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Mycobacterium tuberculosis RpfB drives Th1-type T cell immunity via a TLR4-dependent activation of dendritic cells

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ABSTRACT

The failure of *Mycobacterium bovis* BCG as a TB vaccine against TB reactivation suggests that latency-associated proteins should be included in alternative TB vaccine development. Further, antigens known to generate protective immunity against the strong Th1 stimulatory response to reactivated TB should be included in novel vaccine design. Recent studies have emphasized the importance of Rpfs from *Mycobacterium tuberculosis* in the reactivation process and cellular immunity. However, little is known about how RpfB mediates protective immunity against *M. tuberculosis*. Here, we investigated the functional roles and signaling mechanisms of RpfB in DCs and its implications in the development of T cell immunity. DCs treated with RpfB displayed features of mature and functional status, with elevated 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). Activation of DCs was mediated by direct binding of RpfB to TLR4, followed by MyD88/TRIF-dependent signaling to MAPKs and NF-κB signaling pathways. Specifically, we found that the RpfB G5 domain is the most important part in RpfB binding to TLR4. RpfB-treated DCs effectively polarized naïve $CD4^+$ and $CD8^+$ T cells to secrete IFN-γ and IL-2. Importantly, RpfB induced the expansion of memory $CD4^+$/$CD8^+CD44^{high}CD62L^{low}$ T cells in the spleen of *M. tuberculosis*-infected mice. Our data suggest that RpfB regulates innate immunity and activates adaptive immunity through TLR4, a finding that may help in the design of more effective vaccines. ***J. Leukoc. Biol.*** **94: 733–749; 2013.**

Introduction

M. tuberculosis is an outstanding pathogen that successfully adapts to its host [1]. More than one-third of the world's population is infected with *M. tuberculosis*, and 1.7 million people die from TB annually, making *M. tuberculosis* a major public health threat [2]. Although the only available TB vaccine, BCG, has been administered to >3 billion people worldwide, an apparent lack of efficacy of BCG in reactivated populations and TB-endemic regions has raised concerns [3, 4]. In fact, it is generally believed that BCG antigens do not last for a sufficient time to generate long effector memory T cells, which ultimately affect BCG efficiency [5]. The variable efficacy of the BCG vaccine motivates ongoing efforts to develop alternative vaccines for the control of TB [6–9]. In this regard, antigens capable of activating APCs toward the Th1-type immune response offer an attractive alternative to BCG [5]. Unfortunately, the majority of antigens fails to initiate a protective T cell response, as a result of their poor ability as APC stimulators [5]. Furthermore, there is a need to include latency antigens to protect against reactivated TB.

Practical screening for the antigens capable of developing effective and long-lasting protective immunity is necessary, as these act as protective antigens necessary for the generation of effector and memory T cells responsible for protective immunity [10–13]. It still remains to be determined if such protective antigens of *M. tuberculosis* can be exploited as vaccines [13]. In fact, the antigens isolated from in vitro cultures have failed to generate considerable protective immunity, indicating that there are huge differences in antigen expression in vitro

Abbreviations: $^{-/-}$=knockout, BCG=Bacille Calmette Guerin, BMDC=bone marrow-derived DC, CB=Coomassie blue, CD62L=CD62 ligand, DUF=domain of unknown function, ESAT-6=6-kd early secreted antigenic target, GATA-3=GATA-binding protein 3, HBHA=heparin-binding HA, HspX=heat-shock protein X, iDC=immature DC, LAL=Limulus amoebocyte lysate, Ni-NTA=nickel-nitrilotriacetic acid, PBS/T=PBS containing 0.1% Tween-20, PmB=polymyxin B, RpfB=resuscitation-promoting factor B, TB=tuberculosis, T-bet, T-box expressed in T cells, TRIF=Toll/IL-1R homology domain-containing adapter-inducing IFN-β

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and in vivo. In an effort to develop an improved TB vaccine, it is also important to characterize the vaccine potential of antigens possibly associated with the reactivation of a latent *M. tuberculosis* infection [14].

Recent publications have emphasized the importance of Rpfs, which are implicated in the reactivation of TB, as latency and reactivation are central features of *M. tuberculosis* pathogenesis [15–18]. It is generally thought that Rpf proteins possess cell-wall-modifying enzymatic activity, which results in cell-wall remodeling through hydrolysis of the glycan backbone of peptidoglycan [19–21]. Among the five Rpf proteins, RpfB has been highlighted most frequently because of its functional roles in immune responses and pathogenesis [19]. In fact, the lack of *rpfB* alone in mice produced delayed reactivation kinetics following immune suppression, but deletion of the other *rpf* genes did not result in significant growth defects of *M. tuberculosis* Erdman in vitro and in vivo [22]. In addition, all of the *M. tuberculosis* Rpfs, with the exception of RpfC, are immunogenic, as they elicit the production of antibodies in mice and stimulate the proliferation of LN T cells, suggesting that they modulate the immune response during infection and may serve as useful vaccine candidates [17, 23]. Furthermore, immunization with the *rpfB*-encoding DNA vaccine resulted in elevated cellular immune responses and significant protection against intratracheal *M. tuberculosis* challenge [16]. These results indicate that RpfB is the most promising candidate among the five rpf-like proteins of *M. tuberculosis* in terms of its immunogenicity and protective efficacy. In addition, RpfB-induced activation of lymphocytes did not increase IL-10, in contrast to other Rpf proteins [24]. Moreover, a recent report demonstrated that RpfB is involved in innate immunity through interactions with macrophages [22]. However, the precise mechanisms through which RpfB elicits innate immunity, especially in DCs, and how innate and adaptive immune responses are linked, remain unknown.

Although the exact reason for the generation of protective immunity by RpfB cannot be explained, it is obvious that DCs are involved in RpfB-induced T cell immunity via APCs, as only mature DCs generate T cell immunity. DCs are potent APCs that can stimulate resting T cells in the primary immune response [25]. iDCs have the ability to capture and recognize antigens, the so-called PAMPs, via PRRs [26], and the immunoregulatory role of DCs mainly relies on the ligation of these specific receptors, such as TLRs that initiate and modulate DC maturation [25]. Upon PAMP-PRRs interaction, activation of appropriate immune responses to TB can be initiated to maintain well-regulated immunologic homeostasis. TLRs are the most well-known PRRs, playing a crucial role in the activation of the cellular immune response against *M. tuberculosis* infection [27]. Activation of signaling through Toll/IL-1R homology domains results in recruitment of the adaptor molecules MyD88 and/or TRIF, ultimately leading to activation of MAPKs and NF-κB [27]. This signaling process, referred to as DC maturation or activation, enables DCs to become potent APCs to initiate robust innate immunity by up-regulating the expression of surface molecules, including CD80, CD86, MHC class I and II, and a number of cytokines, such as IL-1β, IL-6, IL-12, and TNF-α [26]. Upon maturation or activation, DCs acquire the capacity to present antigens to naive T cells [26]. When DCs initiate T cell-mediated adaptive immune responses, they also play important roles in Th1/2 polarization [26]. Recently, several studies have reported that *M. tuberculosis* antigens induced the maturation and activation of DCs in a TLR2-dependent manner. For example, *M. tuberculosis* antigens, such as Rv1196, Rv0978c, and Rv0754, recognize TLR2 and induce the maturation and activation of DCs [28–30]. Recently, we identified a *M. tuberculosis* antigen, Rv0577, that induces maturation of DCs in a TLR2-dependent manner and drives the Th1 immune responses [31]. Additionally, only mature DCs can present antigens to naïve T cells, making activation of DCs a prerequisite to initiate the proper adaptive immunity [32]. With their unique role in T cell priming, DCs may be the most important cell type for triggering proper immunity against TB, thus antigens that induce Th1-polarized immune responses via DC maturation are crucial for the development of an effective TB vaccine.

Although comparative analyses suggest that the *rpfB* gene product may be important to the pathogenesis of *M. tuberculosis*, especially in the reactivation process, the immunological function of this protein has not been elucidated, especially with respect to its role in innate and adaptive immunity. A better understanding of the mycobacterial RpfB protein and its role in the host immune response may improve our understanding of the immunological processes induced by this important human pathogen and host-pathogen interactions and may help in the rational design of more effective vaccines or vaccine adjuvants. Thus, in this study, we attempted to clarify the precise mechanism by which RpfB induces the Th1-type immune response via DC activation.

MATERIALS AND METHODS

Animals

Specific pathogen-free female C57BL/6 (H-2K^b and I-A^b), BALB/c (H-2K^d and I-A^d), C57BL/6J TLR2$^{-/-}$ mice (B6.129-Tlr2^{tm1Kir}/J), C57BL/10 TLR4$^{-/-}$ (C57BL/10ScNJ), and C57BL/6 OT-1 TCR transgenic mice, 5–6 weeks of age, were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). For the animal-infection study, a low-dose infection model described by Phyu et al. [33] was used with minor modifications to estimate the longevity of effector/memory T cell populations responding to RpfB. This low-dose model has been widely accepted as an analog of human latent TB through the demonstration that the bacterial load remains stable for many months. Briefly, 6-week-old BALB/c or C57BL/6 mice were i.v.-infected with a low dose of *M. tuberculosis* H37Rv (ATCC27294; 2×10^2 CFUs), and CFUs were counted from the lungs and spleen at 2-week intervals until 30 weeks after infection. This protocol produced steady-state chronic infection in the lungs and spleen, as determined by the stable bacillary load in these organs 10 weeks postinfection and the development of severe disease 20 weeks postinfection. Five to six mice were killed at 6 (early persistence) and 24 (progressive disease) weeks postinfection, and their spleens were collected for subsequent experiments, including MLRs. All infection-related experiments were performed in a Biosafey Level 3 Biohazard Animal Facility at the Yonsei University Medical Research Center, according to the regulations set forth by the Institutional Animal Care and Use Committee of the Yonsei University Health System.

Antibodies and reagents

Mouse rGM-CSF, mouse rIL-4, and the FITC-Annexin V/PI 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). LPS (from *Escherichia coli* O111:B4; Sigma) and palmitoyl-3-Cys-Ser-(Lys)4 were purchased from InvivoGen (San Diego, CA, USA). 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 synthesized the OT-I peptide ($OVA_{257–264}$) and OT-II peptide ($OVA_{323–339}$). Antiphosphorylated ERK1/2 mAb, anti-ERK1/2 polyclonal antibody, antiphosphorylated JNK mAb, anti-JNK polyclonal antibody, antiphosphorylated p38 mAb, anti-p38 polyclonal antibody, anti-NF-κB (p65) polyclonal antibody, antiphosphorylated IκB-α mAb, anti-IκB-α mAb, anti-Lamin B mAb (C-20), anti-GATA-3 antibody, and anti-T-bet antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). HRP-conjugated anti-mouse IgG antibody and HRP-conjugated anti-rabbit antibody 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, anti-GATA-3 antibody, and anti-T-bet antibody; PerCP-cy5.5-conjugated mAb to $CD4^+$ and $CD8^+$; PE-conjugated mAb to IL-4, CD80, CD86, MHC class I, MHC class II, CD11c, CD44, and CXCR3; and Alexa 647-conjugated anti-CCR3 mAb and PE-Cy7-conjugated mAb to IL-2 were purchased from eBioscience (San Diego, CA, USA). ELISA kits for IL-6, IL-1β, TNF-α, IL-2, IL-4, and IFN-γ were obtained from eBioscience, and IL-12p70 and IL-10 ELISA kits were obtained from BD Biosciences (San Diego, CA, USA).

