Mycobacterium tuberculosis Rv0577, a novel TLR2 agonist, induces maturation of dendritic cells and drives Th1 immune response

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ABSTRACT **Tuberculosis (TB) caused by *Mycobacterium tuberculosis* constitutes an ongoing threat to global health. An antigen that can induce dendritic cell (DC) maturation and lead to enhanced cellular immunity is crucial to the development of an effective TB vaccine. Here, we investigated the functional roles and the related signaling mechanism of the Rv0577 protein, a *M. tuberculosis* complex-restricted secreted protein involved in the methylglyoxal detoxification pathway. Rv0577 recognizes Toll-like receptor 2 (TLR2) and functionally induces DC maturation by augmenting the expression of cell surface molecules (CD80, CD86, and MHC class I and II) and proinflammatory cytokine production (TNF-α, IL-1β, IL-6, and IL-12p70) in DCs on MyD88-dependent signaling, mitogen-activated protein kinases, and nuclear factor κB signaling pathways. In addition, Rv0577-treated DCs activated naive T cells, effectively polarized $CD4^+$ and $CD8^+$ T cells to secrete IFN-γ and IL-2, and induced T-cell proliferation, indicating that this protein possibly contributes to Th1-polarization of the immune response. More important, unlike LPS, Rv0577-treated DCs specifically induced the proliferation of memory $CD4^+/CD8^+CD44^{high}$ $CD62L^{low}$ T cells in the spleen of *M. tuberculosis*-infected mice in a TLR2-dependent manner. Taken together, these findings suggest that Rv0577 may regulate innate and adaptive immunity by interacting with TLR2, a finding that could be helpful in the design of new TB vaccines.—Byun, E.-H., Kim, W. S., Kim, J.-S., Jung, I. D., Park, Y.-M., Kim, H.-J., Cho, S.-N., Shin, S. J. *Mycobacterium tuberculosis* Rv0577, a novel TLR2 agonist, induces maturation of dendritic cells and drives Th1 immune response. *FASEB J.* 26, 2695–2711 (2012). www.fasebj.org**

Key Words: Toll-like receptor · tuberculosis · surface molecules · proinflammatory cytokines · memory T cell

Mycobacterium tuberculosis causes tuberculosis (TB) and is one of the most successful intracellular pathogens in humans, with approximately one-third of the world's population infected (1). The host immune response has been found to play a critical role in both control of and protective immunity against the pathogen by targeting antigens (Ags) secreted by *M. tuberculosis* (2). Consequently, identification and characterization of *M. tuberculosis* Ags is essential to understanding host–pathogen interactions and facilitating the development of prospective vaccine candidates (2, 3).

Dendritic cells (DCs) are potent antigen-presenting cells (APCs) that play a pivotal role in controlling immune responses to *M. tuberculosis* (4). Many studies regarding the function of DCs on *M. tuberculosis* infection have mainly focused on the ability of these cells by interacting with *M. tuberculosis* or its Ags to express major histocompatibility complex (MHC) class II and costimulatory molecules, including CD80 and CD86,

Abbreviations: Ag, antigen; APC, antigen-presenting cell; BMDC, bone marrow-derived dendritic cell; BSA, bovine serum albumin; CFSE, carboxyfluorescein diacetate succinimidyl ester; DC, dendritic cell; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte-macrophage colony-stimulating factor; IFN-γ, interferon-γ; IgG, immunoglobulin G; IκB-α, inhibitory κB-α; IL, interleukin; LAL, *Limulus* amoebocyte lysate; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PE, phycoerythrin; PmB, polymyxin B; PRR, pattern recognition receptor; SDS-PAGE, sulfate-polyacrylamide gel electrophoresis; TB, tuberculosis; TCR, T-cell receptor; TIR, Toll/interleukin-1 receptor homology; TLR, Toll-like receptor; TNF-α, tumor necrosis factor-α; TRIF, TIR domain containing adapter inducing interferon-β; WT, wild type

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which induce T cell proliferation (5, 6). Immature DCs (iDCs) have the ability to capture and internalize Ags *via* pattern recognition receptors (PRRs), recognizing conserved molecular moieties that distinguish a broad specificity of microbial products, the so-called pathogen-associated molecular patterns (PAMPs) (6). On PAMP ligation by PRRs, activation of appropriate immune responses to TB can be initiated to maintain well-regulated immunological homeostasis (7). Among various PRRs, Toll-like receptors (TLRs) play a crucial role in the activation of the cellular immune response against *M. tuberculosis* infection (8). Recognition of microbial components by TLRs plays a central role in whether the immune system will respond to a particular microbial infection (8). The intracellular signal transduction activated *via* TLRs is initially mediated through the Toll/interleukin-1 receptor homology (TIR) domains. Activation of signaling through TIR domains results in recruitment of the adaptor molecule myeloid differentiation factor 88 (MyD88) and/or TIR domain containing adapter inducing interferon-β (TRIF), ultimately leads to activation of mitogen-activated protein kinases (MAPKs) and nuclear factor κB (NF-κB) (8). This signaling process enables DCs to become potent APCs to initiate robust innate immunity by up-regulating the expression of surface molecules including CD80, CD86, and MHC class I and II and a number of cytokines such as interleukin (IL)-1β, IL-6, IL-12, and tumor necrosis factor-α (TNF-α), and losing endocytic/phagocytic receptors (6). Moreover, these mature DCs migrate to draining lymph nodes where they present these Ags and stimulate naive T lymphocytes to initiate an immune response (6). As described above, the maturation of DCs may be particularly important for the elicitation of both primary and secondary immune responses to pathogens. Recently, several studies have reported that *M. tuberculosis* Ags induced the maturation and activation of DCs in a TLR2-dependent manner. For example, *M. tuberculosis* Ags, such as Rv1196, Rv0978c, and Rv0754, recognize TLR2 and induce maturation and activation of DCs (9–11). In addition, DCs matured by *M. tuberculosis* Ags relocate to the lymph nodes, where they produce proinflammatory cytokines and the development of an adaptive immune response that includes helper ($CD4^+$) T cells, cytotoxic ($CD8^+$) T cells, γδ T cells, and the production of interferon-γ (IFN-γ) and TNF-α (key cytokines in immunity to TB; refs. 12, 13).

M. tuberculosis Rv0577 (also known as CFP32 and TB27.3) is a complex-restricted secreted protein absent from environmental mycobacteria but expressed during the course of infection (14). Notably, *M. tuberculosis* clinical isolates appear to express more Rv0577 than laboratory-adapted *M. tuberculosis* strains and other *M. tuberculosis* complex subspecies (including the *Mycobacterium bovis* BCG strain; ref. 14). Recently, CFP32 was recognized as the enzymatic mediator of *M. tuberculosis*-specific neutral red dye cytochemical staining, a classic test used to differentiate virulent *M. tuberculosis* from nontuberculous mycobacteria (15). Moreover, native Rv0577 has been detected in the sputum of *M. tuberculosis*-infected individuals and may be useful as a possible diagnostic potential (14). Furthermore, a recent study has suggested that Rv0577 is associated with the methylglyoxal detoxification pathway as a potential glyoxylase (16), which suggests that it is a candidate target of pyrimidine-imidazoles. Although comparative analyses suggest that the *rv0577* gene product may be important to the pathogenesis of *M. tuberculosis* and a biological function for Rv0577 has been identified, the immunological function of this protein has not been elucidated, especially its role in innate and adaptive immunity. In the present study, we investigated the molecular basis of DC maturation and functional changes induced by a novel Rv0577 protein.

MATERIALS AND METHODS

Animals

Specific pathogen-free female C57BL/6 ($H\text{-}2K^b$ and $I\text{-}A^b$), BALB/c ($H\text{-}2K^d$ and $I\text{-}A^d$), C57BL/6J TLR2-knockout mice ($TLR2^{-/-}$; B6.129-$Tlr2^{tm1Kir}$/J), C57BL/10 TLR4 knockout mice ($TLR4^{-/-}$; C57BL/10ScNJ), C57BL/6 OT-1 and OT-2 T-cell receptor (TCR) transgenic mice, at 5-6 wk of age were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and maintained under barrier conditions in a biohazard animal room at the Medical Research Center, Chungnam National University. The animals were fed a sterile commercial mouse diet and provided with water ad *libitum*. The animal experiments complied with the ethical and experiment regulations for animal care at Chungnam National University.

Animal infection study was performed in a BL-3 biohazard animal facility at Yonsei University Medical Research Center. Briefly, 6-wk-old BALB/c mice were exposed to a predetermined dose of *M. tuberculosis* H37Rv (ATCC27294) for 60 min in the inhalation chamber of an airborne infection apparatus (Glas-Col, Terre Haute, IN, USA) (17). Bacteria were counted 1 d after exposure, and ~200 viable *M. tuberculosis* were found to have been delivered to the lungs. At 8 wk postinfection, 5–6 mice were euthanized, and their spleens were collected to conduct the subsequent experiments, including mixed lymphocyte reactions (MLRs). All infection-related experiments were done according to the regulation of the Yonsei University Health System Institutional Animal Care and Use Committee.

Abs and reagents

Recombinant mouse granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin-4 (IL-4), and the fluorescein isothiocyanate (FITC)-annexin V/propidium iodide kit were purchased from R&D Systems (Minneapolis, MN, USA). Dextran-FITC (molecular mass, 40,000 Da) was obtained from Sigma (St. Louis, MO, USA). Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 and palmitoyl-3-Cys-Ser-(Lys)4 (Pam3) were purchased from Sigma and Invitrogen (San Diego, CA, USA), respectively. An endotoxin filter (END-X) and an endotoxin removal resin (END-X B15) were acquired from Associates of Cape Cod (East Falmouth, MA, USA). Peptron (Daejeon, South Korea)synthesized the OT-I peptide ($OVA_{257–264}$) and OT-II peptide ($OVA_{323–339}$). Anti-phosphorylated ERK1/2 monoclonal Ab, anti-ERK1/2 poly-

clonal Ab, anti-phosphorylated JNK monoclonal Ab, anti-JNK polyclonal Ab, anti-phosphorylated p38 monoclonal Ab, anti-p38 polyclonal Ab, anti-NF-κB (p65) polyclonal Ab, anti-phosphorylated inhibitory κB-α (IκB-α) monoclonal Ab, anti-IκB-α monoclonal Ab, and anti-lamin B polyclonal Ab were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). HRP-conjugated anti-mouse immunoglobulin G (IgG) Ab and HRP-conjugated anti-rabbit Ab were obtained from Calbiochem (San Diego, CA, USA), and anti-β-actin mAb (AC-15) was purchased from Sigma. FITC-conjugated mAb to CD11c, CD62L, and IFN-γ; APC-conjugated mAb to IL-12p70; Alexa647-conjugated mAb to CCR3; PerCP-cy5.5-conjugated mAb to $CD4^+$ and $CD8^+$; phycoerythrin (PE)-conjugated mAb to IL-4, CD80, CD86, MHC class I, MHC class II, CD44, and CXCR3; PE-cy5-conjugated mAb to $CD4^+$ and $CD8^+$; and PE-cy7-conjugated mAb to IL-2 were purchased from eBioscience (San Diego, CA, USA). IL-6, IL-1β, TNF-α, IL-2, IL-4, and IFN-γ enzyme-linked immunosorbent assay (ELISA) kits were obtained from eBioscience, and IL-12p70 and IL-10 ELISA kit were obtained from BD Biosciences (San Diego, CA, USA).

