal (pH 7.2) or acidic (pH 6.0) with hypoxic or anoxic conditions was measured by semi-quantitative RT-PCR (A). Based on the intensity of the bands, the results are expressed as fold-induction relative to the value for normal culture conditions (B). Lane 1, normal conditions (pH 7.2 and 21% oxygen tension); lane 2, hypoxic condition (pH 7.2 and 13% oxygen tension); lane 3, anoxic condition (pH 7.2 and 0% oxygen tension); lane 4, mildly acidic and hypoxic condition (pH 6.0 and 13% oxygen tension); lane 5, mildly acidic and anoxic condition (pH 6.0 and 0% oxygen tension).

Figure 2. SDS-PAGE analysis of dialyzed Rv2041c protein. Purified Rv2041c fusion protein was dialyzed against PBS and then resolved by 12% SDS-PAGE. The protein bands were visualized by staining with Coomassie brilliant blue. Lane 1 is a molecular weight marker (kDa). Lanes 2, 3, and 4 represent 2, 4, and 6 μg dialyzed recombinant Rv2041c protein, respectively.

Figure 4. IL-6, IL-12p40, and TNF-α production in response to purified Rv2041c recombinant protein in BMDMs from mice. BMDMs were treated with recombinant Rv2041c protein at concentrations ranging from 0.5 to 5 μg/ml. The supernatants were harvested after 18 h to assess cytokines by ELISA. Lane 1, non-treated (negative control); lane 2, LPS (positive control, 1 μg/ml); lane 3, rRv2041c (0.5 μg/ ml); lane 4, rRv2041c (1 μg/ml); lane 5, rRv2041c (5 μg/ml). Values represent the mean ± SD of triplicate samples. $*p < 0.05$, $** p < 0.01$; compared to LPS antigen (Student's t-test).

media with hypoxic or anaerobic conditions was measured by semi-quantitative RT-PCR. Based on the intensity of the bands, the results were expressed as a fold-induction relative to the value for normal culture conditions (pH 7.2, 21% oxygen tension). The results depicted in Fig. 3 revealed that four genes of the *Rv2038c ~ Rv2041c* operon (*Rv2038c*, *Rv2039c*, *Rv2040c*, and *Rv2041c*) were expressed during the lifecycle of *M. tb*. When altered by oxygen tension or pH, the transcripts of *Rv2041c* increased significantly.

Also, other sugar-binding protein genes such as *lpqY*, *ugpB*, and *uspC* were expressed during the lifecycle of *M. tb* (Fig. 3). When altered by oxygen tension or pH, the transcripts of *Rv2041c* increased; however, the transcripts of other sugar-binding protein genes showed no significant change.

Cytokine production in murine BMDMs in response to Rv2041c protein

Because macrophages play pivotal roles in mycobacterial infections by secreting pro-inflammatory cytokines to activate a variety of immune effector cells, cytokine production by stimulation with rRv2041c in macrophages was exploited.

The production of proinflammatory cytokines such as TNF-α, IL-6, and IL-12 during *M. tb* infection is essential for the activation of effector cells for innate resistance (22~24). When BMDMs from mice were treated with varying concentrations (0.5, 1, 5 μg/ml) of purified rRv2041c for 18 h, the secretion of TNF-α, IL-6, and IL-12p40 in the culture medium increased significantly compared to those treated with 1 μg/ml LPS as a positive control (Fig. 4). Significant differences ($p < 0.05$; $p < 0.01$) compared to

LPS are depicted in Fig. 4.

IFN-γ and TNF-α production in response to Rv2041c in latent and active TB mice

Vaccination against *M. tb* has been shown to require activated Th1 cells that induce the expression of Th1-type cytokines (e.g., IL-12, IFN-γ, TNF-α). IFN-γ and TNF-α are important macrophage activators produced by T cells when exposed to microbial products derived from *M. tb* (23, 25). To analyze the cell-mediated responses of latent and active TB to the Rv2041c protein, lymphocytes of spleen from infected mice in a Cornell model were stimulated with purified rRv2041c protein, and then IFN-γ,