Expression and purification of rRpfB deletion mutants

To produce rRpfB deletion mutant proteins, the corresponding gene was amplified by PCR using genomic DNA from *M. tuberculosis* H37Rv ATCC 27294 as the template and the following primers (R1): forward 5′-*CATATGTGCAAAACGGTGACGTTGACC*-3′ and reverse 5′-*AAGCTTCTCGGTACCGGGCTTGGTGCC*-3′; (R2): forward 5′-*CATATGTCGACGGTGGACGAGGCGCTG*-3′ and reverse 5′-*AAGCTTCACCTCGGTACCGGGCTTGGT*-3′; and (R3): forward 5′-*CATATGACCCGCAATCGGATCAAGAAG*-3′ and reverse 5′-*AAGCTTGCGCGCACCCGCTCGTGCAGC*-3′. The products of *rpfB* deletion mutants were inserted into the pET-22b (+) vector (Novagen, Madison, WI, USA) by cutting with *Nde*I and *Hin*dIII. The recombinant plasmids containing *rpfB* deletion mutants were transformed into *E. coli* BL21 cells by heat-shock for 1 min at 42°C. The overexpressed RpfB mutants were prepared as described previously. Each purification step was analyzed by 13.5% SDS-PAGE with brilliant CB staining and immunoblot analysis using anti-His antibodies (Santa Cruz Biotechnology). To remove endotoxin contamination, the dialyzed recombinant protein was incubated with PmB-agarose (Sigma) for 6 h at 4°C. Finally, the purified endotoxin-free recombinant protein was filter-sterilized and frozen at −70°C. The protein concentration was calculated using a BCA protein assay kit (Pierce, Rockford, IL, USA). The amount of residual LPS in the RpfB preparation was estimated using the LAL test (Lonza, Basel, Switzerland), according to the manufacturer's instructions. The purity of RpfB was evaluated by CB staining and Western blot analysis using an antihistidine antibody.

Generation and culture of DCs

Murine BMDCs were prepared and cultured as described recently [34]. To obtain highly purified populations (>95% cell purity) in some experiments, 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.

Measurement of cytokines

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 [34].

Intracellular cytokine assays

Cells were first blocked with 10% (vol/vol) normal goat serum for 15 min at 4°C and then stained with PE-conjugated $CD11c^+$ and PerCP-Cy5.5-conjugated $CD4^+$ and $CD8^+$ antibodies 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), 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 by flow cytometry (FACSCanto; BD Biosciences) using the CellQuest (BD Biosciences) program.

Analysis of surface molecule expression by flow cytometry

Cell surface staining was performed with specifically labeled fluorescent-conjugated mAb, as described recently [34]. The fluorescence was measured by flow cytometry (FACSCanto; BD Biosciences), and the data were analyzed using CellQuest data analysis software.

Antigen uptake ability of BMDCs facilitated by RpfB

BMDCs (2×10^5 cell) were equilibrated at 37°C or 4°C for 30 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 system. Nonspecific binding of Dextran to the DCs was determined by incubating the DCs with FITC-conjugated Dextran at 4°C, and the resulting background value was subtracted from the specific binding values.

Generation of mouse anti-RpfB R2 antibodies

Female BALB/c (The Jackson Laboratory) mice, aged 6–8 weeks, were immunized by i.p. injection using CFA (Sigma) and boosted three times with purified rRpfB R2. The details of the immunizations are as follows. Mice were immunized with rRpfB R2 i.p. injection of 100 μl, containing 20 μg antigens in 50 μl PBS, mixed with 50 μl CFA. Control mice received an initial i.p. injection of 100 μl containing 50 μl PBS without antigen mixed with 50 μl CFA. The animals were then boosted three times by i.p. injection of 100 μl containing 20 μg antigen in 50 μl PBS mixed with 50 μl IFA or 100 μl containing 50 μl PBS mixed with 50 μl IFA at intervals of 2 weeks. The blood was then centrifuged at 2000 rpm for 10 min and the sera collected and stored at −70°C until tested.

Binding assay

WT, $TLR2^{-/-}$, and $TLR4^{-/-}$ DCs were then treated for 1 h at 37°C with irrelevant normal mouse IgG as an isotype, nontreated control, or purified His-tagged RpfB or RpfB mutant. After Alexa 488-conjugated anti-His mAb was added to the cell suspensions, they were incubated for 1 h at 4°C, washed three times with PBS, and fixed with 4% PFA. Samples were analyzed using the FACSCanto system (BD Biosciences) with CellQuest analysis software.

Confocal laser-scanning microscopy

DCs were plated overnight on poly-L-lysine or 0.1% gelatin-coated glass coverslips. After treatment with 2 μg/ml RpfB, DCs were fixed in 4% PFA, permeabilized in 0.1% Triton X-100, and then blocked with 2% BSA in PBS/T for 2 h before being incubated with 2% BSA in PBS/T containing antihistidine antibody or anti-p65 mAb for 2 h at room temperature. After washing with PBS/T, the cells were reincubated with FITC-conjugated secondary antibody in a dark room for 1 h and were then stained with 1 μg/ml DAPI for 10 min at room temperature. Cell morphology and fluorescence intensity were observed using a confocal laser-scanning microscope (LSM510 Meta; Carl Zeiss, Welwyn Garden City, UK). Images were acquired using LSM510 Meta software and processed using the LSM image examiner.

Immunoblotting analysis

After stimulation with 2 μg/ml RpfB, DCs were lysed in 100 μl lysis buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1

mM EDTA, 50 mM NaF, 30 mM Na_4PO_7, 1 mM PMSF, 2 μg/ml aprotinin, and 1 mM pervanadate. Immunoblotting was carried out as described previously [34]. Epitopes on target proteins, including MAPKs and NF-κB, recognized specifically by antibodies, were visualized using the ECL Advance kit (GE Healthcare, Little Chalfont, UK).

Nuclear extract preparation

Nuclear extracts from cells were prepared by first treating DCs 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 DTT, 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, 1 mM PMSF] and incubated on ice for 30 min. Following centrifugation at 12,000 rpm for 10 min, the supernatant containing nuclear extracts was collected and stored at −80°C until required.

MLR

Responder T cells, which participate in naïve T cell reactions, were isolated from total mononuclear cells prepared from BALB/c mice using a MACS column (Miltenyi Biotec). OVA-specific $CD8^+$ and $CD4^+$ responder T cells were obtained from the splenocytes of OT-1 and OT-2 mice, respectively. A MLR was performed, as described previously [31]. DCs (2×10^5 cells/well), treated with OVA peptide in the presence of 2 μg/ml RpfB for 24 h, were cocultured with 1 μM CFSE-stained $CD8^+$ and $CD4^+$ T cells (2×10^6; Invitrogen, Carlsbad, CA, USA) at DC:T cell ratios of 1:10. After 3 or 4 days of coculture, T cells were stained with PerCP-Cy5.5-conjugated anti-$CD4^+$ mAb, PerCP-Cy5.5-conjugated anti-$CD8^+$ mAb, Alexa 647-conjugated anti-CCR3 mAb, or PE-conjugated anti-CXCR3 mAb and analyzed by flow cytometry (FACSCanto; BD Biosciences). Supernatants were harvested, and the production of IFN-γ, IL-2, and IL-4 was measured by ELISA.

Analysis of the expansion 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 collected 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 WT, $TLR2^{-/-}$, and $TLR4^{-/-}$ C57BL/6 mice, treated with RpfB for 24 h, were washed extensively and then cocultured with 2×10^6 responder allogeneic T cells (*M. tuberculosis*-infected T cells) at DC:T cell ratios of 1:10. On Day 4 of coculture, the cells were stained with PerCP-Cy5.5-conjugated anti-$CD4^+$ mAb, PerCP-Cy5.5-conjugated anti-$CD8^+$ mAb, Alexa 488-conjugated anti-CD62L mAb, and PE-conjugated anti-CD44 mAb and analyzed by flow cytometry. T-bet and GATA-3 expression in T cells isolated from *M. tuberculosis*-infected mice were assessed by immunoblotting using specific anti-T-bet and anti-GATA-3 mAb.

Statistical analysis

All experiments were repeated at least three times with consistent results. The levels of significance for comparison between samples were determined by one-way ANOVA, followed by Tukey's multiple comparison test using statistical software (GraphPad Prism Software, version 4.03; GraphPad Software, San Diego, CA, USA). The data in the graphs are expressed as the mean ± SEM. $^*P<0.05$, $^{**}P<0.01$, or $^{***}P<0.001$ was considered statistically significant.

RESULTS

Purification of rRpfB protein and cytotoxicity

Soluble rRpfB protein was first produced and characterized in the present study. The recombinant protein was extracted after cell disruption by sonication and purification using Ni-NTA resin and then dialyzed. SDS-PAGE and immunoblotting with an antihistidine antibody was then used to confirm the purity of the rRpfB protein and a molecular mass of ~40 kDa (Supplemental Fig. 1A). The purity of RpfB was quantified using Quantity One software (Bio-Rad, Hercules, CA, USA), as described previously [34]. The rRpfB was found to have >98% purity after passing 20 μg of the purified protein through a Ni-NTA column twice.

We next examined RpfB-induced cytotoxicity in DCs by treating cells with increasing amounts of RpfB protein up to 2.0 μg/ml for 24 h, followed by staining with anti-CD11c, Annexin V, and PI, to assess cell viability. Treatment with RpfB protein up to 2.0 μg/ml did not result in cellular toxicity in DCs (Supplemental Fig. 1B). Importantly, these findings indicate that our rRpfB protein was not cytotoxic to DCs and when used at concentrations below 2.0 μg/ml, does not contain significant amounts of endotoxin that would interfere with our studies.

Induction of DC maturation by RpfB protein

The maturation step of DCs is critical to their functional role as APCs in mediating immune responses to pathogens [25]. Thus, to investigate whether RpfB protein induces DC maturation, we first measured the expression of DC maturation markers, including CD80, CD86, and MHC classes I and II, by flow cytometry. BMDCs were cultured for 6 days in RPMI 1640, supplemented with GM-CSF and IL-4 under standard conditions, followed by 1 day of growth in the presence of 0.5, 1.0, or 2.0 μg/ml RpfB or LPS (as a positive control). We found that RpfB protein prompted DC maturation by significantly enhancing the expression of the costimulatory molecules, CD80 and CD86, as well as MHC class I and II, in a dose-dependent manner, suggesting that RpfB protein has the ability to induce DC maturation (**Fig. 1A**).

Cytokine profile of DCs stimulated with RpfB protein

The polarization of T cells is affected by a variety of cytokines secreted from DCs [35]. Thus, we sought to determine whether the RpfB-mediated maturation of DCs is coupled with the secretion of pro- or anti-inflammatory cytokines by stimulating DCs with various concentrations of RpfB (0.5, 1.0, or 2.0 μg/ml). RpfB stimulated DCs to secrete high levels of TNF-α, IL-6, and IL-1β, whereas untreated DCs secreted negligible amounts of these cytokines (Fig. 1B). We then investigated the production of IL-12p70 and IL-10, which are associated with the development of Th1 and Th2 cells, respectively. RpfB significantly induced the secretion of IL-12p70 but not IL-10 (**Fig. 2B**). We also found that the level of IL-12p70 production in RpfB-treated DCs was approximately tenfold higher than that of untreated DCs (Fig. 1C).