Expression and purification of recombinant Rv0577

To produce recombinant Rv0577 protein, the corresponding gene was amplified by PCR using *M. tuberculosis* H37Rv ATCC27294 genomic DNA as template and the following primers: forward, 5′-GGGCCCCATATGCCCAAGAGAAGC-GAATACAGC-3′, and reverse, 5′-GGGCCCAAGCTTCTATT-GCTGCGGTGCGGG-3′. The product of *rv0577* was cut with *Nde*I and *Hind*III. Both the products were inserted into pET28a (+) vector (Novagen, Madison, WI, USA), and the resultants were sequenced. The recombinant plasmids containing *rv0577* were transformed into *E. coli* BL21 cells by heat shock for 1 min at 42°C. The overexpressed Rv0577 was prepared with slight modifications, as described previously (18). Briefly, *E. coli* containing recombinant plasmid were grown at 37°C until the optical density (OD) at 600 nm was 0.4 to 0.5 and then induced with 1 mM isopropyl-D-thiogalactopyranoside (IPTG; ELPIS-Biotech, Daejeon, South Korea). The bacterial cells were then harvested by centrifugation and suspended in 20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 20 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride (Sigma); and lysed by sonication. The recombinant Rv0577 was purified by nickel-nitrilotriacetic acid (Ni-NTA) agarose chromatography in accordance with the manufacturer's instructions (Qiagen, Chatsworth, CA, USA). Each purification step was analyzed by 13.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie brilliant blue stain and immunoblot using anti-His antibodies (Santa Cruz Biotechnology). The purified protein was pooled, concentrated, and dialyzed against phosphate-buffered saline (PBS; pH 7.4). To remove endotoxin contamination, the dialyzed recombinant protein was incubated with polymyxin B (PmB)-agarose (Sigma) for 6 h at 4°C. Lastly, purified endotoxin-free recombinant protein was filter-sterilized and frozen at −70°C. The protein concentration was estimated with the bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as the standard. Residual LPS in the Rv0577 preparation was determined using the *Limulus* amoebocyte lysate (LAL) test (Lonza, Basel, Switzerland), according to the manufacturer's instructions. The purity of Rv0577 was evaluated by Coomassie blue staining, silver nitrate staining, and Western blot using an anti-histidine antibody. In addition, the purity of Rv0577 was quantified by Quantity One software (Bio-Rad, Hercules, CA, USA) and calculated by dividing the intensity per square millimeter of the Rv0577-specific band by that of all the protein bands in the preparation lane.

Generation of polyclonal antibody to Rv0577

The polyclonal antibodies against Rv0577 were generated in BALB/c mice by s.c. injection of 1 mg purified Rv0577 proteins emulsified with equal volume of incomplete Freund's adjuvant (Sigma). The first immunization was carried out with incomplete Freund's adjuvant followed by 2 booster immunizations with the same procedure at 10-d intervals. The antibody titers in the serum were determined by ELISA after 2 wk postfinal immunization.

Generation and culture of DCs

Bone marrow cells isolated from C57BL/6 mice were lysed with red blood cell (RBC) lysing buffer (ammonium chloride, 4.15 g/500 ml, and 0.01 M Tris-HCl buffer, pH 7.5±2) and washed with RPMI 1640 medium. Obtained cells were plated in 6-well culture plates (10^6 cells/ml, 3 ml/well) and cultured at 37°C in the presence of 5% CO_2 using RPMI 1640 medium supplemented with 100 U/ml penicillin/streptomycin (Lonza), 10% fetal bovine serum (Lonza), 50 μM mercaptoethanol (Lonza), 0.1 mM nonessential amino acid (Lonza), 1 mM sodium pyruvate (Sigma), 20 ng/ml GM-CSF, and 20 ng/ml IL-4. On d 6 or 7 of culture, nonadherent cells and loosely adherent proliferating DC aggregates were harvested for analysis or stimulation, or in some experiments they were replated into 60-mm dishes (10^6 cells/ml, 5 ml/dish). On d 6, >80% of the nonadherent cells expressed CD11c. To obtain highly purified populations for subsequent analyses, the DCs were labeled with bead-conjugated anti-CD11c mAb (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by positive selection on paramagnetic columns (LS columns; Miltenyi Biotec) according to the manufacturer's instructions. The purity of the cell fraction selected was >95%.

Cytotoxicity analysis

The Rv0577 (10 μg/ml) was added to cultures of isolated bone marrow-derived DCs (BMDCs) in 12-well plates (0.5×10^6 cells/ml). To investigate the cytotoxic effect of Rv0577 on BMDCs, the cell-death pattern of BMDCs was analyzed after treatment with 10 μg/ml Rv0577. After 24 h of treatment, harvested DCs were washed with PBS and stained by FITC-annexin V and propidium iodide (BD Biosciences). Thereafter, cytotoxicity of DCs was analyzed by FACSCanto flow cytometry (BD Biosciences).

Measurement of cytokines

A sandwich ELISA was used for detecting IL-6, IL-1β, TNF-α, IFN-γ, IL-4, IL-2, IL-12p70, and IL-10 in culture supernatants, as described previously (19). Assays of cytokines in culture medium were performed as recommended by the manufacturers (eBioscience and BD Biosciences). The levels of cytokines released into culture medium were determined by measuring absorbance at 450 nm with a microplate reader. Cytokine concentrations in the samples were calculated using standard curves generated from recombinant cytokines, and the results were expressed in picograms per milliliter.

Intracellular cytokine assays

Cells were first blocked with 10% (v/v) normal goat serum for 15 min at 4°C and then stained with FITC-conjugated $CD11c^+$ antibody for 30 min at 4°C. Cells stained with the appropriate isotype-matched Ig were used as negative controls. The cells were fixed and permeabilized with a Cytofix/

Cytoperm kit (BD Biosciences) used according to the manufacturer's instructions. Intracellular IL-12p70, IL-10, IL-2, IL-4, and IFN-γ were detected with fluorescein-conjugated antibodies (BD Biosciences) in a permeation buffer. The cells were analyzed with a flow cytometry using the CellQuest (BD Biosciences) program.

Analysis of the expression of surface molecules by flow cytometry

On d 6, BMDCs were harvested, washed with PBS, and resuspended in fluorescence-activated cell sorter washing buffer (2% FBS and 0.1% sodium azide in PBS). The cells were preincubated with 0.5% BSA in PBS for 30 min and washing with PBS. The cells were stained with PE-conjugated anti-H-2Kb (MHC class I), anti-I-Ab (MHC class II), anti-CD80, and anti-CD86 along with FITC-conjugated anti-CD11c for 45 min at 4°C. Cells were washed 3 times with PBS and resuspended in 500 μl PBS. The fluorescence was measured by flow cytometry, and the data were analyzed using CellQuest data analysis software.

Ag uptake ability of BMDCs by Rv0577

BMDCs (2×10^5 cell) were equilibrated at 37°C or 4°C for 45 min and then pulsed with fluorescein-conjugated dextran at a concentration of 1 mg/ml. Cold staining buffer was added to stop the reaction. The cells were washed three times, stained with PE-conjugated anti-CD11c antibodies, and then analyzed with the FACSCanto. Nonspecific binding of dextran to DCs was determined by incubation of DCs with FITC-conjugated dextran at 4°C, and the resulting background value was subtracted from the specific binding values.

Confirmation of LPS-decontamination for Rv0577

To confirm the maturation of DCs induced by Rv0577 was not due to contaminating endotoxins or LPS in the protein preparations, the pretreatment with PmB (Sigma), heat-denaturation, and digestion of proteinase K (Sigma) assays were performed. DCs were preincubated with 50 μg/ml PmB for 1 h at room temperature prior to treating with 100 ng/ml LPS and 10 μg/ml Rv0577. For heat denaturation, LPS or Rv0577 was incubated at 100°C for 1 h. For digestion of proteinase K, LPS or Rv0577 was digested for 1 h at 37°C with soluble proteinase K at the concentration of 10 μg/ml, followed by heating for 15 min at 100°C to deactivate the enzyme, and subsequently was added to BMDC cultures. After 24 h, TNF-α and IL-6 levels in the supernatant of BMDCs were analyzed using ELISA.

Confocal laser scanning microscopy

DCs were plated overnight on poly-L-lysine-coated glass coverslips. After treatment with Rv0577, cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and then blocked with 2% BSA in PBS containing 0.1% Tween-20 (PBS/T) for 2 h before being incubated with 2% BSA in PBS/T containing anti-Rv0577 antibody for 2 h at room temperature. After washing with PBS/T, the cells were reincubated with Cy-3 conjugated secondary antibody in the dark room for 1 h, and then were stained with 1 μg/ml of DAPI for 10 min at room temperature. Cell morphology and fluorescence intensity were observed using a confocal laser scanning microscope (Zeiss LSM510 Meta; Carl Zeiss Ltd, Welwyn Garden City, UK). Images were acquired using the LSM510 Meta software and processed using the LSM image examiner.

Immunoprecipitation

DCs (1×10^7) were incubated with 10 μg/ml Rv0577 for 6 h, and cell pellets were lysed with lysis buffer (10 mM Tris-HCl, pH 7.4; 1% Nonidet P-40; 0.25% sodium deoxycolate; 150 mM NaCl; 1 mM EDTA; 1 mM PMSF; 1 μg/ml each aprotinin, leupeptin, and pepstatin; 1 mM Na_3VO_4; and 1 mM NaF). To avoid nonspecific binding, the cell lysates were precleared by adding 50 μl of normal serum (Santa Cruz Biotechnology) and 100 μl of 50% protein A or G sepharose bead slurry (Invitrogen) to 1 mg of cell lysates. After the 2 h incubation at 4°C, the mixture of bead and cell lysates was centrifuged at 10,000 *g* for 5 min at 4°C, and the supernatant was collected for the subsequent experiment. Rv0577 (His)-, TLR2-, and TLR4-associated proteins were immunoprecipitated by incubation with protein A or G Sepharose for 24 h at 4°C after incubation with anti-rat IgG as control Ab for anti-TLR2 and anti-TLR4, anti-mouse IgG as control Ab for anti-Rv0577 (His) for 1 h at 4°C. The beads were harvested, washed, and boiled in 5× sample buffer for 5 min. The proteins were separated on 10% SDS-PAGE followed by transfer of proteins to polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membranes were further probed with anti-TLR2, anti-TLR4, and anti-His Abs as indicated.

Immunoblotting analysis

After stimulation with 10 μg/ml Rv0577, DCs were lysed in 100 μl lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton-X100, 1 mM EDTA, 50 mM NaF, 30 mM Na_4PO_7, 1 mM phenylmethanesulfonyl fluoride, 2 μg/ml aprotinin, and 1 mM pervanadate. Whole-cell lysate samples were resolved on SDS-polyacrylamide gels and then transferred onto a nitrocellulose membrane. The membranes were blocked in 5% skim milk and incubated with the antibody for 2 h, followed by incubation with HRP-conjugated secondary Abs for 1 h at room temperature. Epitopes on target proteins including MAPKs and NF-κB recognized specifically by Abs were visualized by using the ECL advance kit (GE Healthcare, Little Chalfont, UK).

