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*Correspondence to

Yong Taik Lim
SKKU Advanced Institute of Nanotechnology (SAINT), Department of Nano Engineering, School of Chemical Engineering, Sungkyunkwan University, 2066 Seobu-ro, Jangan-gu, Suwon 16419, Korea.
Email: yongtaik@skku.edu

Sung Jae Shin
Department of Microbiology, Yonsei University College of Medicine, 50-1 Yonsei-ro, Seodaemun-gu, Seoul 03722, Korea.
Email: sjshin@yuhs.ac

Sang-Jun Ha
Department of Biochemistry, College of Life Science & Biotechnology, Yonsei University, 50 Yonsei-ro, Seodaemun-gu, Seoul 03722, Korea.
Email: sjha@yonsei.ac.kr

[†]Kee Woong Kwon and Tae Gun Kang contributed equally to this work.

Protective Efficacy and Immunogenicity of Rv0351/Rv3628 Subunit Vaccine Formulated in Different Adjuvants Against *Mycobacterium tuberculosis* Infection

Kee Woong Kwon [1,†], Tae Gun Kang [2,3,†], Ara Lee [2,3], Seung Mo Jin [4], Yong Taik Lim [4,*], Sung Jae Shin [1,5,*], Sang-Jun Ha [2,3,*]

[1]Department of Microbiology, Graduate School of Medical Science, Brain Korea 21 Project, Yonsei University College of Medicine, Seoul 03722, Korea
[2]Department of Biochemistry, College of Life Science & Biotechnology, Yonsei University, Seoul 03722, Korea
[3]Brain Korea 21 (BK21) FOUR Program, Yonsei Education & Research Center for Biosystems, Yonsei University, Seoul 03722, Korea
[4]SKKU Advanced Institute of Nanotechnology (SAINT), Department of Nano Engineering, School of Chemical Engineering, Sungkyunkwan University (SKKU), 2000066 Seobu-ro, Jangan-gu, Suwon 16419, Korea
[5]Institute for Immunology and Immunological Disease, Yonsei University College of Medicine, Seoul 03722, Korea

ABSTRACT

Bacillus Calmette-Guerin (BCG) vaccine is the only licensed vaccine for tuberculosis (TB) prevention. Previously, our group demonstrated the vaccine potential of Rv0351 and Rv3628 against *Mycobacterium tuberculosis* (Mtb) infection by directing Th1-biased CD4^{+} T cells co-expressing IFN-γ, TNF-α, and IL-2 in the lungs. Here, we assessed immunogenicity and vaccine potential of the combined Ags (Rv0351/Rv3628) formulated in different adjuvants as subunit booster in BCG-primed mice against hypervirulent clinical Mtb strain K (Mtb K). Compared to BCG-only or subunit-only vaccine, BCG prime and subunit boost regimen exhibited significantly enhanced Th1 response. Next, we evaluated the immunogenicity to the combined Ags when formulated with four different types of monophosphoryl lipid A (MPL)-based adjuvants: 1) dimethyldioctadecylammonium bromide (DDA), MPL, and trehalose dicorynomycolate (TDM) in liposome form (DMT), 2) MPL and Poly I:C in liposome form (MP), 3) MPL, Poly I:C, and QS21 in liposome form (MPQ), and 4) MPL and Poly I:C in squalene emulsion form (MPS). MPQ and MPS displayed greater adjuvancity in Th1 induction than DMT or MP did. Especially, BCG prime and subunit-MPS boost regimen significantly reduced the bacterial loads and pulmonary inflammation against Mtb K infection when compared to BCG-only vaccine at a chronic stage of TB disease. Collectively, our findings highlighted the importance of adjuvant components and formulation to induce the enhanced protection with an optimal Th1 response.