A loss of endocytic activity is one of the features of DC maturation. We investigated the role of RpfB protein in the regulation of DC endocytic activity. To test whether RpfB protein-stimulated DCs had the reduced endocytic activity characteristic of functionally mature DCs, we exposed DCs to RpfB protein in the presence of Dextran-FITC and determined the percentage of double-positive cells ($CD11c^+$ and Dextran-FITC-positive) using flow cytometry. A much lower percentage of active cells was present in LPS-treated DCs than in un-

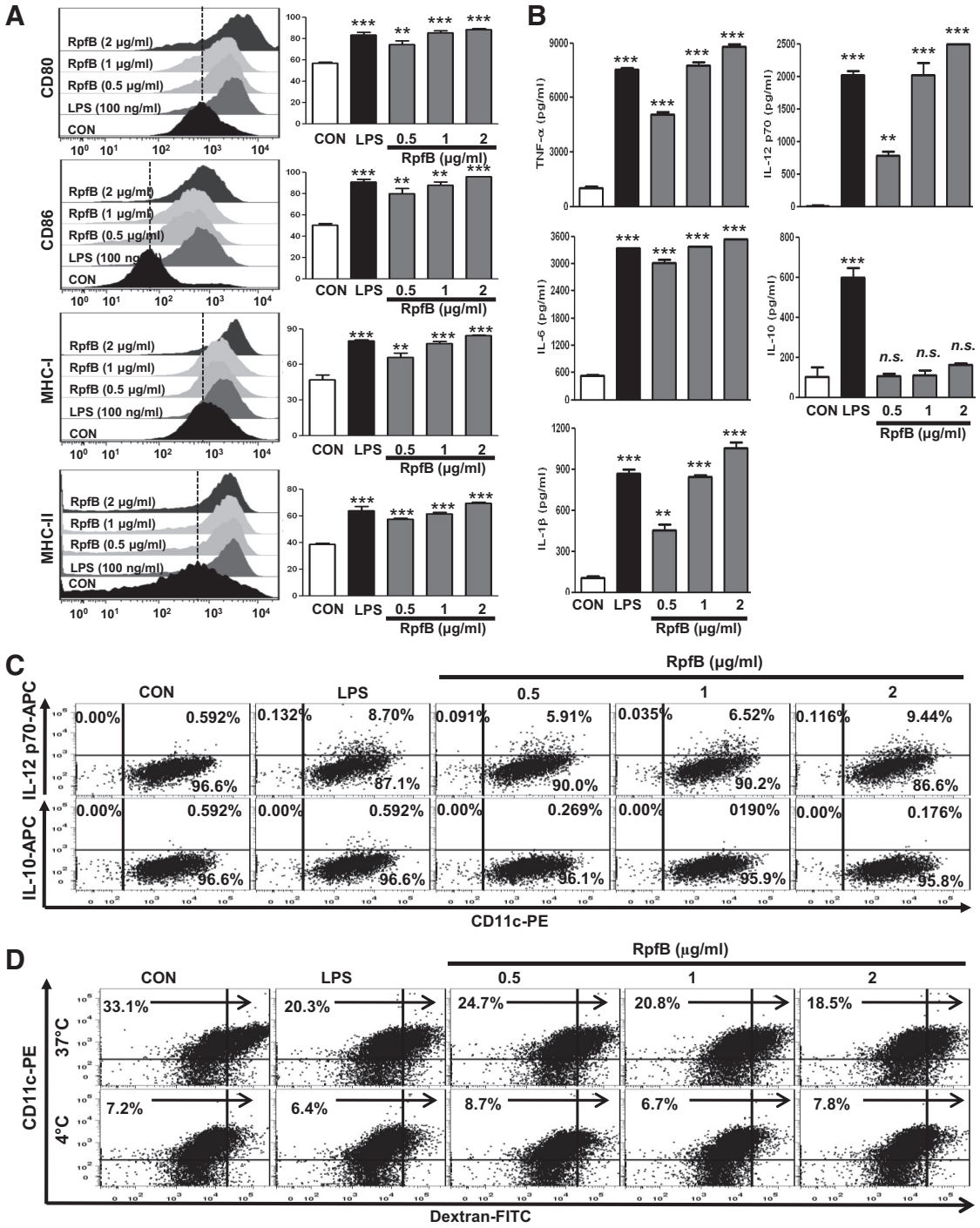

Figure 1. RpfB induces phenotypical and functional maturation of DCs. (A) iDCs (1×10^6 cells/ml) were cultured with GM-CSF and IL-4 alone [control (CON)] or GM-CSF, IL-4, or 0.5, 1, or 2 μg/ml RpfB or GM-CSF, IL-4, or 100 ng/ml LPS for 24 h and analyzed for surface marker expression by two-color flow cytometry. The cells were gated on CD11c cells. DCs were stained with anti-CD80, anti-CD86, or anti-MHC class I or anti-MHC class II. The percentage of positive cells is shown for each panel. The bar graphs show the mean ± SEM of the percentages of each surface molecule on $CD11c^+$ cells for three independent experiments. (B) Cytokine production by RpfB protein-treated DCs. DCs were generated by stimulating iDCs with 100 ng/ml LPS or 0.5, 1, or 2 μg/ml RpfB for 24 h. The amount of TNF-α, IL-6, IL-1β, IL-10, and IL-12p70 in the culture supernatant was measured by ELISA. All data are expressed as the means ± SD (n=3), and statistical significance (**P<0.01, or ***P<0.001) is shown for treatments compared with the controls. (C) Dot plots of intracellular IL-12p70 and IL-10 in $CD11c^+$ DCs. The percentage of positive cells is shown in each panel. APC, Allophycocyanin. (D) Reduced endocytic capacity of DCs induced by RpfB. DCs were treated with 100 ng/ml LPS or 0.5, 1, or 2 μg/ml RpfB for 24 h and stained with a PE-conjugated anti-$CD11c^+$ antibody. Endocytic activity at 37°C or 4°C was assessed by flow cytometry analysis of Dextran-FITC uptake. The percentages of Dextran-FITC-positive and $CD11c^+$-positive cells are indicated.

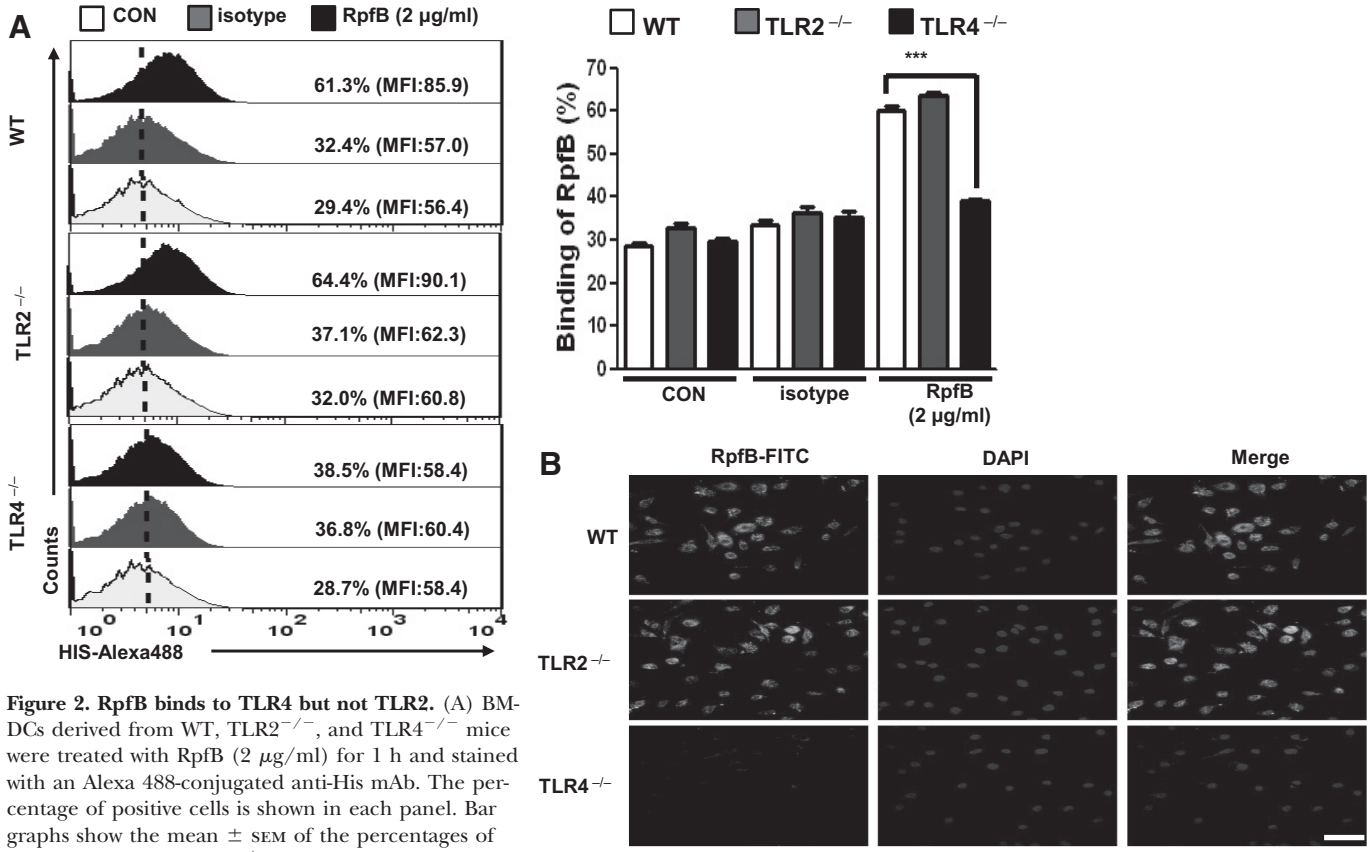

Figure 2. RpfB binds to TLR4 but not TLR2. (A) BM-DCs derived from WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice were treated with RpfB (2 μg/ml) for 1 h and stained with an Alexa 488-conjugated anti-His mAb. The percentage of positive cells is shown in each panel. Bar graphs show the mean ± SEM of the percentages of RpfB-Alexa 488 in CD11c^{+} cells from three independent experiments. Statistical significance (***P<0.001) is indicated for RpfB-treated TLR2$^{-/-}$ DCs compared with RpfB-treated WT DCs. MFI, Mean fluorescence intensity. (B) Fluorescence intensities of anti-RpfB bound to RpfB-treated DCs. DCs derived from WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice were treated with RpfB (2 μg/ml) for 1 h, fixed, and stained with DAPI and a FITC-conjugated anti-His antibody (original scale bar: 10 μm).

treated DCs (Fig. 1D). In addition, the same was true of RpfB protein-treated DCs when compared with untreated DCs. Thus, the RpfB protein-treated DCs had reduced endocytic activity, indicating more functional maturity. These experiments were also repeated at 4°C, and the results showed that the uptake of Dextran-FITC by DCs was inhibited at low temperatures. Taken together, these results strongly suggest that RpfB induces the secretion of proinflammatory cytokines and that RpfB-matured DCs induce Th1 polarization.