Nuclear extract preparation

Nuclear extracts from cells were prepared as follows. DCs were treated with 100 μl lysis buffer [10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.5% Nonidet P-40; 1 mM dithioreitol (DTT); and 0.5 mM PMSF] on ice for 10 min. Postcentrifugation at 4000 rpm for 5 min, the pellet was resuspended in 100 μl extraction buffer (20 mM HEPES, pH 7.9; 400 mM NaCl; 1 mM EDTA; 1 mM DTT; and 1 mM PMSF) and incubated on ice for 30 min. Postcentrifugation at 12,000 rpm for 10 min, the supernatant containing nuclear extracts was collected and stored at −80°C until required.

Treatment of DCs with pharmacological inhibitors of signaling pathways

All the pharmacological inhibitors were purchased from Calbiochem. Dimethyl sulfoxide (DMSO; Sigma) was added to cultures at 0.1% (v/v) as a solvent control. DCs were washed with PBS and pretreated with inhibitors in RPMI 1640 medium containing glutamine for 1 h prior to treatment with Rv0577 for 24 h. Inhibitors were used at following concentrations: U0126, 10 μM; SB203580, 20 μM; SP600125, 10 μM; and Bay11-7082, 20 μM. In all experiments with inhibitors, a tested concentration was used after careful titration experiments assessing the viability of the DCs using MTT assay.

MLR

Responder T cells, which participate in naive T-cell reactions, were isolated using a MACS column (Miltenyi Biotec) from total mononuclear cells prepared from BALB/c mice. Both OVA-specific $CD8^+$ and $CD4^+$ T cells, responders, were obtained from splenocytes of OT-1 and OT-2 mice, respectively. These T cells were stained with 1 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen) as described previously (20). DCs (2×10^5 cells/well) treated with OVA peptide in the presence of 10 μg/ml of Rv0577 for 24 h were cocultured with CFSE-stained $CD8^+$ and $CD4^+$ T cells (2×10^6) at DC:T-cell ratios of 1:10. On 3 or 4 d of coculture, T cells were stained with PerCP-cy5.5-conjugated anti-$CD4^+$ mAb, PE-cy5-conjugated anti-$CD4^+$ mAb, PE-cy5-conjugated anti-$CD8^+$ mAb, Alexa647-conjugated anti-CCR3 mAb, or PE-conjugated anti-CXCR3 mAb and analyzed by flow cytometer. Supernatants were harvested, and the production of IFN-γ, IL-2, and IL-4 was measured by ELISA.

Analysis of the activation of effector/memory T cells

As explained above, responder T cells, which participate in allogeneic T-cell reactions, were isolated using a MACS column (Miltenyi Biotec) from total mononuclear cells prepared from *M. tuberculosis*-infected BALB/c mice. Staining with APC-conjugated anti-CD3 mAb (BD Biosciences) revealed that the preparation consisted mainly of $CD3^+$ cells (>95%). DCs (2×10^5 cells/well) prepared from wild-type (WT), $TLR2^{-/-}$, and $TLR4^{-/-}$ C57BL/6 mice were treated with Rv0577 for 24 h followed by extensive washing and were cocultured with 2×10^6 responder allogeneic T cells (*M. tuberculosis*-infected T cells) at DC:T cell ratios of 1:10. On d 4 of coculture, the cells were stained with PerCP-cy5.5-conjugated anti-$CD4^+$ mAb, PerCP-cy5.5-conjugated anti-$CD8^+$ mAb, FITC-conjugated anti-CD62L mAb, and PE-conjugated anti-CD44 mAb, and analyzed by flow cytometer.

Statistical analysis

All experiments were repeated ≥3 times with consistent results. The levels of significance for comparison between samples were determined by Tukey's multiple comparison test distribution using statistical software (Prism 4.03; GraphPad, San Diego, CA, USA). The data in the graphs are expressed as means ± SE. Values of $P<0.05$were considered to be statistically significant.

RESULTS

Purification of recombinant Rv0577 protein and cytotoxicity

Rv0577 is an *M. tuberculosis* complex-restricted secreted protein involved in the methylglyoxal detoxification pathway (14). However, the immunological contribution of Rv0577, especially the effect on the maturation and functions of DCs, remains unknown.

Recombinant Rv0577 protein was therefore produced and characterized. The purity of Rv0577 was first assessed by SDS-PAGE and Western blot analysis. The purified recombinant Rv0577 protein had a molecular mass of ~32 kDa (Supplemental Fig. S1*A*). Endotoxin content was measured by an LAL assay and was <15 pg/ml (<0.1 UE/ml) in Rv0577 preparations. Rv0577 had 96% purity when 20 μg of the protein preparation was stained with silver nitrate (data not shown). We next examined the Rv0577 protein-induced cytotoxicity in DCs by treating cells with 10 μg/ml Rv0577 for 24 h, then staining with anti-CD11c, annexin V, and propidium iodide to assess cell viability. As shown in Supplemental Fig.

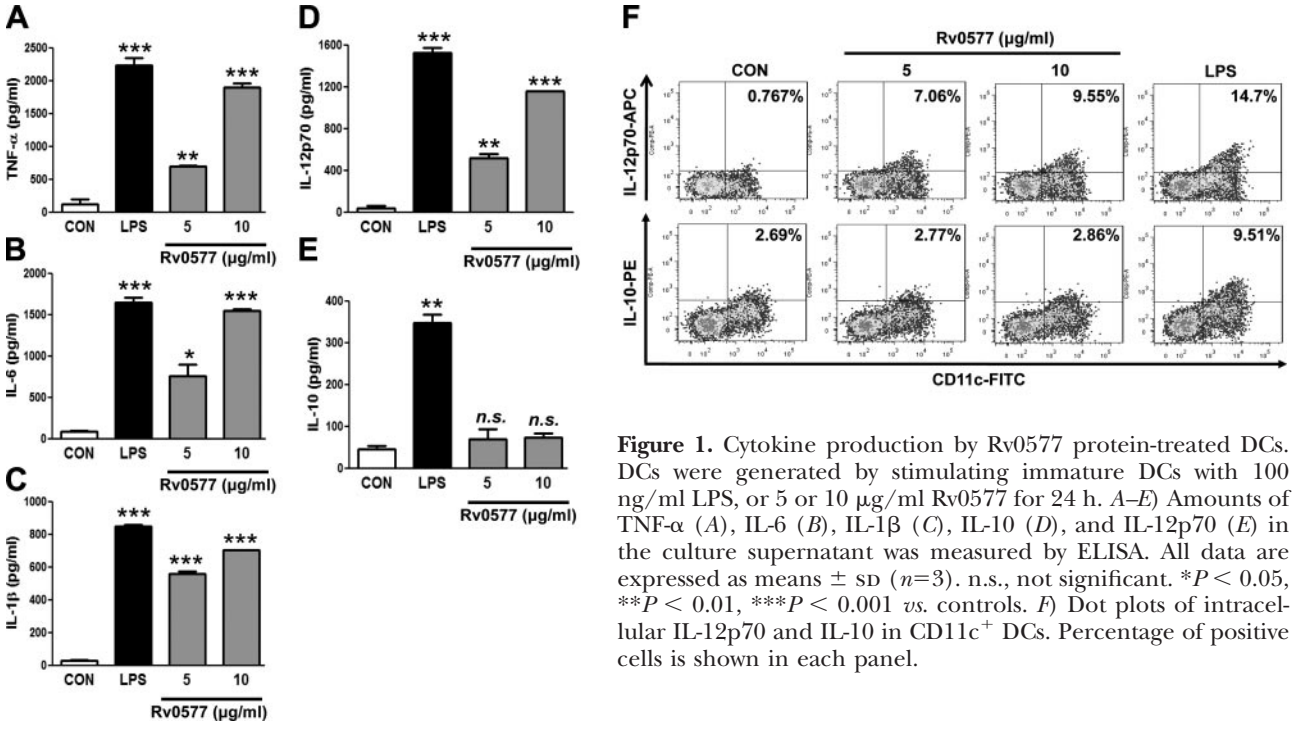
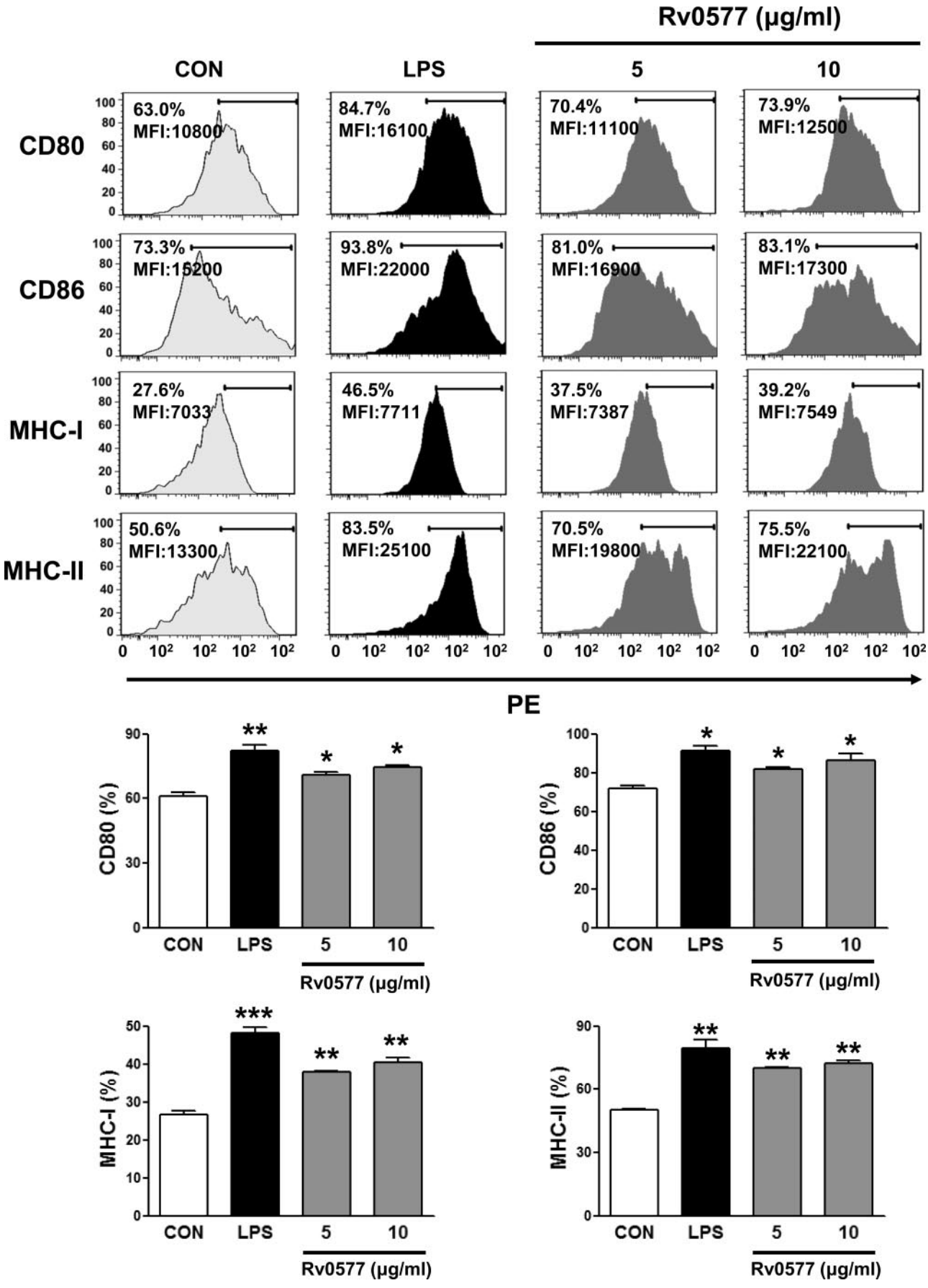