and TNF-α production was measured by ELISA. A peak of IFN-γ and TNF-α secretion was observed after 4 and 1 day(s) of stimulation, respectively. Thus, IFN-γ and TNF-α concentration between latent and active TB mice were compared at 1 and 4 days. IFN-γ responses at 4 days in both latent and active TB mice exceeded the responses from Con A as a positive control (Fig. 5; $p < 0.01$). As shown in Fig. 5A and B, the spleen cells in active TB produced much higher levels of IFN-γ than did the splenocytes in latent TB. And, TNF-α production at day 1 was also significantly induced by rRv2041c protein in latent and active TB mice (Fig. 5; $p < 0.01$). Similar to IFN-γ secretion, splenocytes in active TB produced much higher levels of TNF-α than
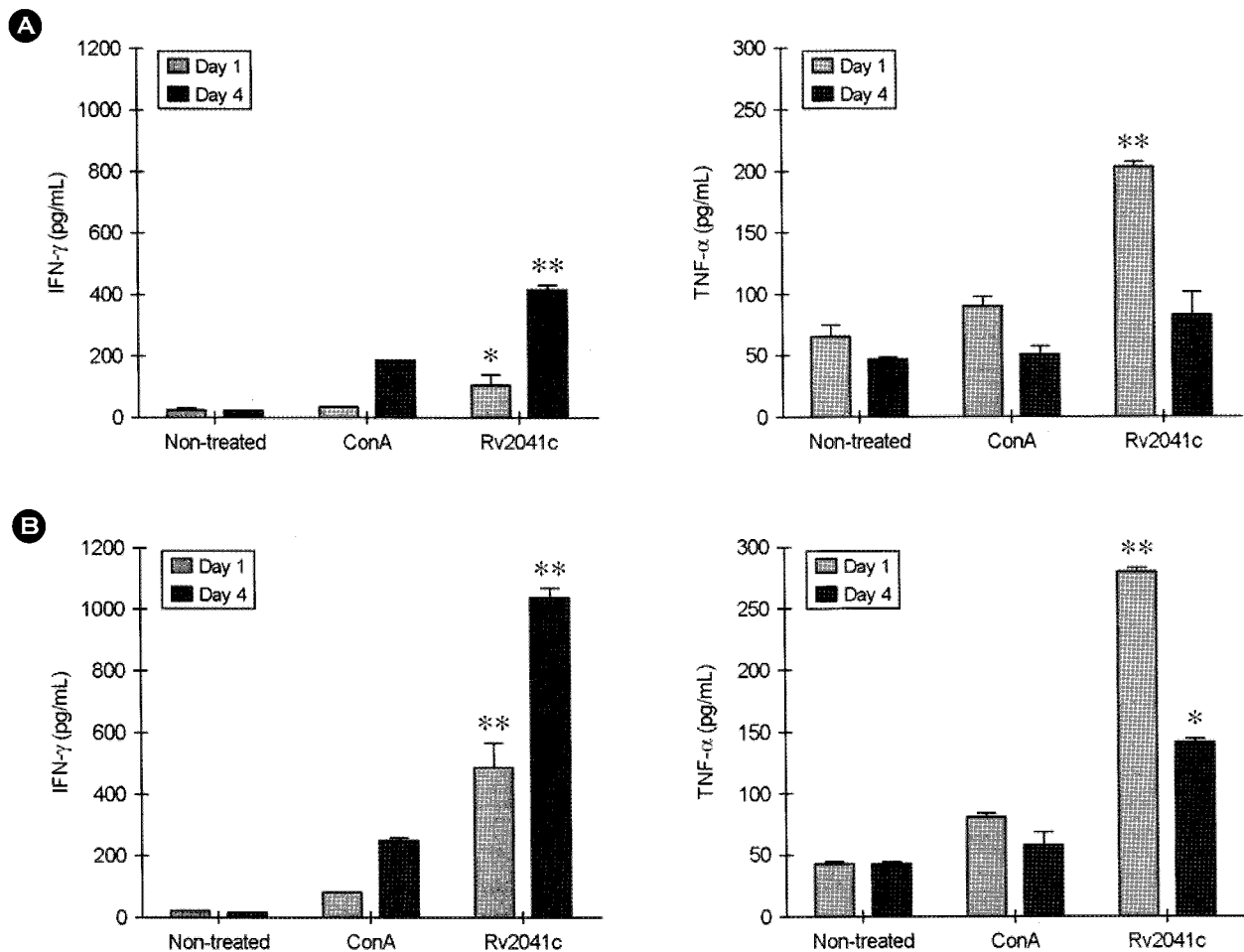
Figure 5. IFN-γ and TNF-α production in response to purified Rv2041c recombinant protein in a latent and active TB model. The recombinant Rv2041c protein or Con A was used to treat lymphocytes of spleen from latent (**A**) and active (**B**) TB mice at a concentration of 10 μg/ml. The supernatants were harvested after 1 (gray bar) or 4 days (black bar) to assess cytokines by ELISA. Non-treated and Con A-treated cells were used as negative and positive controls, respectively. Values represent the mean ± SD of triplicate samples. * $p < 0.05$, ** $p < 0.01$; compared to the Con A antigen (Student's *t*-test).

did splenocytes in latent TB. Therefore, the Rv2041c protein is a strong stimulator of IFN-γ and TNF-α production.

DISCUSSION

Mycobacterial lipoproteins are crucial for synthesizing the unique mycobacterial cell envelope, the sensing of and protection from environmental stress, and participation in host-pathogen interactions (26). Due to their contribution to virulence, lipoproteins and the enzymes of the lipoprotein synthesis pathway represent promising drug targets. In addition, some lipoproteins confer a protective immune response and thus may qualify as subunit vaccines.