Engagement of TLR4 signaling in RpfB-induced DC maturation

Next, we examined whether RpfB could be recognized by, and act through, TLRs in DCs. To identify TLRs on DCs that interact with RpfB, WT, TLR2$^{-/-}$, and TLR4$^{-/-}$, DCs were stimulated with RpfB, and then RpfB on the cell surface was detected with an Alexa 488-conjugated anti-RpfB polyclonal antibody. Anti-RpfB bound to the cell surface of WT and TLR2$^{-/-}$ DCs, but not TLR4$^{-/-}$ DCs (Fig. 2A). This observation was also confirmed by confocal microscopy. Interestingly, we found that RpfB interacts preferentially with WT and TLR2$^{-/-}$ DCs but not TLR4$^{-/-}$ DCs (Fig. 2B).

To test the ability of RpfB to activate DCs via TLR4, we measured the expression of surface molecules and proinflammatory cytokine production in RpfB-treated WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ DCs. The expression of surface molecules (**Fig. 3A**) and proinflammatory cytokine secretion (Fig. 3B) was enhanced in WT and TLR2$^{-/-}$ DCs after RpfB treatment. In contrast, these effects were strongly diminished in TLR4$^{-/-}$ DCs, indicating that RpfB is an agonist for TLR4 in DCs. To further investigate the importance of the MyD88- and TRIF-dependent pathways in RpfB-induced cytokine production by DCs, we compared DC-based cytokine production in WT, MyD88$^{-/-}$, and TRIF$^{-/-}$ mice. RpfB-induced production of TNF-α, IL-6, and IL-1β was reduced significantly the absence of MyD88 and TRIF (Fig. 3C). Our results suggest that MyD88 and TRIF are crucial for an optimal RpfB-induced cytokine response. Taken together, these findings clearly demonstrate that RpfB induces DC maturation in a TLR4-dependent manner, causing increased expression of cell-surface molecules and proinflammatory cytokines.

Role of MAPK and NF-κB pathways in RpfB-induced maturation of DCs

We examined the activation of NF-κB and MAPKs in response to RpfB. DCs were stimulated with RpfB at a concentration of 2 μg/ml, and the phosphorylation of MAPKs (including ERK1/2, JNK, and p38), the phosphorylation/degradation of

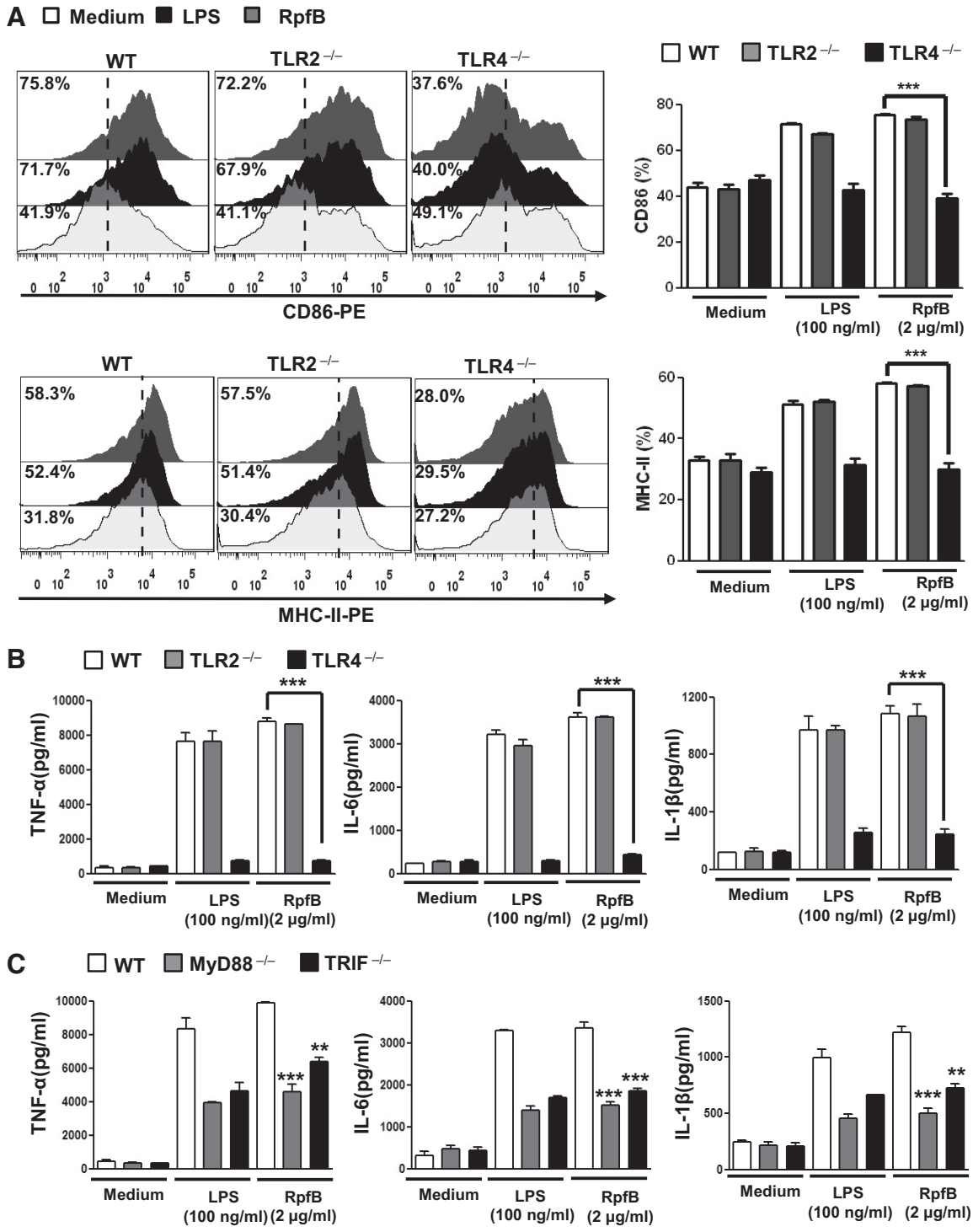

Figure 3. RpfB induces the activation of DCs via the interaction with TLR4. (A) Histograms showing CD86 or MHC II expression of RpfB-treated, $CD11c^+$-gated DCs derived from WT, $TLR2^{-/-}$, and $TLR4^{-/-}$ mice. DCs derived from WT, $TLR2^{-/-}$, and $TLR4^{-/-}$ mice were treated with RpfB (2 μg/ml) for 24 h. The percentage of positive cells is shown for each panel. Bar graphs show the mean ± SEM of the percentages for each surface molecule on $CD11c^+$ cells in three independent experiments. Statistical significance (***$P<0.001$) is indicated for RpfB-treated $TLR4^{-/-}$ versus RpfB-treated WT DCs. (B) DCs derived from WT, $TLR2^{-/-}$, and $TLR4^{-/-}$ mice were treated with RpfB or LPS for 24 h. TNF-α, IL-6, and IL-1β production in RpfB- 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 RpfB (2 μg/ml) and LPS (100 ng/ml) for 24 h. TNF-α, IL-6, and IL-1β production in RpfB- or LPS-treated DCs, derived from WT, $MyD88^{-/-}$, and $TRIF^{-/-}$ mice were measured by ELISA. All data are expressed as the means ± SD (n=3), and statistical significance (**$P<0.01$, or ***$P<0.001$) is indicated for treatments compared with RpfB-treated WT DCs.

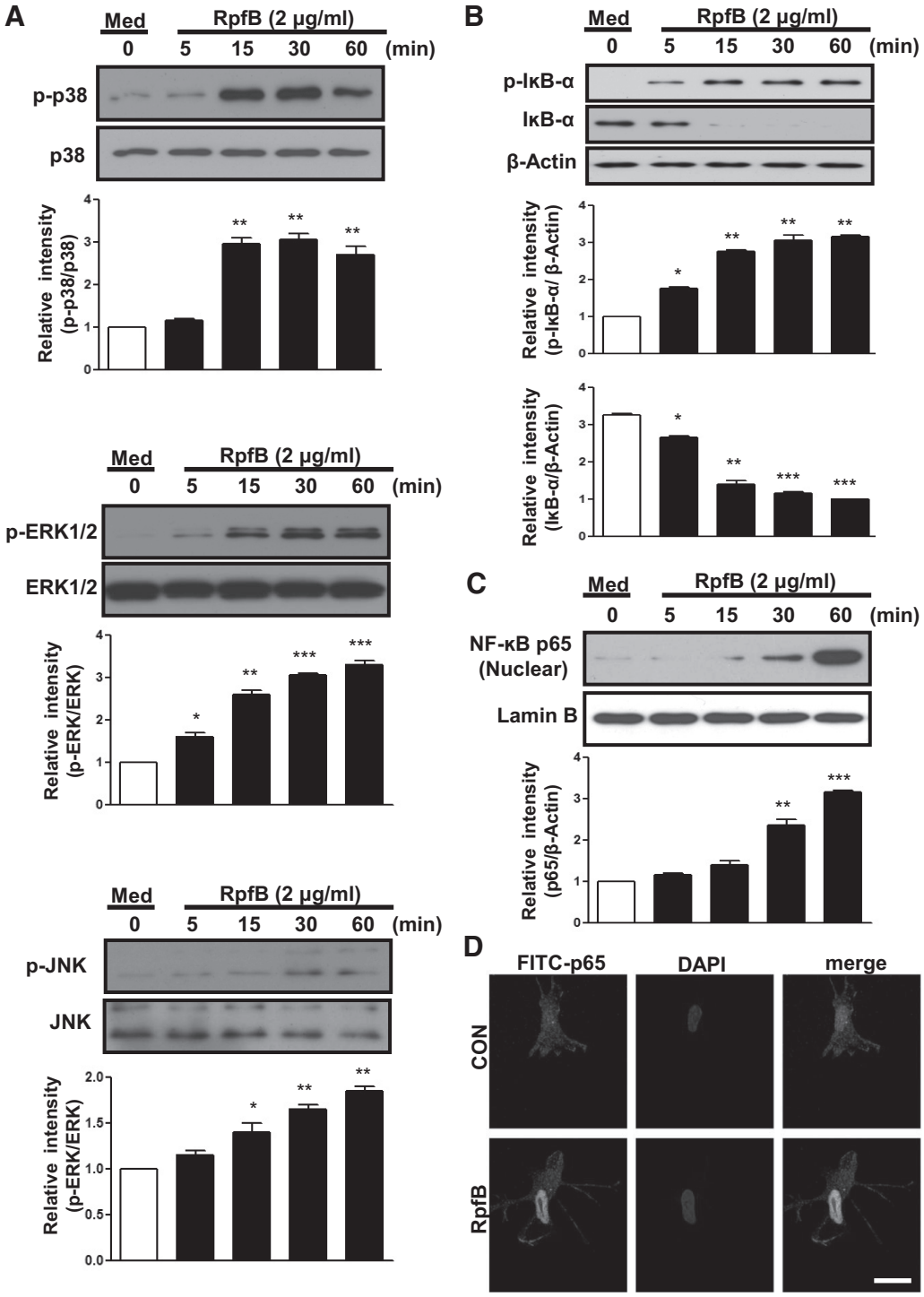

Figure 4. DC maturation triggered by RpfB involves activation of MAPKs and NF-κB. (A–C) DCs were treated with 10 μg/ml RpfB, and protein expression is shown over time. Cell lysates were subjected to SDS-PAGE, and immunoblot analysis was performed using antibodies specific to phospho (p)-p38, p38, phospho-ERK1/2, ERK1/2, phospho-JNK, JNK, phospho-IκB-α, IκB-α, and p65 NF-κB. β-Actin and Lamin B were used as loading controls for cytosolic and nuclear fractions, respectively. The relative band intensity of each protein is expressed as a percentage when compared with the value of untreated controls. The results shown are typical of three experiments performed under each condition. The data are shown as means ± SD (n=3), and statistical significance (*P<0.05, **P<0.01, or ***P<0.001) is indicated for treatments versus untreated DCs. Med, Medium. (D) Effect of RpfB on cellular localization of the p65 subunit of NF-κB in DCs. DCs were plated on covered glass chamber slides and treated with RpfB for 1 h. After stimulation, immunoreactivity of the p65 subunit of NF-κB in cells was determined by immunofluorescence, as described in the Materials and Methods (original scale bar: 5 μm).