Figure 1. Cytokine production by Rv0577 protein-treated DCs. DCs were generated by stimulating immature DCs with 100 ng/ml LPS, or 5 or 10 μg/ml Rv0577 for 24 h. *A–E*) Amounts of TNF-α (*A*), IL-6 (*B*), IL-1β (*C*), IL-10 (*D*), and IL-12p70 (*E*) in the culture supernatant was measured by ELISA. All data are expressed as means ± SD ($n=3$). n.s., not significant. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs.* controls. *F*) Dot plots of intracellular IL-12p70 and IL-10 in $CD11c^+$ DCs. Percentage of positive cells is shown in each panel.


Rv0577 (μg/ml)
CON
LPS
5
10
CD80
63.0%
MFI:10800
84.7%
MFI:16100
70.4%
MFI:11100
73.9%
MFI:12500
CD86
73.3%
MFI:15200
93.8%
MFI:22000
81.0%
MFI:16900
83.1%
MFI:17300
MHC-I
27.6%
MFI:7033
46.5%
MFI:7711
37.5%
MFI:7387
39.2%
MFI:7549
MHC-II
50.6%
MFI:13300
83.5%
MFI:25100
70.5%
MFI:19800
75.5%
MFI:22100
PE
CD80 (%)
CD86 (%)
MHC-I (%)
MHC-II (%)
Rv0577 (μg/ml)

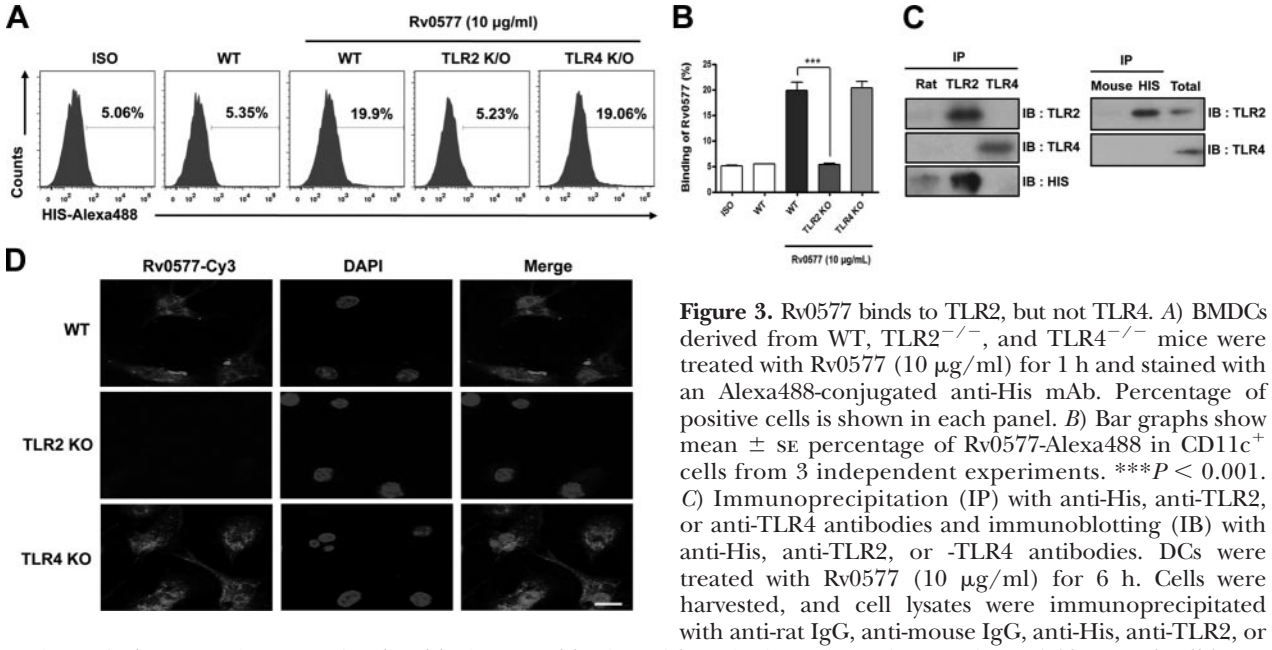


Figure 3. Rv0577 binds to TLR2, but not TLR4. *A*) BMDCs derived from WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice were treated with Rv0577 (10 μg/ml) for 1 h and stained with an Alexa488-conjugated anti-His mAb. Percentage of positive cells is shown in each panel. *B*) Bar graphs show mean ± SE percentage of Rv0577-Alexa488 in CD11c^{+} cells from 3 independent experiments. ***$P < 0.001$. *C*) Immunoprecipitation (IP) with anti-His, anti-TLR2, or anti-TLR4 antibodies and immunoblotting (IB) with anti-His, anti-TLR2, or -TLR4 antibodies. DCs were treated with Rv0577 (10 μg/ml) for 6 h. Cells were harvested, and cell lysates were immunoprecipitated with anti-rat IgG, anti-mouse IgG, anti-His, anti-TLR2, or anti-TLR4; then proteins were visualized by immunoblotting with anti-His, anti-TLR2, or anti-TLR4 Abs. Total cell lysate was used as an input control. *D*) Fluorescence intensities of anti-Rv0577 bound to Rv0577-treated DCs. DCs derived from WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice were treated with Rv0577 (10 μg/ml) for 1 h, fixed, and stained with DAPI and a Cy3-conjugated anti-Rv0577 antibody. Scale bar = 10 μm.

S1*B*, up to 10 μg/ml of Rv0577 protein displayed no cellular toxicity against DCs. Notably, this suggests that recombinant Rv0577 protein was not cytotoxic to DCs and when used in concentrations <10 μg/ml would not contain significant amounts of endotoxin to interfere with our studies.

Rv0577 induces the secretion of proinflammatory cytokines by DCs toward a Th1 type immune response

Previous studies have reported that DC-derived cytokines play a critical role in the polarization of T cells and in mediating inflammatory responses (21). We therefore analyzed whether Rv0577-mediated maturation of DCs was coupled with the secretion of pro- or anti-inflammatory cytokines by stimulating DCs with various concentrations of Rv0577 (5–10 μg/ml). As demonstrated in **Fig. 1*A–C***, Rv0577 significantly stimulated DCs to secrete high levels of TNF-α, IL-6, and IL-1β, whereas untreated DCs secreted negligible amounts of these cytokines. We then investigated the production of IL-12p70 and IL-10, which have an important effect on the development of T cell-mediated immune responses. Unlike LPS, Rv0577 significantly induced the secretion of IL-12p70, but not IL-10 (Fig. 1*D*, *E*). We also analyzed the production of intracellular IL-12p70 and IL-10 in Rv0577-treated DCs. As shown in Fig. 1*F*, DCs treated with Rv0577 increased the percentage of IL-12p70-positive cells compared to the results obtained for untreated DCs, while no change was found in IL-10-positive cells. These results strongly suggest that Rv0577 induces the secretion of proinflammatory cytokines from DCs and these Rv0577-matured DCs may promote a Th1 type immune response.

Rv0577 induces maturation of DCs

Maturation of DCs plays a major role in mediating immune responses to mycobacteria (4). Therefore, to investigate whether Rv0577 induces DC maturation, we first measured the expression of DC maturation markers such as CD80, CD86, and MHC classes I and II. BMDCs were cultured for 24 h in the presence of 5 or 10 μg/ml Rv0577 and analyzed for the expression of surface markers. LPS was used as a positive control. As shown in **Fig. 2**, Rv0577 enhanced the expression of costimulatory molecules such as CD80 and CD86, and cell surface markers such as MHC classes I and II at concentrations of 5

Figure 2. Rv0577 induces the expression of costimulatory and MHC molecules on DCs in a dose-dependent manner. Immature DCs (1×10^6 cells/ml) were cultured with GM-CSF and IL-4 alone (control) or GM-CSF, IL-4, or 5 or 10 μg/ml Rv0577 or GM-CSF, IL-4, or 100 ng/ml LPS for 24 h and analyzed for the expression of surface markers by 2-color flow cytometry. Cells were gated on CD11c cells. DCs were stained with anti-CD80, anti-CD86, or anti-MHC class I or anti-MHC class II. Percentage of positive cells is shown for each panel. Bar graphs show mean ± SE percentage of each surface molecule on CD11c^{+} cells for 3 independent experiments*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ *vs.* controls.