In a previous study, we verified the up-regulation of *Rv2041c* under hypoxic conditions and mildly acidic pH in *M. tb* by DECAL. *Rv2041c* is expressed weakly *in vitro* under normal culture conditions (pH 7.2 and 21% O_2 pressure); however, a high level of Rv2041c expression was induced when *M. tb* was cultivated at low pH under hypoxic conditions that mimic *in vitro* inside macrophages (12). The purpose of this study was to investigate the significant induction of Rv2041c transcripts in *M. tb* growing *in vitro*, as well as assess its immunostimulatory capabilities in macrophages and *in vivo* TB model.

First, we successfully expressed and purified the recombinant Rv2041c protein in an *E. coli* system. The availability of sufficient amounts of Rv2041c is an essential part of studying the immunological characters of the protein.

Next, we confirmed that *Rv2041c* was induced under mild acidic and hypoxic conditions (in contrast to other genes included in the *Rv2038 ~ Rv2041* operon, which is an ABC transporter for an undetermined sugar) and that the other three SBP genes were involved in the sugar transporter of *M. tb* by semi-quantitative RT-PCR. As shown in Fig. 3, *Rv2041c* was up-regulated significantly. A possible role for *Rv2041c* is that it may be important for the maintenance of intracellular survival during infection (12).

The rRv2041c induced pro-inflammatory cytokines such as IL-6 and IL-12p40 in murine BMDMs in a dose-dependent manner. It has been known that mycobacterial lipoproteins recognized by TLR2 on macrophages can

activate innate immune responses such as TNF-α and IL-12 production (27, 28). IL-6 has been associated with protection against *M. tb* with a role in granuloma maintenance. It is clear that the maintenance of granulomas requires antigen-specific T cells (22). IL-6 acts as a regulator of the T cell responses that are critical for the maintenance of granulomas, especially during infection. IL-6 was higher in TB patients than in healthy controls (29). In addition, IL-12 has been shown to be crucial in the development of protective immunity against TB, and in the maintenance of latency. Persistent IL-12 production is necessary for the maintenance of Th1-dependent host resistance to *M. tb* (24). This is important in vaccine design.

Rv2041c protein can increase IL-6 and IL-12p40 secretions in macrophages, suggesting that Rv2041c protein can induce the protective immune response. The proinflammatory cytokines are essential to the activation of the anti-mycobacterial activity of macrophages and T lymphocytes. Therefore, rRv2041c might be used as a vaccine or diagnostic candidate antigen.

In the present study, we also demonstrated increased IFN-γ and TNF-α production in rRv2041c stimulated splenocytes from active TB mice compared to latent TB mice. IFN-γ and TNF-α response by splenocytes from *M. tb*-infected mice exceeded the responses from the Con A control. Therefore, rRv2041c acts as a stimulator of IFN-γ and TNF-α production. It is well established that Th1 cytokines, in particular IFN-γ and TNF-α, are important in the control of *M. tb* infection (23, 25). IFN-γ produced by T cells and NK cells stimulates a mycobactericidal response in macrophages characterized by the induction of nitric oxide synthase (NOS). In synergy with IFN-γ, TNF-α activates macrophages to kill intracellularly replicating *M. tb* (30). During the early stages of mycobacterial infection, macrophages and T cells actively interact to control the infection. TB patients generally had higher production of cytokines such as IFN-γ and TNF-α than controls and this was observed in both of the CD4+ and CD8+ T cell subsets (31). Recent evidence suggests that TB disease is associated with an increase in proportion of T lymphocytes capable of producing TNF-α in combination with IFN-γ. It seems

reasonable that IFN-γ and TNF-α was more elevated in active TB mice compared to latent TB mice in this study. Our data indicate that an increased pattern of cytokine production against TB antigen between latent and active TB may be associated with an increased proportion of IFN-γ and TNF-α producing cells in active TB disease progression.

Proteins are covalently modified with lipids via multiple mechanisms in the cytoplasm or on the cytoplasmic face of membranes and protein lipidation affects the function and location of proteins (32, 33). The rRv2041c protein was amplified from E. coli. The lipidation of rRv2041c protein may be different from protein produced from M. tb. Maybe the innate immune responses induced by non-lipidated protein can be different from lipidated protein.

In summary, the Rv2041c protein is thought to be important for the maintenance of intracellular survival during infection (12). In addition, rRv2041c induces a host protective immune response with ex vivo stimulation of mouse. The rational development of an effective subunit vaccine requires the identification of candidate antigens of M. tb and a protective immune response in the host-pathogen interaction. We identified Rv2041c of M. tb for intracellular survival in a previous study and have characterized the immunoreactivity of Rv2041c in this study. Therefore, Rv2041c may be a candidate anti-TB vaccine in the future.

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