IκB-α, and the nuclear translocation of p65 were analyzed over time. RpfB triggered the phosphorylation of MAPKs, including p38 JNK and ERK1/2, in DCs (see Fig. 6A). In addition, we found that RpfB induced the phosphorylation and degradation of IκB-α and significant translocation of p65 from the cytosol to the nucleus (**Fig. 4B–D**).

Next, to elucidate the functional roles of these kinases in the activation of DCs induced by RpfB, we used highly specific pharmacological inhibitors and measured RpfB-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 a NF-κB inhibitor (Bay 11-0782) for 1 h before being exposed to RpfB. Interestingly, we found that these pharmacological inhibitors significantly abrogated the RpfB-induced expression of costimulatory molecules CD80 and

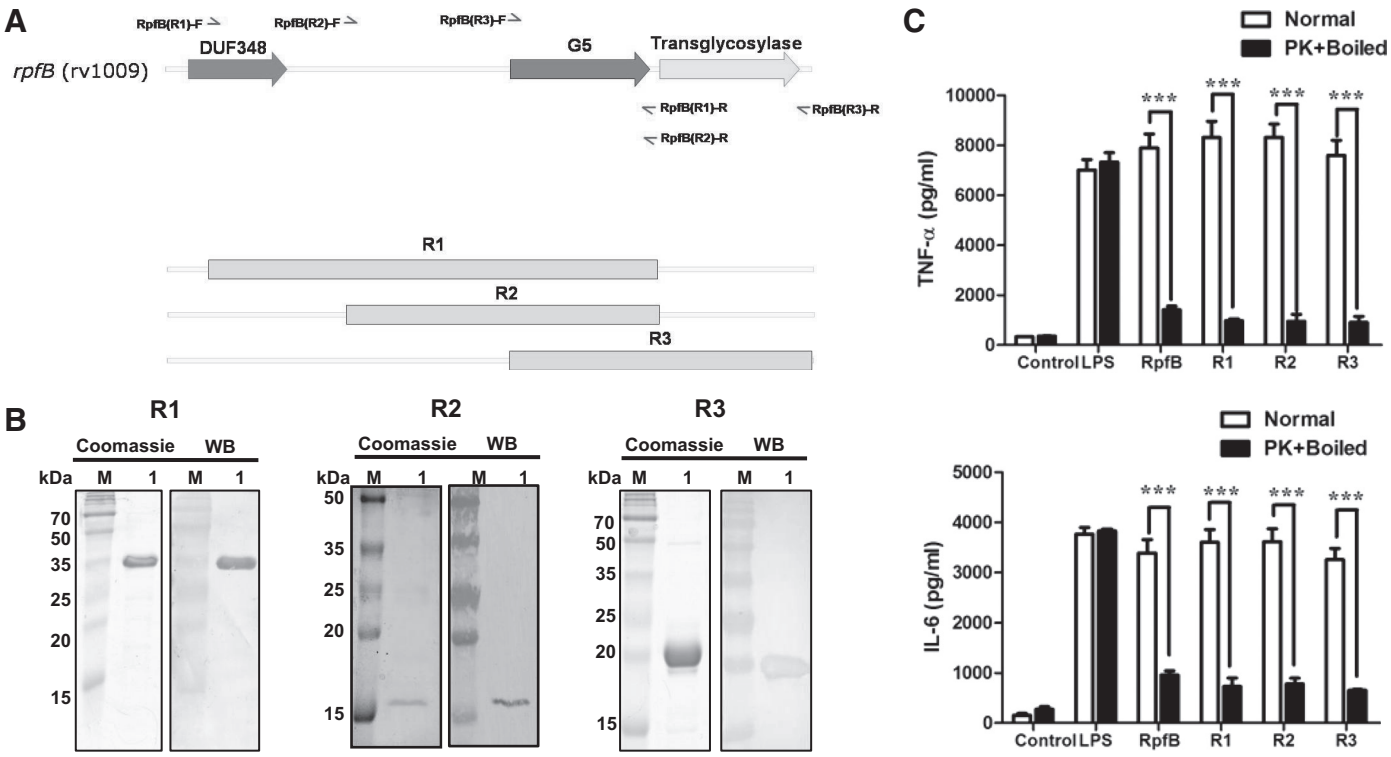

Figure 5. Purification of RpfB deletion mutants. (A) Schematic diagram of RpfB and of its deletion mutants. (B) rRpfB mutants were purified with NTA resin. The purified proteins were then subjected to SDS-PAGE and Western blot (WB) analysis, using 1:1000 mouse anti-His antibodies and CB staining. M, Marker. (C) Confirmation of endotoxin decontamination in purified RpfB mutant preparations and cytokine production by RpfB mutant-treated DCs. DCs were generated by stimulating iDCs with 100 ng/ml LPS or 2 μg/ml RpfB, R1, R2, or R3 mutant for 24 h. The amount of TNF-α and the culture supernatant was measured by ELISA. To ensure that RpfB mutant-induced DC maturation was not a result of endotoxin contamination during protein preparation, RpfB, R1, R2, and R3 mutants (2 μg/ml) were digested with proteinase K (PK; 10 μg/ml) for 1 h at 37°C. Then, the samples were boiled for 30 min. All data are expressed as the means ± SD (n=3), and statistical significance (***P<0.001) is indicated for treatments compared with RpfB, R1, R2, and R3, respectively.

CD86 on the surface of DCs (Supplemental Fig. 2A), production of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1β (Supplemental Fig. 2B), and translocation of p65 from the cytosol to the nucleus (Supplemental Fig. 2C). Based on 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 RpfB.

Confirmation of endotoxin-free, purified RpfB preparations

To ensure that RpfB-induced DC activation was not a result of endotoxin or LPS contamination in the protein preparations, all experiments were performed with purified RpfB preparations that were passed three times through a PmB agarose column. Endotoxin content was measured by a LAL assay and was found to be below 15 pg/ml (<0.1 EU/ml) in the RpfB preparations. Furthermore, we assessed endotoxin or LPS contamination by heat denaturation and treatment with Proteinase K or PmB. Heat denaturation and Proteinase K pretreatment abrogated the activity of RpfB to trigger DC maturation (Supplemental Fig. 3A and B). PmB treatment did not affect the viability of RpfB but did inhibit that of LPS significantly (Supplemental Fig. 3C).

We further confirmed the absence of LPS contamination of RpfB by trypsin digestion. The trypsin-treated RpfB was digested completely at 3 h (Supplemental Fig. 3D). The trypsin-digested RpfB abrogated MAPK activation and production of proinflammatory cytokines (Supplemental Fig. 3E and F). These results confirm that DC maturation was in fact induced by intact RpfB and not by contaminating endotoxins.

Identification of interacting regions in RpfB

RpfB has a DUF348, a G5 domain, and a transglycosylase-like domain. To identify the domains of RpfB interacting with TLR4, several deletion mutants of RpfB were designed (**Fig. 5A**). There was a total of three His fusion RpfB mutants containing the DUF348 and G5 domains (RpfB R1), the G5 domain (RpfB R2), and the G5 and transglycosylase-like domains (RpfB R3; Fig. 5B). We then treated DCs with the three mutant forms of RpfB (R1, R2, and R3) and measured TNF-α and IL-6 secretion levels. The results showed that all RpfB mutants induced production of TNF-α and IL-6 similar to WT RpfB treatment. However, boiled protei-

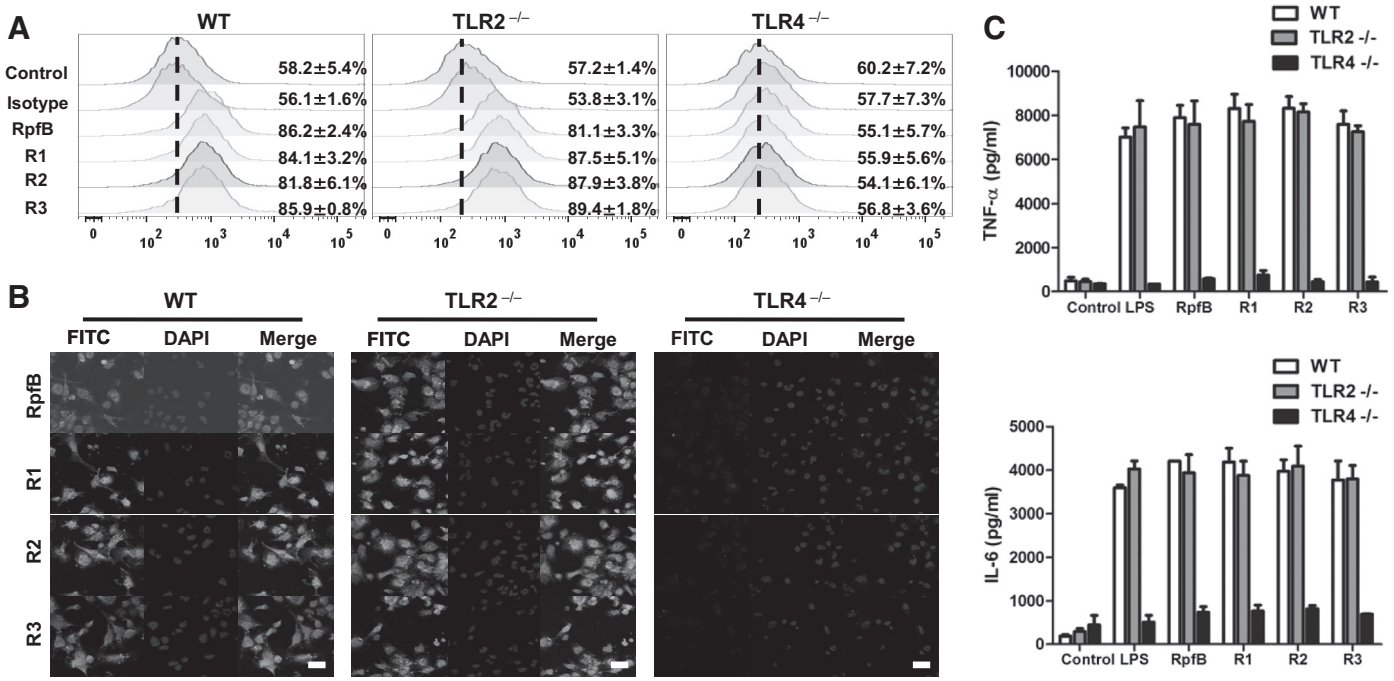

Figure 6. RpfB mutants bind to TLR4 but not TLR2. (A) BMDCs derived from WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice were treated with RpfB (2 μg/ml), R1, R2, and R3 for 1 h and stained with an Alexa 488-conjugated anti-His mAb. The mean percentage ± SEM in CD11c^{+} cells from three independent experiments is shown. (B) Fluorescence intensities of anti-His bound to RpfB-, R1-, R2-, and R3-treated DCs. DCs derived from WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice were treated with RpfB (2 μg/ml) for 1 h, fixed, and stained with DAPI and a FITC-conjugated anti-His antibody (original scale bar: 20 μm). (C) DCs derived from WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice were treated with RpfB, R1, R2, or R3 (2 μg/ml) or LPS (100 ng/ml) for 24 h. TNF-α and IL-6 in RpfB-, R1-, R2-, R3-, or LPS-treated DCs derived from WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ mice were measured by ELISA.

nase K-treated RpfB mutants did not increase TNF-α and IL-6 secretion (Fig. 5C).