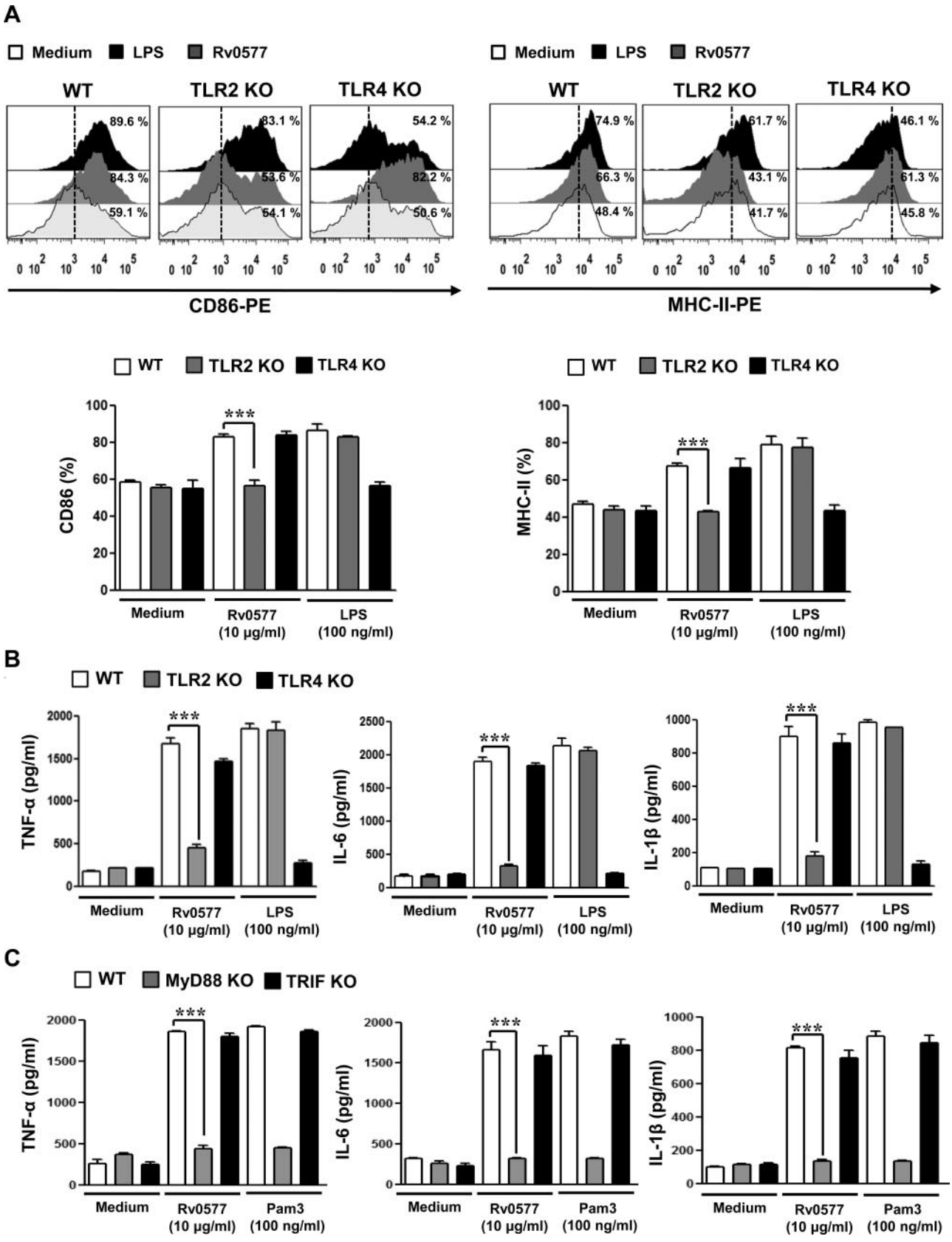


Figure 4. Rv0577 induces the activation of DCs *via* the interaction with TLR2. *A*) Histograms showing CD86 or MHCII expression on Rv0577-treated CD11c$^+$-gated DCs derived from WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice. DCs derived from WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice were treated with Rv0577 (10 μg/ml) for 24 h. Percentage of positive cells is shown for each panel. Bar graphs show mean ± SE percentage for each surface molecule on CD11c$^+$ cells in 3 independent experiments.

(*continued on next page*)

and 10 μg/ml, suggesting that Rv0577 efficiently induces DC maturation.

Rv0577 reduces the endocytic activity of DCs

The expression of surface molecules and changes in IL-12 production show that exposure to Rv0577 leads to profound induction of the phenotypic maturation of DCs. In this study, we investigated the role of Rv0577 in the regulation of DC endocytic activity. To test whether Rv0577 protein-stimulated DCs had the reduced endocytic activity, we exposed DCs to Rv0577 in the presence of dextran-FITC and assessed the percentage of double-positive cells ($CD11c^+$ and dextran-FITC-positive). As shown in Supplemental Fig. S2, a much lower percentage of active cells was present in LPS-treated DCs than in untreated DCs. In addition, the same was true of Rv0577-treated DCs when compared to untreated DCs. Our results showed that Rv0577-treated DCs had reduced endocytic activity, indicating more functional maturity.

Confirmation of endotoxin-free purified Rv0577 preparations

To ensure that Rv0577-induced DC maturation was not due to endotoxin or LPS contamination, the purified Rv0577 preparations were passed through a PmB-agarose column. Furthermore, we assessed LPS contamination by treatment with proteinase K or heat denaturation, which abrogated the ability of Rv0577 to trigger DC maturation (Supplemental Fig. S3*A*, *B*). PmB treatment did not affect the viability of Rv0577, whereas that of LPS was significantly inhibited by PmB (Supplemental Fig. *S3C*). These results confirmed that DC maturation was in fact induced by intact Rv0577 and not by contaminating LPS.

Rv0577 interacts with TLR2 and induced DC maturation

Pattern recognition receptors, such as TLRs, have the ability to recognize pathogen-associated molecular patterns from whole mycobacterial cells or mycobacterial cell wall components (7). Thus, we examined whether Rv0577 could be recognized by, and act through, TLRs in DCs. To identify TLRs on DCs that interact with Rv0577, WT, $TLR2^{-/-}$, and $TLR4^{-/-}$ DCs were stimulated with Rv0577; then Rv0577 on the cell surface was detected with an Alexa488-conjugated anti-Rv0577 polyclonal Ab (**Fig. 3*A***). Anti-Rv0577 bound to the cell surface of WT and $TLR4^{-/-}$ DCs but not $TLR2^{-/-}$ DCs (Fig. 3*B*). To confirm the interaction between Rv0577 and TLR, we performed immunoprecipitation studies with TLR2 or TLR4 and Rv0577 in DCs. Rv0577 bound to TLR2 but not TLR4 (Fig. 3*C*). This observation was also confirmed by confocal microscopy. Interestingly, we found that Rv0577 interacts preferentially with WT and $TLR4^{-/-}$ DCs but not $TLR2^{-/-}$ DCs (Fig. 3*D*).

To test the ability of Rv0577 to activate DCs *via* TLR2, we measured the expression of surface molecules and proinflammatory cytokine production in Rv0577-treated WT, $TLR2^{-/-}$, and $TLR4^{-/-}$ DCs. The expression of surface molecules (**Fig. 4*A***) and proinflammatory cytokine secretion (Fig. 4*B*) were enhanced in WT or $TLR4^{-/-}$ DCs by Rv0577. In contrast, these effects were strongly diminished in $TLR2^{-/-}$ DCs, indicating that Rv0577 is an agonist for TLR2 in DCs.

TLRs are critical in provoking innate immune responses through the activation of signaling cascades *via* TIR domain-containing adaptors, such as MyD88 and TRIF (22). MyD88 is common to all the TLRs, whereas TRIF is essential for TLR4-mediated activation of the MyD88-independent signaling pathway (22). Thus, MyD88 and TRIF play a potential role in the control of TLR2- and TLR4-mediated signaling pathways. To investigate the importance of the MyD88- and TRIF-dependent pathways in Rv0577-induced cytokine production by DCs, we compared DC-based cytokine production in WT, $MyD88^{-/-}$, and TRIF-deficient mice. Rv0577-induced production of TNF-α, IL-6, and IL-1β was significantly reduced in the absence of MyD88, whereas this effect was not observed in the absence of TRIF (Fig. 4*C*). These findings clearly demonstrated that Rv0577 induced DC maturation in a TLR2-dependent manner, causing increased expression of cell-surface molecules and proinflammatory cytokines.

Expression of the NF-κB and MAPK pathways in Rv0577 protein-induced maturation of DCs

NF-κB and MAPKs are critical factors mediating cellular responses such as innate immunity, cellular proliferation, and survival, and recent studies have suggested that NF-κB and MAPKs are essential for the maturation of DCs (22, 23). We therefore examined the activation of NF-κB and MAPKs in response to Rv0577. DCs were stimulated with Rv0577 recombinant protein at a concentration of 10 μg/ml, and the phosphorylation of MAPKs (including ERK1/2, p38, and JNK), the phosphorylation/degradation of IκB-α, and the nuclear translocation of p65 were analyzed over time (**Fig. 5**). As shown in Fig. 5*A*, the Rv0577 triggered the phosphorylation of MAPKs, such as ERK1/2, p38, and JNK. In addition, we found that the Rv0577 induced the phosphorylation and degradation of IκB-α and nuclear translocation of p65 from cytosol (Fig. 5*B*, *C*). These results

$^{***}P < 0.001$. *B*) DCs derived from WT, $TLR2^{-/-}$, and $TLR4^{-/-}$ mice were treated with Rv0577 or LPS for 24 h. TNF-α, IL-6, or IL-1β production in Rv0577- or LPS-treated DCs derived from WT, $TLR2^{-/-}$, and $TLR4^{-/-}$ mice was measured by ELISA. *C*) DCs derived from WT, $MyD88^{-/-}$, and $TRIF^{-/-}$ mice were treated with Rv0577 (10 μg/ml) and Pam3 (100 ng/ml) for 24 h. TNF-α, IL-6, or IL-1β production in Rv0577- or Pam3-treated DCs derived from WT, $MyD88^{-/-}$, and $TRIF^{-/-}$ mice was measured by ELISA. All data are expressed as means ± SD (n=3). $^{***}P < 0.001$.

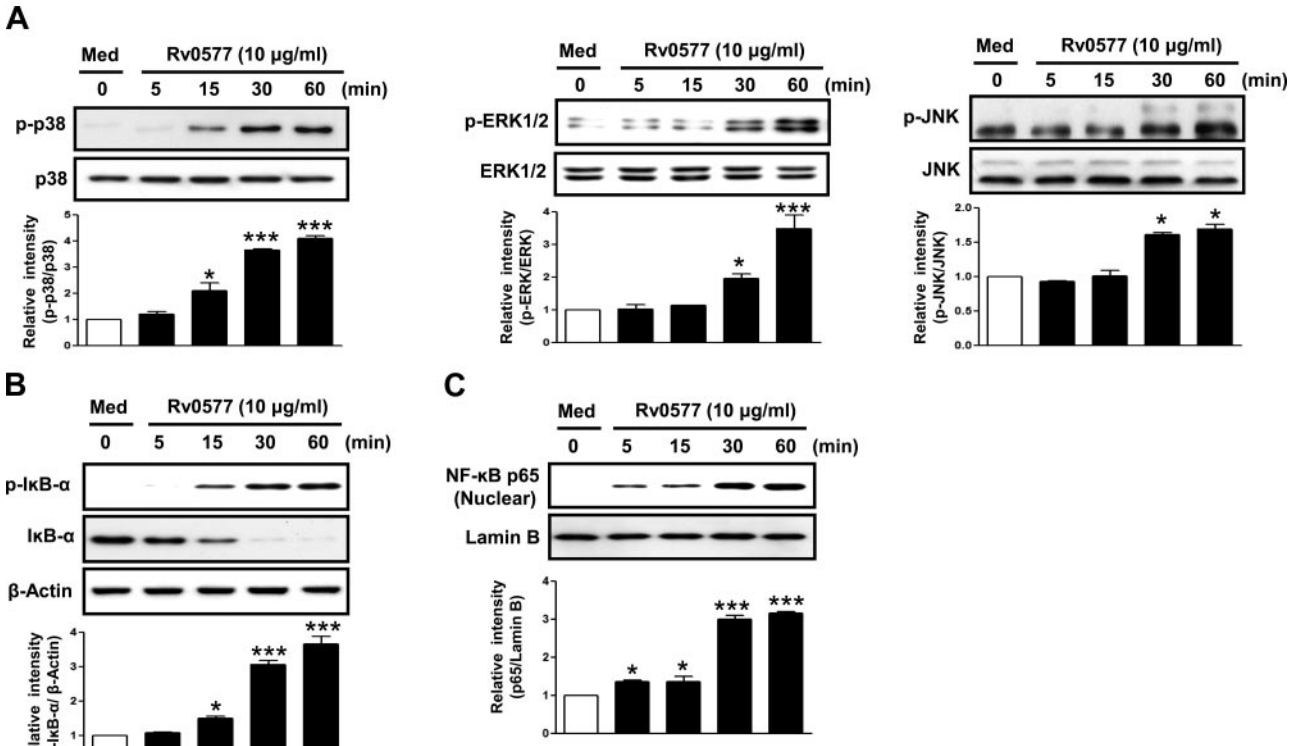


Figure 5. DC maturation triggered by Rv0577 involves activation of MAPKs and NF-κB. *A–C*) DCs were treated with 10 μg/ml Rv0577 protein, and protein expression is shown over time. Cell lysates were subjected to SDS-PAGE, and immunoblot analysis was performed using each specific antibody to phospho-p38 (p-p38), p38, phospho-ERK1/2 (p-ERK1/2), ERK1/2, phospho-JNK (p-JNK), and JNK (*A*); phosphor-IκB-α (p-IκB-α) and IκB-α (*B*); and p65 NF-κB (*C*). β-Actin (*B*) and lamin B (*C*) were used as loading controls for cytosolic and nuclear fractions, respectively. Relative band intensity of each protein is expressed as a percentage compared to the value of untreated controls. Results shown are typical of 3 experiments done under each condition. Data are shown as means ± SD (n=3), $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ *vs.* untreated DCs.

suggest that Rv0577 protein-induced DC activation might be mediated by both the NF-κB and MAPK pathways.