Next, we examined domains of RpfB that bind to TLR4 in DCs. To do this, WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ DCs were each stimulated with the RpfB mutant preparations, and then RpfB mutants on the cell surface were detected with an Alexa 488-conjugated anti-His mAb. All three RpfB mutants bound to the cell surface of WT and TLR2$^{-/-}$ DCs but not TLR4$^{-/-}$ DCs (**Fig. 6A**). This observation was confirmed by confocal microscopy (Fig. 6B). We also measured the proinflammatory cytokine production in RpfB mutant-treated WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ DCs. Secretion of proinflammatory cytokines was enhanced in WT and TLR2$^{-/-}$ DCs after treatment with all RpfB mutants. However, these effects were diminished significantly in TLR4$^{-/-}$ DCs (Fig. 6C).

As all of the RpfB mutant preparations contained the G5 domain, we suspected that the RpfB G5 domain is a key part of TLR4 interaction. To confirm this hypothesis, we made mouse anti-RpfB R2 polyclonal antibodies. The immunoblot assay showed that anti-RpfB R2 antibodies bind strongly to RpfB, R1, R2, and R3 (**Fig. 7A**). We studied the effects of anti-RpfB R2 antibodies on the production of proinflammatory cytokines by DCs treated with RpfB, R1, R2, and R3. As shown in Figs. 7B and C, anti-RpfB R2 antibodies significantly inhibited the production of proinflammatory cytokines and activation of ERK1/2 and p38 by RpfB, R1, R2, and R3. We further investigated the effects of the anti-RpfB R2 antibodies on binding of RpfB, R1, R2, and R3 mutants to DCs by FACS analysis. The results show that the anti-RpfB R2 antibodies considerably inhibited the binding of RpfB, R1, R2, and R3 mutants to DCs (Fig. 7D). Taken together, these findings demonstrate that the RpfB G5 domain is the most important region in RpfB binding to TLR4.

Polarization of naïve CD4^{+} T and CD8^{+} cells to Th1-type T cell immunity via RpfB-treated DCs

The maturation of DCs is necessary to initiate proper adaptive immunity, as only mature DCs can present antigens to naïve T cells. To characterize precisely the effect of RpfB protein 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 RpfB-treated DCs pulsed with $OVA_{257–264}$ or $OVA_{323–339}$ proliferated to a significantly greater extent than the same T cells cocultured with DCs without RpfB treatment pulsed with $OVA_{257–264}$ or $OVA_{323–339}$ (**Fig. 8A**). In addition, naïve CD4^{+} and CD8^{+} T cells primed with RpfB-treated DCs produced significantly (P<0.05–0.01) higher IFN-γ and IL-2 levels than did those from untreated DCs, whereas a comparable level of IL-4 secretion was detected regardless of RpfB stimulation (Fig. 8B).

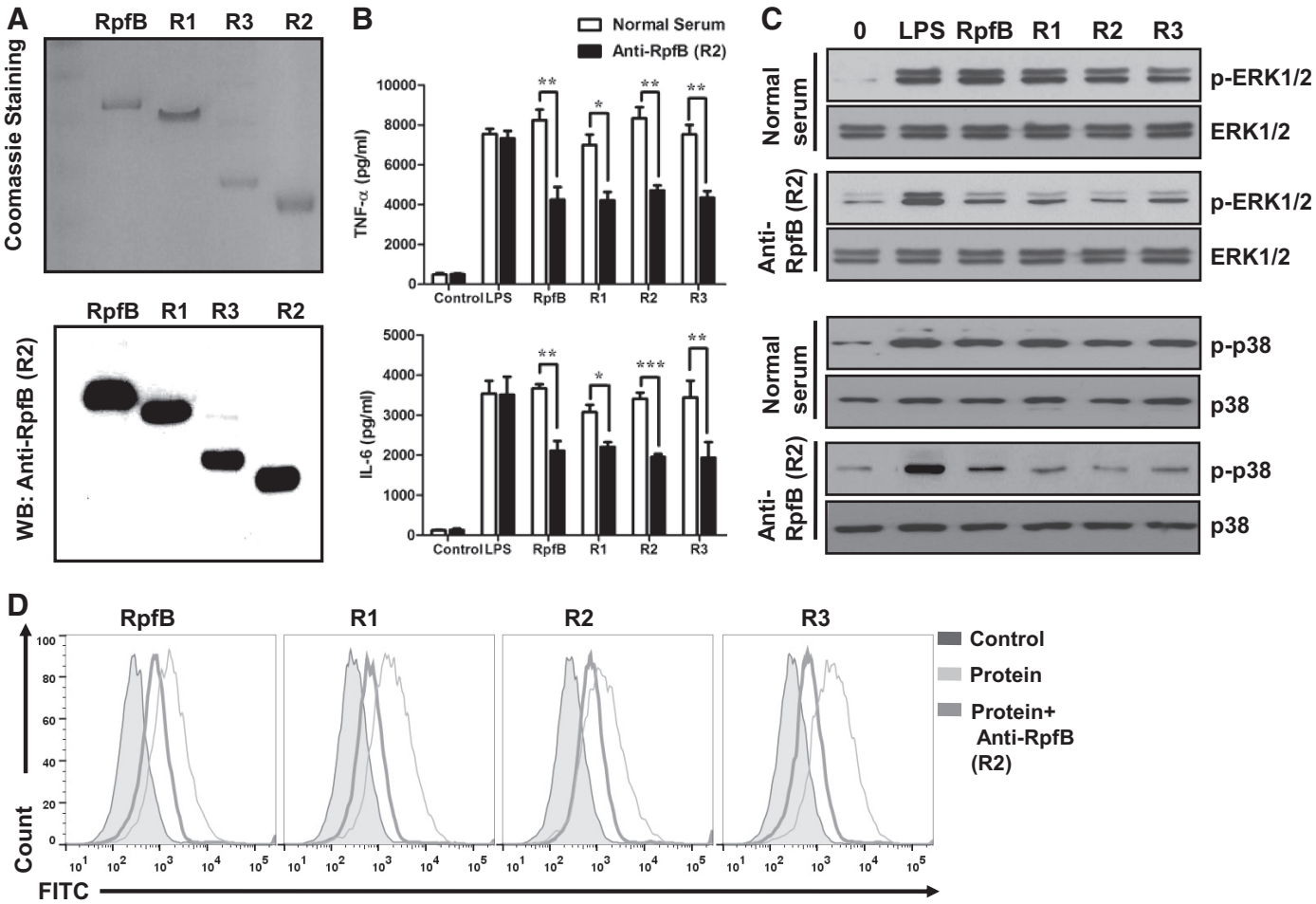

Figure 7. Effects of neutralization antibody on the RpfB-, R1-, R2-, and R3-treated DCs activation. (A) Western blot analysis of sera from BALB/c mice immunized with rRpfB R2 proteins. Sera from mice immunized and boosted three times with RpfB R2 proteins were diluted 1:5000 and used to probe RpfB, R1, R2, and R3 proteins that were separated on a SDS-PAGE gel. The secondary antibody was HRP-labeled goat anti-mouse IgG. (B) After pretreatment with 1 μg/ml anti-RpfB R2 antibodies for 1 h, DCs were treated with 2 μg RpfB, R1, R2, and R3 for 24 h. The effect of anti-RpfB R2 antibodies on cytokine production by the RpfB-, R1-, R2-, and R3-treated DCs was determined using ELISA, as described in Materials and Methods. All data are expressed as the mean ± SD (n = 3) and statistical significance (*P<0.05, **P<0.01, or ***P<0.001) is shown for treatments compared to the normal serum. (C) After pretreatment with 1 μg/ml anti-RpfB R2 antibodies for 1 h, DCs were treated with 2 μg RpfB, R1, R2, and R3 for 1 h. Cells were then lysed, and 30 μg proteins were subjected to SDS-PAGE, transferred to PVDF membranes, and probed with the indicated antibodies. (D) DCs were pretreated with anti-RpfB R2 antibodies (1 μg/ml) for 1 h, and cells were treated with 2 μg RpfB, R1, R2, and R3 for 1 h. Then, cells were stained with an Alexa 488-conjugated anti-His mAb.

We then investigated the expression of chemokine receptors CXCR3 and CCR3, which are associated with T cell polarization in $CD4^+$ T cells using flow cytometry. CXCR3 is known to be preferentially expressed on Th1 cells, whereas Th2 cells favor the expression of CCR3 [36]. As shown in Fig. 8C, $CD4^+$ T cells cocultured with RpfB-treated DCs and pulsed with $OVA_{323–339}$ showed a significant increase in the expression of CXCR3 compared with those cocultured with DCs pulsed with $OVA_{323–339}$. No alterations in the expression of CCR3 induced by RpfB treatment were detected, whereas LPS-treated DCs significantly increased the expression of CCR3 in $CD4^+$ T cells. Furthermore, we found that RpfB-treated DCs elevated the expression of T-bet, which is a Th1-specific transcription factor that maintains the Th1 phenotype in naïve $CD4^+$ T cells, whereas the expression of GATA-3, which is essential for Th2 development, was not observed (Fig. 8D). These results suggest that RpfB directs naïve T cell proliferation toward a Th1 phenotype.