Rv0577 induces the production of proinflammatory cytokines and the expression of costimulatory molecules through NF-κB and MAPKs

Previous studies have demonstrated that activation of the NF-κB and MAPK signaling pathways plays a crucial role in the maturation of DCs induced by mycobacterial Ags (13, 24). The molecular mechanisms involved in the regulation of DCs by Rv0577 protein, however, are not yet fully understood. Thus, to elucidate the functional roles of these kinases in the activation of DCs induced by Rv0577, we used highly specific kinase inhibitors and measured Rv0577-induced proinflammatory cytokine production and costimulatory molecule expression. The cells were pretreated with a p38 inhibitor (SB203580), an ERK1/2 inhibitor (U0126), a JNK inhibitor (SP600125), or NF-κB inhibitor (Bay 11-0782) for 1 h before being exposed to Rv0577. Notably, we found that these pharmacological inhibitors significantly abrogated Rv0577-induced expression of the costimulatory molecules on the surface of DCs (**Fig. 6*A***), and the production of proinflammatory cytokines (Fig. 6*B*). From these findings, we suggest that the NF-κB and MAPK signaling pathways are essential for the production of proinflammatory cytokines and the expression of DC maturation markers induced by Rv0577.

Rv0577 protein-stimulated DCs induce naive T-cell proliferation

Increased expression of the costimulatory molecules, CD80 and CD86, along with MHC classes I and II, on DCs promotes their interaction with and activation to T cells. To precisely characterize Rv0577 activity on DC and T-cell interactions, we performed a syngeneic MLR assay using OT-I TCR transgenic $CD8^+$ T cells and OT-II TCR transgenic $CD4^+$ T cells. Transgenic CFSE-labeled OVA-specific $CD4^+$ and $CD8^+$ T cells cocultured with Rv0577-treated DCs, pulsed with $OVA_{257–264}$ (**Fig. 7*A***) or $OVA_{323–339}$ (Fig. 7*B*) and proliferated to a significantly greater extent than the same T cells cocultured with DCs without Rv0577 treatment, but pulsed with $OVA_{257–264}$ or $OVA_{323–339}$. Furthermore, naive $CD4^+$ and $CD8^+$ T cells primed with Rv0577-treated DCs produced significantly (P<0.05–0.01) higher IFN-γ and IL-2 levels than those with untreated DCs, whereas the comparable level of IL-4 secretion was detected regardless of Rv0577 stimulation (Fig. 7*C*). We then investigated the expression of chemokine receptor CXCR3 and CCR3, which are associated with T-cell polarization, in $CD4^+$ T cells using flow cytometry.

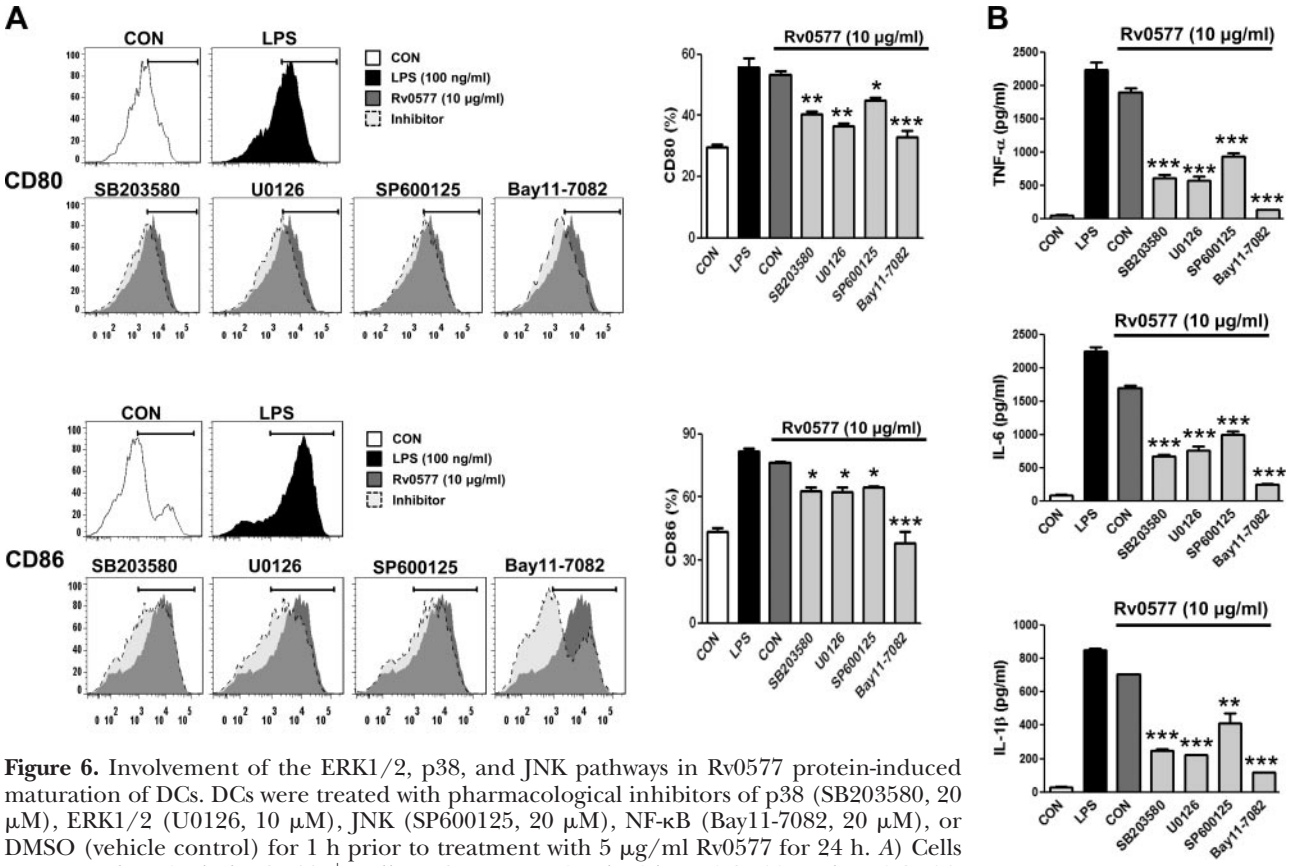


Figure 6. Involvement of the ERK1/2, p38, and JNK pathways in Rv0577 protein-induced maturation of DCs. DCs were treated with pharmacological inhibitors of p38 (SB203580, 20 μM), ERK1/2 (U0126, 10 μM), JNK (SP600125, 20 μM), NF-κB (Bay11-7082, 20 μM), or DMSO (vehicle control) for 1 h prior to treatment with 5 μg/ml Rv0577 for 24 h. *A*) Cells were gated to include $CD11c^+$ cells. DCs were stained with anti-CD80 and anti-CD86. Expression of CD80 and CD86 was analyzed by flow cytometry. Bar graphs show mean ± SE percentage of each surface molecule on $CD11c^+$ cells from 3 separate experiments. *B*) Amounts of TNF-α, IL-6, and IL-1β in the culture medium were measured by ELISA. Values are means ± SE from 3 independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ *vs.* Rv0577-treated controls.

CXCR3 is known to be expressed at a high level on Th1 cells, whereas Th2 cells up-regulate the expression of CCR3 (25). As shown in Fig. 7*D*, $CD4^+$ T cells cocultured with Rv0577-treated DCs, pulsed with $OVA_{323–339}$ showed a significant increase in the expression of CXCR3 compared to those cocultured with DCs pulsed with $OVA_{323–339}$ only, whereas no alteration in the expression of CCR3 by Rv0577 was investigated. These results suggest that the Rv0577-treated DCs direct naive T-cell proliferations toward a Th1 phenotype.

Rv0577 induces the development of effector/memory T cells *via* TLR2-mediated DC activation

To assess whether the maturation of DCs by Rv0577 is reflected in their ability to specifically stimulate $CD4^+$ and $CD8^+$ T cells from *M. tuberculosis*-infected mice, we analyzed the surface expression of CD62L and CD44 on $CD4^+$ and $CD8^+$ splenic T cells using flow cytometry. Naive T cells were previously reported to express a $CD62L^{high}CD44^{low}$ phenotype, whereas effector/memory T cells exhibit a $CD44^{high}CD62L^{low}$ phenotype (26). $CD4^+$ and $CD8^+$ splenic T cells from *M. tuberculosis*-infected mice were cocultured with Rv0577-treated DCs derived from WT, $TLR2^{-/-}$, or $TLR4^{-/-}$ mice. The Rv0577-treated WT and $TLR4^{-/-}$ DCs specifically induced the formation of effector/memory T cells by displaying significantly down-regulated CD62L and up-regulated CD44 expression in $CD4^+$ and $CD8^+$ splenic T cells from *M. tuberculosis*-infected mice compared to control DCs, Pam3-treated DCs, and LPS-treated DCs, while such an effect of Rv0577 was abrogated in $TLR2^{-/-}$ DCs (**Fig. 8*A***). In addition, the percentages of CD4-IFN-γ/CD4-IL-2 and CD8-IFN-γ/CD8-IL-2 double-positive cells among T cells cocultured with Rv0577-WT or $TLR4^{-/-}$ DCs were higher than that of those cocultured with untreated DCs, Pam3-pulsed DCs, or LPS-pulsed DCs, whereas the elevation of theses cytokines was not observed in T cells cocultured with Rv0577-treated $TLR2^{-/-}$ DCs (Fig. 8*B*). In addition, IL-4 expression of $CD4^+$ T cells among T cells treated with Rv0577-pulsed DCs, Pam3-pulsed DCs, or LPS-pulsed DCs remained at baseline levels (Fig. 8*B*). These data suggest that Rv0577-mediated DC activation induces the development of effector/memory T cells in *M. tuberculosis* infection in a TLR2-dependent manner.