Development and maintenance of effector/memory T cells responding to RpfB via TLR4-mediated DC activation

To assess whether mature DCs treated with RpfB can specifically induce effector/memory T cell populations in *M. tuberculosis*-infected mice during the course of infection, we analyzed the surface expression of CD62L and CD44 on $CD4^+$ and $CD8^+$ T cells using flow cytometry. As clinical latency in humans is characterized by stable, low bacillary loads, a low-dose persistence model via the i.v. route of infection was established in this study. $CD4^+$ and $CD8^+$ T cells from the spleens of *M.*

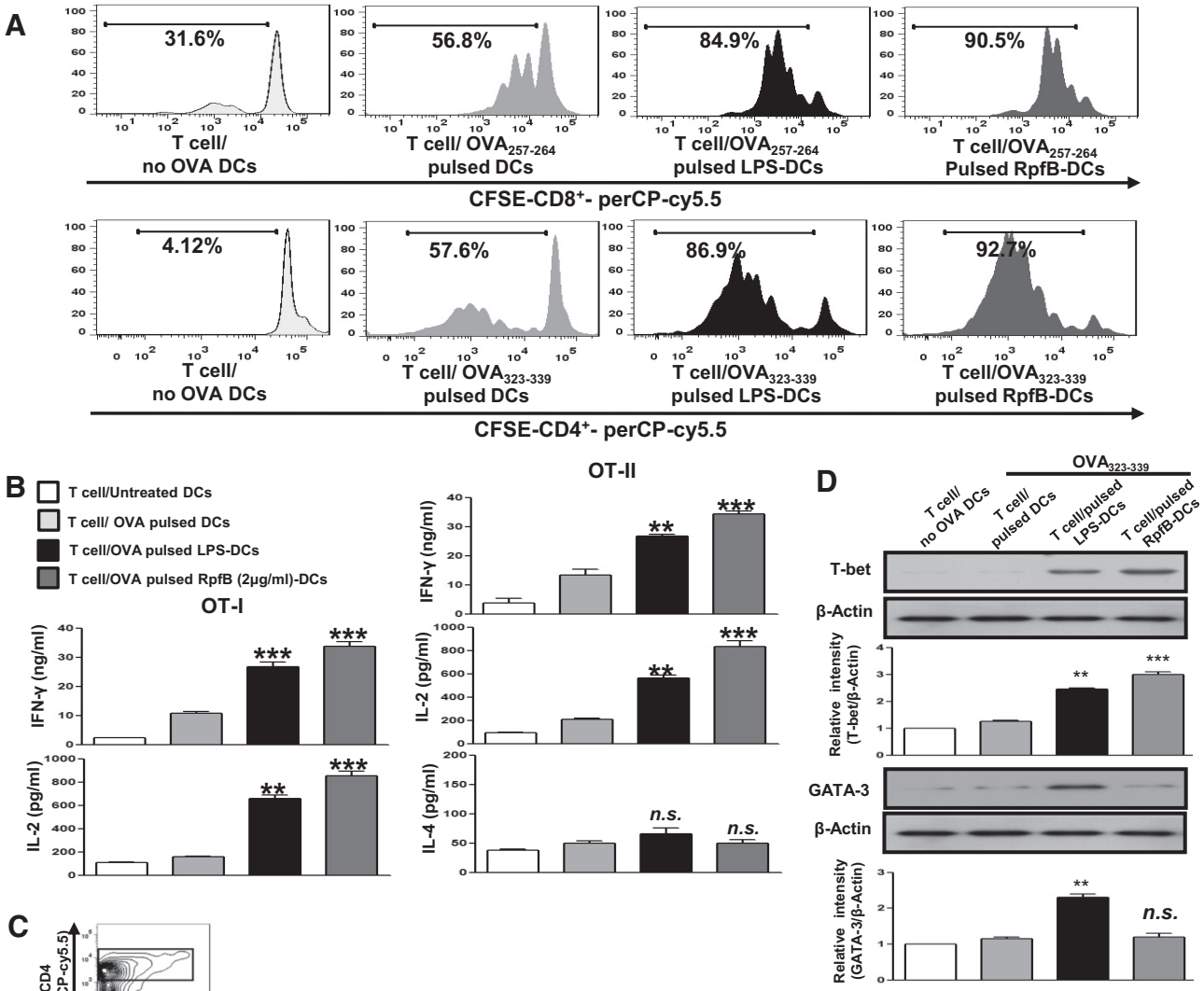

Figure 8. RpfB protein-treated DCs stimulate T cells to produce Th1 cytokines. (A) Transgenic OVA-specific $CD8^+$ T cells and transgenic OVA-specific $CD4^+$ T cells were isolated, stained with CFSE, and cocultured for 96 h with DCs treated with RpfB (2 μg/ml) or LPS (100 ng/ml) and then pulsed with $OVA_{257–264}$ (1 μg/ml) for OVA-specific $CD8^+$ T cells or $OVA_{323–339}$ (1 μg/ml) for OVA-specific $CD4^+$ T cells. T cells only and T cells cocultured with untreated DCs served as controls. The proliferation of OT-I^+ and OT-II^+ T cells was then assessed by flow cytometry. (B) The culture supernatants obtained from the conditions described in A were harvested after 24 h, and IFN-γ, IL-2, and IL-4 were measured by ELISA. (C) Splenocytes were stained with anti-CXCR3 mAb or anti-CCR3 mAb. The 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. (D) T-bet and GATA-3 expression in the OVA-specific $CD4^+$ T cells was assessed by immunoblotting using specific anti-T-bet and anti-GATA-3 mAb. The mean ± SEM is shown for three independent experiments, and statistical significance (*$P<0.05$, **$P<0.01$, or ***$P<0.001$) is shown for treatments compared with the appropriate controls (T cell/$OVA_{257–264}$-pulsed DCs or T cell/$OVA_{323–339}$-pulsed DCs). Treatments with no significant effect are indicated as *n.s.*

tuberculosis-infected mice at 6 and 25 weeks postinfection were cocultured with RpfB-treated DCs derived from WT, TLR2$^{-/-}$, or TLR4$^{-/-}$ mice. As shown in **Fig. 9A** and Supplemental Fig. 4A, the RpfB-treated WT and TLR2$^{-/-}$ DCs specifically induced the formation of effector/memory T cells by significantly down-regulating CD62L and up-regulating CD44 expression in CD4$^+$ and CD8$^+$ T cells from the spleens of *M. tuberculosis*-infected mice compared with control DCs and LPS-treated DCs. In contrast, this effect of RpfB was abrogated in TLR4$^{-/-}$ DCs. 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 RpfB-pulsed WT or TLR2$^{-/-}$ DCs were higher than those among T cells cocultured with untreated DCs or LPS-pulsed DCs, whereas elevation of these cytokines was not observed in T cells cocultured with RpfB-treated TLR4$^{-/-}$ DCs. Additionally, IL-4 expression by CD4$^+$ cells among T cells treated with RpfB-pulsed DCs or LPS-pulsed DCs remained at baseline levels (Fig. 9B and Supplemental Fig. 4B). Furthermore, we found that RpfB-treated DCs elevated the expression of T-bet in effector/memory T cells, whereas the expression of GATA-3 was not observed (Fig. 9C). The percentages of CD4-IFN-γ/CD4-IL-2 and CD8-IFN-γ/CD8-IL-2 double-positive cells among T cells treated directly with RpfB were higher than those among infected T cells only, and the percentages of CD4-IFN-γ/CD4-IL-2 and CD8-IFN-γ/CD8-IL-2 double-positive cells were higher in T cells cultured with RpfB-pulsed DCs than without DCs (Supplemental Fig. 4C). These data suggest that RpfB-mediated DC activation induces the activation of effector/memory T cells in a TLR4-dependent manner and drives Th1 immune responses in chronic infection persistence and severe disease progression.

DISCUSSION

In the present study, we established the molecular basis for DC maturation and its functional roles in response to RpfB. Mechanistic investigations demonstrated that the activation of DCs was medicated by direct binding of RpfB to TLR4, followed by MyD88/TRIF signaling, which is the downstream signaling pathway of TLR4, to MAPK and NF-κB signaling pathways. Specifically, the G5 domain of RpfB has an important role in TLR4 binding. Furthermore, RpfB-treated DCs activated naïve 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 contributes to Th1 polarization of T cell immunity. Notably, unlike with LPS, RpfB-treated DCs specifically expanded a population of CD44^{high}CD62L^{low} CD4$^+$/CD8$^+$ effector/memory cells from splenic T cells collected from *M. tuberculosis*-infected mice during the course of infection, indicating that RpfB acts as a specific recall antigen.

Although a number of antigens in *M. tuberculosis* have been described for their interaction with host cells, these antigens were not sufficient for the development of a protective immune response that restricts and controls infection. One major reason for this may be their poor expression or immunogenicity [4, 37]. In fact, many antigens selected from in vitro studies have impaired in vivo T cell priming by APCs along with the generation of considerable protective immunity [28–30]. It may be possible to grow bacilli in vivo for different disease statuses to express discrete molecules from in vitro culture, indicating that protective antigens, driven by *M. tuberculosis*, growing inside of macrophages, are necessary for the generation of effector and memory T cells responsible for protective immunity [13, 38]. RpfB is an abundantly expressed antigen during in vivo growth, and transcriptional profiling studies have shown that *rpfB* is preferentially expressed in vivo [15]. In addition, RpfB is readily accessible to APCs, as it is thought to be a secreted or cell-wall-associated antigen [17]. As described previously, many of the cell-wall-associated or secreted antigens of *M. tuberculosis* are targets of host immune responses as a result of their accessibility for interactions with DCs.

Regarding the adaptive immune responses induced by *RpfB*, a recent study noted that specific IFN-γ expression in response to *RpfB* was observed in CD4$^+$CD45RO$^+$ memory T cells of individuals with a latent *M. tuberculosis* infection [16, 39]. In addition, elevated levels of IFN-γ production were detected as early as 4 weeks after *M. tuberculosis* infection or BCG vaccination in spleen cell cultures from C57BL/6 and BALB/c mice in response to treatment with rRpfB [16, 39]. These results are consistent with those from our study. In the present study, RpfB-treated DCs specifically induced the proliferation of effector/memory cells from splenic T cells of *M. tuberculosis*-infected mice with a high level of IFN-γ and elevated T-bet expression, although a low bacterial burden (~10^3 CFUs/total organ) was detected in the spleen and lungs, 6 weeks postinfection.

Although T cell responses need to be maintained for *M. tuberculosis* to be controlled during chronic disease, the degree to which *M. tuberculosis* antigens recognized by T cells change, according to the TB phases, remains unclear. Some antigenic proteins, such as Ag85B, are expressed primarily during the early stages of *M. tuberculosis* infection, and T cells recognizing Ag85B and other antigens, expressed with similar kinetics, may not contribute to protection during chronic infection [40]. Other antigenic proteins (such as ESAT-6) seem to be recognized by antigen-specific T cells during acute and chronic stages of disease [41]. T cells recognizing ESAT-6 express cell surface markers with characteristics of effector T cells and undergo high levels of proliferation, both of which are suggestive of chronic antigenic stimulation [42]. However, T cells recognizing *M. tuberculosis* antigens, expressed during latent infection but not active TB, are not well-characterized. In addition, the vaccine efficacy of the antigens differs according to the stages of infection. For example, the Ag85A subunit vaccine was protective against *M. tuberculosis* infection 30 days postchallenge, whereas the HspX antigen (the latency-associated antigen) vaccine only induced significant protective efficacy 90 days postchallenge [43, 44]. Interestingly, Ag85A and HspX combined had protective efficacy against *M. tuberculosis* challenge during the early and late stage of infection [43, 44]. Thus, characterization of *M. tuberculosis* antigen expression during latent infection could be instrumental in the development of immune interventions, effective against latent infection and the reactivation of TB.