DISCUSSION

Rv0577, also known as CFP32, is one of the major culture filtrate (CF) proteins that have emerged as

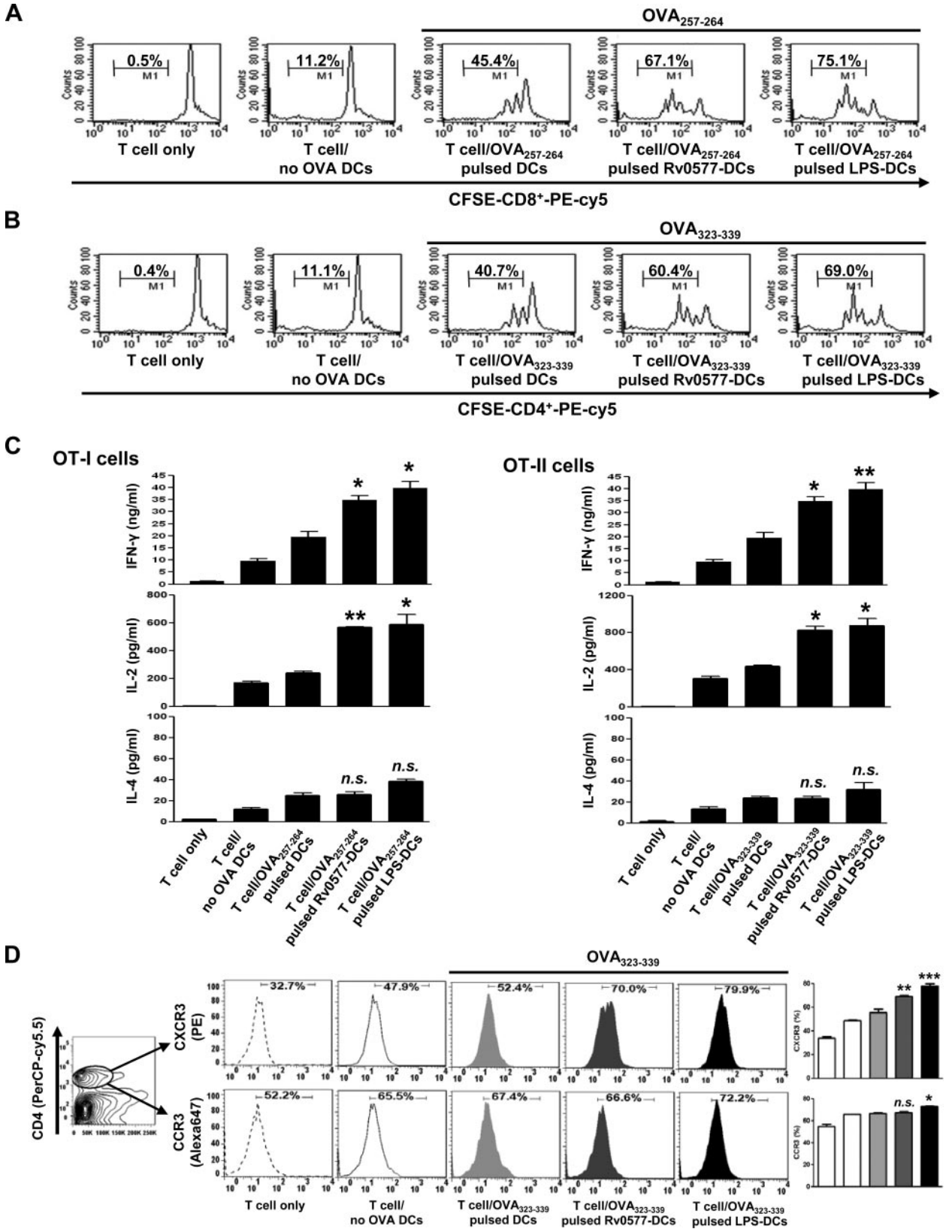


Figure 7. Rv0577 protein-treated DCs stimulate T cells to produce Th1 cytokines. *A*, *B*) Transgenic OVA-specific $CD8^+$ T cells and transgenic OVA-specific $CD4^+$ T cells were isolated, stained with CFSE, and cocultured for 96 h with DCs treated with Rv0577 (10 μg/ml) or LPS (100 ng/ml), then pulsed with $OVA_{257–264}$ (1 μg/ml) for OVA-specific $CD8^+$ T cells (*A*) or $OVA_{323–339}$ (1 μg/ml) for OVA-specific $CD4^+$ T cells (*B*). T cells only and T cells cocultured with untreated DCs served as controls. Proliferation of OT-I^+ (*A*) and OT-II^+ (*B*) T cells was then assessed by flow cytometry. *C*) Culture supernatants obtained from experiments in *A* and *B* were harvested after 24 h, and IFN-γ, IL-2, and IL-4 were measured by ELISA. *D*)

(*continued on next page*)

potential candidates for the diagnosis and drug targeting of TB (14). The gene encoding Rv0577 is found exclusively in the *M. tuberculosis* complex. Unlike other CF proteins, such as 85 Ag complex, Rv0577 is uniquely present in members of the *M. tuberculosis* complex and has not been identified in environmental mycobacteria (14). Furthermore, transfer of the rv*0577* gene into *Mycobacterium smegmatis* has recently been demonstrated by Andreu *et al.* (27) to confer the virulence-related neutral red character typical of virulent mycobacteria, suggesting an association of this gene with virulence. In addition, Rv0577 levels in the lungs of patients with active TB have been positively correlated with the progression of the disease (14). Moreover, Bertholet *et al.* (28) reported that Rv0577 provided a partial protection as a T cell Ag against challenge with *M. tuberculosis* in the mouse model. Recent research on the proteomic analysis in exosomes from *M. tuberculosis*-infected J774 cells also demonstrated that Rv0577 existed in exosomes as a highly antigenic protein (29). These observations suggest that Rv0577 may play a role in TB pathogenesis and likely interacts with the immune system. From this perspective, identification and characterization of novel Ags of *M. tuberculosis* including Rv0577 that interact with host immune responses is an essential step in the development of new drugs and effective vaccines against TB.

Host immune responses are generally sufficient to contain TB infection and many of the cell wall-associated or secreted Ags of pathogenic mycobacteria are targets of host immune responses (2, 3). Among the host immune cells, DCs are principal mediators of initiation as well as activation of host immune responses to TB infection; thus, they are essential for the containment of *M. tuberculosis* infections by inducing cellular immune responses to mycobacteria (4). Recent studies have examined the interaction of DCs with *M. tuberculosis* Ags, and some, such as Rv0754 and Rv0978c, were found to induce maturation and activation of DCs by regulating the NF-κB and MAPK signaling pathways, enhancing the ability of DCs to stimulate $CD4^+$ T cells (11). In addition, *M. tuberculosis* Ags, such as Rv1196 and Rv1917c, were reported to strongly induce the secretion of IL-10, known to favor and drive Th2 immune response (13, 30). Although many *M. tuberculosis* Ags have been discovered, little is known about *M. tuberculosis* Ags that induce Th1-polarized immune responses *via* DC maturation. Recently, the *M. tuberculosis* protein Rv0577 was detected in individuals infected with *M. tuberculosis* (14), and previous work has reported the development of specific Rv0577 serum antibody-capture ELISAs for TB diagnosis (31). Little is known, however, about the functional role of *M. tuberculosis* Rv0577 in the biology of *M. tuberculosis*. In this study, we show that *M. tuberculosis* Rv0577 induces DC maturation by augmenting the expression of CD80, CD86, and MHC class I and MHC class II molecules and produces markedly higher levels of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β. Furthermore, Rv0577 was found to significantly induce the secretion of IL-12p70, a Th1-polarizing cytokine, but not IL-10. IL-12 was previously reported to mediate the expression of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, in DCs (32), and when secreted by APCs is known to induce the secretion of IFN-γ from T cells and to play a critical role in driving Thl immune responses (33). Thus, our findings indicate that Rv0577 contributes to Th1 polarization of the immune response *via* DC maturation.

Cell-surface interactions are one of the most important events for the initiation of biological responses to extracellular stimuli. Among various cell-surface receptors, TLR2 is crucial in both the innate and adaptive immune responses of a host to microbial pathogens (8) and is known to play a pivotal role in the activation of inflammatory immune responses by *M. tuberculosis* Ags (34). *M. tuberculosis* Ags, such as glycolipids and lipoproteins, were shown to stimulate macrophage and DC activation through TLR2, thus stimulating both $CD4^+$ and $CD8^+$ T lymphocytes (35). Rv1818c was shown to interact directly with TLR2, thereby mediating apoptosis and cytokine secretion (36). Rv0978c and Rv0754 were also shown to interact with TLR2, prompting maturation and activation of DCs (11). In addition, lipomannan (LM) from several mycobacterial species was found to induce macrophage activation, which was characterized by TNF-α and nitric oxide secretion through TLR2 (37). These findings suggest that TLR2 may play a significant role in regulating the cellular immune responses of *M. tuberculosis*. Thus, identification of novel TLR2 agonists is needed for use as adjuvants in infectious disease control and in the development of credible vaccine candidates. Although several *M. tuberculosis* Ags have been identified as TLR2 agonists and are known to regulate APC activation by modulating TLR2-mediated signaling, the underlying mechanisms of how the novel *M. tuberculosis* Rv0577 interacts with the cell surface are not understood. Here we show, for the first time, that Rv0577 directly interacts with cell surface TLR2 and induces DC maturation in a TLR2-dependent manner. In addition, we also show that Rv0577 significantly increases the expression of surface molecules and proinflammatory cytokine secretion by activation of the MAPK and NF-κB pathways in DCs under MyD88-dependent signaling pathways. Thus, our findings indicate that Rv0577 is an agonist for TLR2 and that MyD88-mediated signaling is essential for an optimal, Rv0577-induced cytokine response by DCs. MyD88 was previously shown to act as an adaptor molecule essential for signaling of Ags *via* the

Splenocytes were stained with anti-CXCR3 mAb or anti-CCR3 mAb. Percentage of positive cells is shown for each panel. Histograms and bar graphs show $CXCR3^+CCR3^+$ T cells in the OVA-specific $CD4^+$ T cells. Values are means ± SE from 3 independent experiments. n.s., not significant. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ *vs.* corresponding controls.