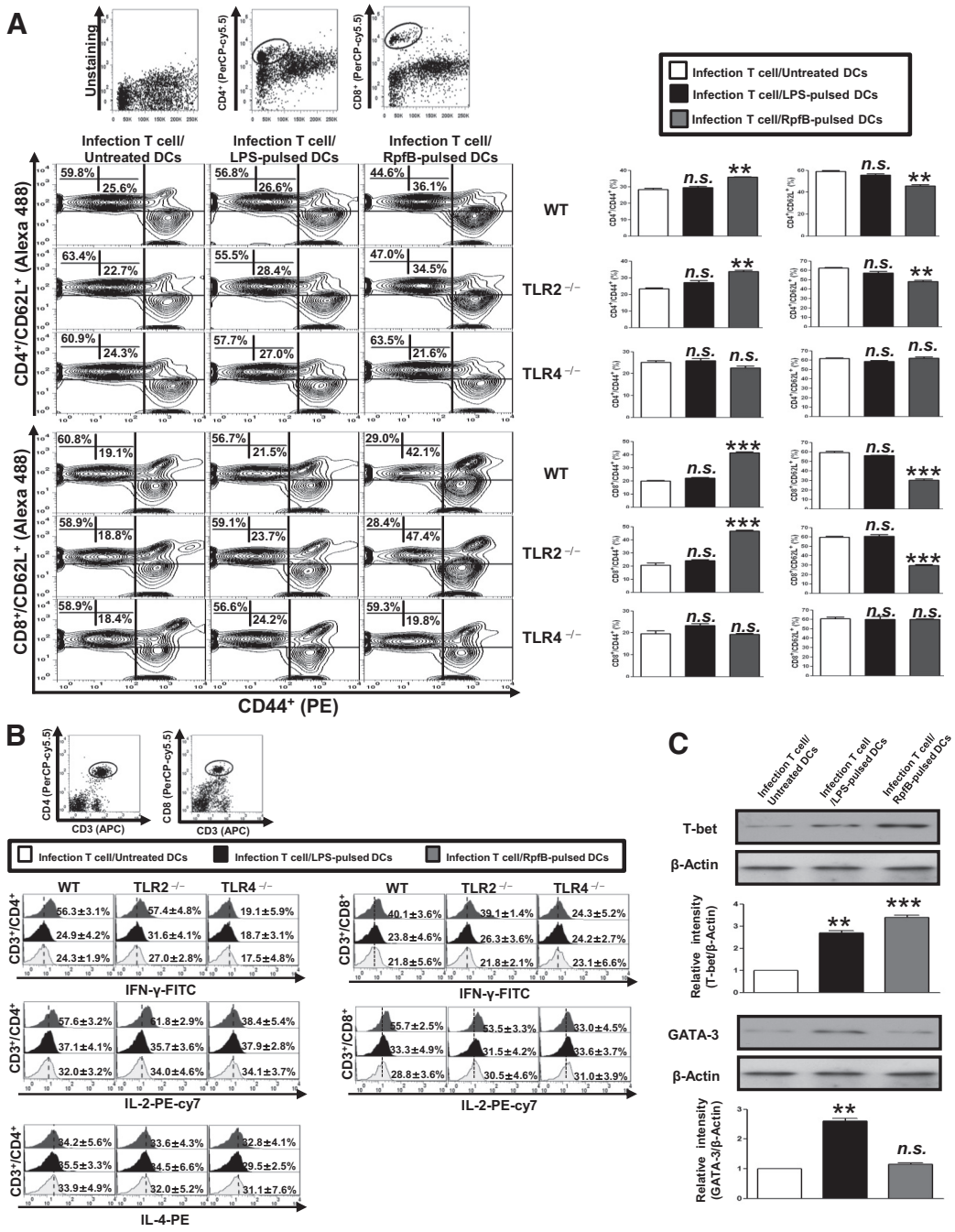

Figure 9. RpfB-stimulated DCs induce the effector/memory T cell proliferation via TLR4 signaling. (A) WT, TLR2$^{-/-}$, and TLR4$^{-/-}$ DCs were cultured with 2 μg/ml RpfB for 24 h. The RpfB-matured DCs were then washed and cocultured with allogeneic T cells at DC:T cell ratios of 1:10 for 3 days. Splenocytes were stained with anti-CD4, anti-CD8 mAb, anti-CD62L, and anti-CD44 mAb. Contour and bar graphs show CD62L$^+$CD44$^+$ T cells in the spleen. Bar graphs show the percentages (mean±SEM) for CD4$^+$/CD44$^+$CD62L$^+$ and CD8$^+$/CD44$^+$CD62L$^+$ T cells from three independent experiments. Statistical significance (**P<0.01 or ***P<0.001) is indicated for treated DCs compared with untreated DCs, and the lack of a significant effect is indicated as n.s. (B) IFN-γ, IL-2, and IL-4 expression in CD3$^+$/CD4$^+$ and CD3$^+$/CD8$^+$ cells was analyzed in T cells cocultured with RpfB-pulsed DCs or LPS-pulsed DCs by intracellular IFN-γ, IL-2, or IL-4 staining. The percentages of double-positive cells among the T cells are indicated in the top-right corner. The results are presented as means ± SD of each group (n=5/group), and this experiment was repeated three times with similar results. (C) WT DCs were cultured with 2 μg/ml RpfB for 24 h. The RpfB-matured DCs were then cocultured with T cells from *M. tuberculosis*-infected mice for 3 days. T-bet and GATA-3 expression in T cells from *M. tuberculosis*-infected mice was assessed by immunoblotting using specific anti-T-bet and anti-GATA-3 mAb. The results shown are typical of three experiments performed under each condition. The data shown are means ± SD (n=5), and statistical significance (**P<0.01, or ***P<0.001) is indicated for treatments versus untreated DCs.

Rapid initiation and maintenance of adaptive immunity have been found to be critical for successful immune protection before *M. tuberculosis* can effectuate an array of pathogen-beneficial regulatory networks [45]. To avert this immune control, virulent *M. tuberculosis* has devised multiple strategies to slow the initial T cell response [45, 46]. In the present study, we adopted a steady-state chronic infection model mimicking human latent TB. This low-dose model maintains the bacterial load at a stable level for 20 weeks postinfection (data not shown). Our study showed that the memory T cells responding to RpfB developed rapidly in the early stage of infection and were maintained in late, active disease progression, indicating that RpfB may induce T cell immunity via rapid DC activation. Although the relative contributions of each of these types of antigens to protective immunity during each stage of infection remain poorly understood, it is important to consider universal antigens, such as RpfB, that induce long-lived memory T cell responses from latent infection to active disease, and this has obvious implications for vaccine design.

DCs play a critical role in priming and modulating T cell-mediated immunity against mycobacterial antigens [25]. When DCs initiate a T cell-mediated adaptive immune response, they also play important roles in Th1/2 polarization, depending on the antigen properties. For example, it has been shown that the *M. tuberculosis* antigens Rv0754 and Rv0978c induce DC maturation and activation and enhance the ability of DCs to stimulate $CD4^+$ T cells [30]. These antigens were also reported to increase the production of proinflammatory cytokines, such as IL-12, IL-6, and IL-8, through the NF-κB and MAPK signaling pathways [30]. In contrast, Rv1917c of *M. tuberculosis* (PPE34) induces the 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 antigen processing of macrophages, resulting in a decrease in IFN-γ production [47].

Obviously, the induction and maintenance of Th1-polarized immune responses are thought to be critical to host protection against *M. tuberculosis* infection [48]. In other contexts, IL-12p70 and IL-2 are clearly essential for differentiation and expansion of naive T cells into IFN-γ-producing effector cells in TB, which provides strong evidence that continuous expression of IL-12p70 and IL-2 is required to confer resistance to TB progression [45]. In addition, parenteral administration of IL-12p70 to *M. tuberculosis*-infected, IL-$12p40^{-/-}$ mice restores $CD4^+$ T cell production of IFN-γ and control of bacterial growth in the lungs and spleen, whereas these effects are lost when administration of IL-12p70 is discontinued [45]. Our results clearly demonstrated that RpfB-treated DCs specifically induced the expansion of $CD4^+/CD8^+CD44^{high}CD62L^{low}$ memory T cells along with a high proportion of cells producing IL-2, which was significantly biased toward a Th1 phenotype, together with complete abrogation of IL-4 secretion. Moreover, we found that RpfB-treated DCs elevated T-bet expression in $CD4^+$ cells but did not induce the expression of GATA-3, indicating that RpfB participates in adaptive immunity by directing T cell immune responses to Thl polarization. Previously, it was reported that T-bet is expressed in developing $CD4^+$ Th1 cells, driving IFN-γ production, Th1 differentiation, and repression of the alternate Th2 program [49].

It appears that distinct mycobacterial components may interact with different members of the TLR family, thus increasing the likelihood that a pathogen will be recognized by several mechanisms. TLRs represent potent targets for the development of various vaccines, including those against mycobacterial diseases [50]. In addition, infection with whole bacilli evokes a more complex activation pattern involving at least TLR2 and TLR4 and leads to differential activation of antibacterial effector pathways, although TLR2 and TLR4 could be redundant in the control of *M. tuberculosis* infection [51–53]. We reported previously that a *M. tuberculosis* antigen, Rv0577, regulates innate and adaptive immune responses against *M. tuberculosis* infection by interacting with TLR2 [31]. Although TLR2 and its related signaling pathways have been more emphasized in the mycobacteria fields than TLR4, TLR4 signaling appears to be required to control the local growth and dissemination of *mycobacterial* infection from target organs. For example, the HBHA, a virulence factor of *M. tuberculosis*, identified recently as a novel TLR4 agonist, promotes the dissemination of tubercle bacilli from the lungs to other tissues in experimental TB models [54]. However, HBHA also induces protective immunity against *M. tuberculosis* by triggering IFN-γ production in $CD4^+$ and $CD8^+$ T lymphocytes and has been considered a good vaccine candidate [54]. Similar to HBHA, RpfB acts as a cell-wall-associated virulence factor in the reactivation of TB [22], but it has attractive vaccine potential, as RpfB links innate and adaptive immunity through DC activation in a TLR4-dependent manner, resulting in induction of a Th1 cell-mediated immune response and memory T cell formation.

RpfB contains a DUF348 domain at its N-terminus, a G5 domain in the middle, and a transglycosylase-like domain at its C-terminus. Initially, we attempted to design mutants with only one of each of the three domains. However, mutants with domain overlap are inevitable as a result of the size and solubility of the recombinant protein after purification. Specifically, it is impossible to purify a mutant containing only the transglycosylase-like domain when making a His-tag recombinant protein, as most of the protein is aggregated as a result of the extremely low solubility. In this investigation, the G5 domain was neutralized in overlapping domain mutants using specific anti-RpfB R2 antibody to confirm that the G5 domain is an essential part of TLR4 binding and to compensate for experimental weaknesses. Our results clearly showed that DC activation and binding were partially repressed by anti-RpfB R2 treatment. However, the antibody treatment did not entirely repress DC activation and binding induced by RpfB and each mutant. The partial repression could have been caused by imperfect neutralization of RpfB by anti-RpfB R2 or by TLR4 stimulation as a result of short-term binding of the antibody. In addition, an insufficient amount of antibody treatment could have been responsible for the partial repression. Further studies are required to understand the exact mechanisms underlying RpfB G5 domain binding to TLR4.

Collectively, immunoprotective subunit vaccines are attractive alternatives, as even single antigens can successfully control TB in experimental models. The novelty of RpfB lies in its

ability to incite innate and adaptive immunity simultaneously with a self-adjuvanting, property-activating DC toward Th1 polarized-naïve/memory T cell expansion in a TLR4-dependent cascade. This antigen succeeded in inducing long-lasting Th1 memory, which is the central feature of a successful TB vaccine. Our study is the first to explain why RpfB has the ability to induce protective immunity against *M. tuberculosis* challenge.

AUTHORSHIP

J-S.K., W.S.K., and H-G.C. designed and performed experiments, analyzed data, and wrote the manuscript. K.L., B.J., and H-G.C. performed research. J-H.P. and H-J.K. contributed to the discussion and interpretation of the results. S-N.C. contributed to the discussion of the results and to writing the paper. S.J.S. initiated the idea, supervised the team, designed the experiments, and critically revised the manuscript. All authors read and approved the final version of the manuscript.

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