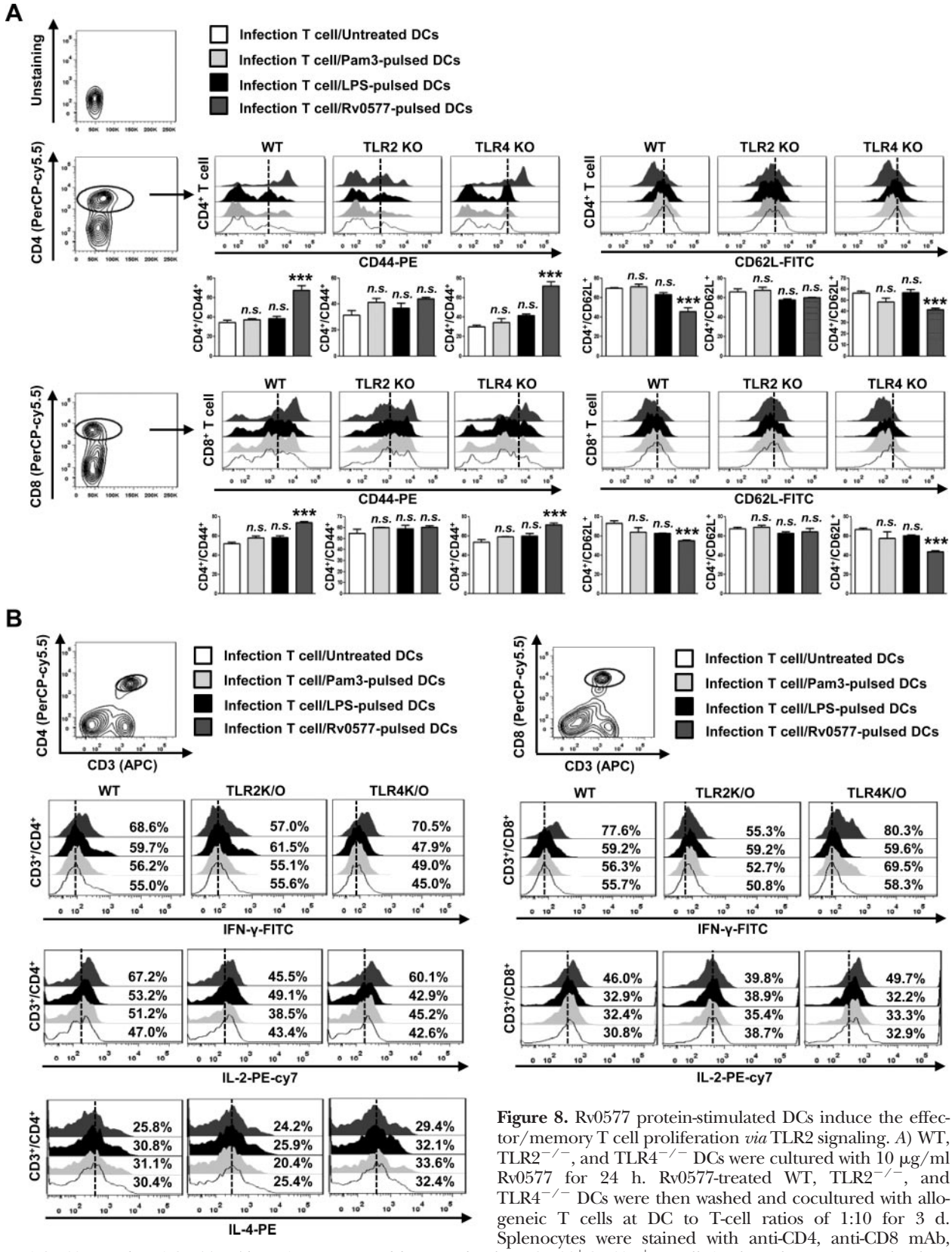


Figure 8. Rv0577 protein-stimulated DCs induce the effector/memory T cell proliferation *via* TLR2 signaling. *A*) WT, $TLR2^{-/-}$, and $TLR4^{-/-}$ DCs were cultured with 10 μg/ml Rv0577 for 24 h. Rv0577-treated WT, $TLR2^{-/-}$, and $TLR4^{-/-}$ DCs were then washed and cocultured with allogeneic T cells at DC to T-cell ratios of 1:10 for 3 d. Splenocytes were stained with anti-CD4, anti-CD8 mAb, anti-CD62L, and anti-CD44 mAbs. Histograms and bar graphs show $CD44^+CD62L^+$ T cells in the spleen. Bar graphs show mean ± SE percentages of $CD4^+/CD44^+CD62L^+$ and $CD8^+/CD44^+CD62L^+$ T cells from 3 independent experiments. n.s., not significant. $^{**}P < 0.01$, $^{***}P < 0.001$ *vs.* untreated DCs. *B*) IFN-γ, IL-2, or IL-4 expression in $CD3^+/CD4^+$ and $CD3^+/CD8^+$ cells was analyzed in T cells cocultured with Rv0577-pulsed DCs, Pam3-pulsed DCs, or LPS-pulsed DCs by intracellular IFN-γ, IL-2, or IL-4 staining. Percentages of double-positive cells among the T cells are indicated in the top right corner; results are representative of 3 independent experiments.

TLR family (8). Furthermore, signaling through MyD88 was reported to play a crucial role in host resistance associated with the regulation of mycobacterial-induced cytokine production (38). Others have shown that MyD88-deficient APCs, like macrophages and DCs, display a marked reduction in the synthesis of IL-12, TNF-α, and nitric oxide when exposed to *M. tuberculosis* (39).

An important finding of this study was that the Rv0577 expressed in *E. coli* mediated TLR2 signaling. In general, TLR2 has been well documented to be recognized by lipidated Ags, including mycobacterial lipoproteins, such as LprA (Rv1280c) and LpqH (Rv3763), and glycolipids, such as phosphatidyl-(myo)-inositol mannosides (PIMs), lipomannans (LMs), lipoarabinomannans (LAMs), and inositol phosphate-capped LAMs (PI-LAMs). Although *Escherichia coli* lacks post-translational modifications such as glycosylation and acylation, several mycobacterial proteins work as TLR2 agonists. For example, PE_PGRS33 expressed in *E. coli* triggers apoptosis in macrophages by directly interacting with TLR2 (36), and *M. tuberculosis* heat shock protein 60 expressed in *E. coli* modulates immune responses by interacting with TLR2 on macrophages (40). Another study showed that early secreted Ag ESAT-6 of *M. tuberculosis* expressed in *E. coli* blocked TLR signaling by direct interaction with TLR2 (24). Originally, *M. tuberculosis* LprG (Rv1411c) was known to require acylation to reflect TLR2 agonist activity (34) but nonacylated LprG retained TLR2 activity, suggesting that certain proteins of *M. tuberculosis* do not require lipidation for their TLR2 activity (41). Thus, the acylation of Rv0577 is not required for TLR2 activity.

Another significant finding of our work was that Rv0577 induced Th1-polarized immune responses, which are thought to be critical to host protection against *M. tuberculosis* infection. Although TLR2-mediated immune responses of cell wall fractions and lipoproteins are essential for the induction of a protective immune response to mycobacteria (42), several TLR2 agonists discovered in *M. tuberculosis*, including PE/PPE family proteins, LprA, and LprG, contribute to escape of the bacterium from host defenses. Rv1917c of *M. tuberculosis* (PPE34), for example, induces selective maturation of human DCs toward Th2 immune responses, with increased levels of IL-10 and lipoproteins (including LprA and LprG) and inhibits MHC II-mediated Ag processing of macrophages, resulting in a decrease in IFN-γ production (13).

T lymphocytes play a critical role in the control of a protective immune response in *M. tuberculosis* infection (43). Under optimal Ag stimulation, DCs first take up Ags and then travel to the lymph nodes where they present processed Ags to naive T cells. These naive T cells are stimulated to proliferate and differentiate into functional effector and memory T cells, a subset of antigen-specific T cells (44). These cells generally do not show direct action during an infection; instead, they enable the body to react quickly and control a recognized pathogen if encountered again (44). Thus, the *M. tuberculosis* vaccine likely acts to affect the quality and quantity of memory T cells.

Recently, several studies have revealed that *M. tuberculosis* Ags induce the activation of $CD4^+$ and $CD8^+$ T cells through TLR-mediated DC activation. This is thought to lead to protective immunity against *M. tuberculosis* by triggering IFN-γ production, which plays a particularly important role in the activation of antigen-presenting cells in $CD4^+$ and $CD8^+$ T cells (12, 13). Based on this, $CD4^+$ and $CD8^+$ T cells have been suggested to play a protective role in immune responses against infection. Notably, we showed that Rv0577 enhances the ability of DCs to stimulate naive T cells and that Rv0577-matured DCs trigger IFN-γ and IL-2 production in $CD4^+$ and $CD8^+$ T cells. In addition, we also found that Rv0577-treated DCs up-regulated CXCR3 expression in $CD4^+$ T cells, whereas CCR3 expression remained at baseline levels, indicating Rv0577 participated in adaptive immunity by directing T cell immune responses to a Thl polarization.

Protection against TB depends on the rapid generation of effector memory T cells capable of producing IFN-γ. These cells are believed to be central components of acquired immunity and are the basis for successful vaccination against TB (43). Furthermore, antigen-bearing DCs regulate the diverse pattern of effector memory $CD4^+/CD8^+$ T cell development in different tissues (45). After *M. tuberculosis* infection, $CD4^+$ and $CD8^+$ T cells expand into effector cells and then differentiate into long-lived memory cells (46). Evidence that these cells potently and specifically inhibit the growth of the intracellular bacteria *M. tuberculosis* has been shown *in vitro,* whereby they displayed macrophage antimycobacterial activity, and *in vivo*, when they promote the survival of infected mice (47). Furthermore, these cells expand in the spleens of mice infected with *M. tuberculosis* (47). The findings have significant implications for the design of vaccines and immunization strategies for the protection against *M. tuberculosis* infection.

In general, effector memory T cells are known to express the $CD44^{high}CD62L^{low}$ surface phenotype. CD44 is a surface protein required for lymphocyte extravasation to inflammatory sites and its up-regulation is a marker for all memory T cells (48). CD62L is a lymph node homing receptor that is down-regulated on activation of T-cell populations (49). In murine models of *M. tuberculosis* and *M. bovis* infection, accumulation of $CD4^+$ T cells with decreased CD62L and increased CD44 expression at the site of infection has been reported (50). Also, IFN-γ producing cells have been described as bearing a $CD44^{high}$ and $CD62L^{low}$ phenotype (50).

In our work, we demonstrated that a population of $CD44^{high}CD62L^{low}$ $CD4^+/CD8^+$ effectors was specifically generated from splenic T cells of *M. tuberculosis*-infected mice in response to DC-bearing Rv0577, indicating that Rv0577 acts as a specific recall antigen. In this regard, Rv0577 could be a potent adjuvant trigger-

ing Th1-mediated immune responses. In addition, TLR2 has a particularly important role in the maintenance of memory Th1 responses in *M. tuberculosis* infection. In addition, the use of TLR2 agonists as T-cell antigens in TB vaccines may be important, since these molecules may directly enhance $CD4^+/CD8^+$ T-cell memory responses and thus confer protective immunity against *M. tuberculosis* (51). Since our study focused on murine DCs *in vitro*, and the TB model for humans is different from the murine model in the aspect of the pathogenesis and immunological events, further work will be needed to extend these findings to human DCs from individuals infected with *M. tuberculosis*.

Nevertheless, these findings conclusively demonstrate that the Rv0577 plays a critical role in the activation of DCs in a TLR2-dependent manner and the initiation of the adaptive immune response by polarizing the development of T cells to a Th1 response. These novel findings about the role of the Rv0577 will be useful in the design of new strategies to prevent many chronic diseases caused by *M. tuberculosis*. FJ

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