Keywords: *Mycobacterium tuberculosis*; Vaccine formulation; Adjuvant; BCG prime subunit booster; Th1 immune response

ORCID iDs
Kee Woong Kwon (iD)
https://orcid.org/0000-0002-6259-855X
Tae Gun Kang (iD)
https://orcid.org/0000-0002-2997-9887
Sung Jae Shin (iD)
https://orcid.org/0000-0003-0854-4582
Sang-Jun Ha (iD)
https://orcid.org/0000-0002-1192-6031

Conflicts of Interest
The authors declare no potential conflicts of interest.

Abbreviations
BCG, Bacillus Calmette-Guerin; DDA, dimethyldioctadecylammonium bromide; DOPC, 1,2-Dioleoyl-sn-glycero-3-phosphocholine; HSP70, heat-shock protein 70; HSV-2, herpes simplex virus type 2; MPL, monophosphoryl lipid A; MPQ, MPL, Poly I:C, and QS21 in liposome form; MPS, MPL and Poly I:C in squalene emulsion form; Mtb, Mycobacterium tuberculosis; Mtb K, Mtb strain K; n.s., not significant; TB, tuberculosis; TDB, trehalose-6,6′-dibehenate; TDM, trehalose dicorynomycolate.

INTRODUCTION

Tuberculosis (TB), caused by infection with *Mycobacterium tuberculosis* (Mtb), is the one of the most global health threats in humans worldwide. According to TB annual report from the World Health Organization (WHO), one-quarter of the worldwide population is estimated to be infected with Mtb (1). Furthermore, about 10 million population progresses to active TB patients, resulting in 1.4 million deaths in 2020 (2). Although the TB mortality has been decreased by the Bacillus Calmette-Guérin (BCG) vaccine and anti-TB drugs, TB remains one of the most severe global health problems because of emergence of multi drug resistant Mtb strains and the lack of BCG's efficacy to pulmonary Mtb infection. Therefore, these current circumstances further demands development of new vaccine and therapeutic agents.

In present, BCG vaccine, which was developed nearly 100 years ago, is the only available TB vaccine to prevent TB. In fact, BCG vaccine is currently the most widely used and provides 0%–80% protection against TB (3). However, the beneficial effect of BCG almost restricted to infants, and it exhibited limitation in prevention of TB after adolescence (4,5). Because of this limitation of BCG, development of many new types of TB vaccines including recombinant fusion protein-based subunit vaccines with novel adjuvant systems has been attempted as a booster to BCG vaccine. For example, the efficacy of H4:IC31 subunit vaccine candidate, composed of fusion protein of Mtb Ags (Ag85B and TB10.4) and IC31® adjuvant, was clinically evaluated as prevention of TB infection in adolescents, who had high risk for transmission (6). In addition, the M72/AS01$_E$ vaccine candidate, composed of immunogenic fusion protein (M72) derived from two Mtb Ags (Mtb32A and Mtb39A) and AS01$_E$ adjuvant, was also clinically validated for a protection of TB disease (7). Both subunit vaccine candidates exhibited enhanced primary BCG-induced Th1 type immune response with prolonged protection efficacy in preclinical models (8,9).

Due to high safety profile, subunit vaccine has been considered one of the most attractive vaccine types. However, since Ag alone is not intrinsically immunogenic, suitable adjuvants are thus required to be incorporated in subunit vaccines to generate sufficient immunogenicity of the vaccine Ags (10). Until now, only a few adjuvants such as aluminum salts, liposomes, and emulsions were approved for clinical use (11). Although most vaccines against human pathogens aim to induce protective Ab responses, a primary focus of the TB vaccines is to generate strong Th1 response, which has been achieved by the use of adjuvants that bind to various TLRs (12-14). For example, AS01 and AS02 were evaluated for Mtb by targeting T cell immune responses and consisted of monophosphoryl lipid A (MPL; TLR 4 agonist) and saponin QS21 with liposome and squalene-containing emulsion formulation, respectively. In clinical trials, M72/AS01 elicited greater Ag-specific Th1 responses than M72/AS02 did (15). More recently, M72/AS01 was validated in the view of vaccine potential when administered as emulsion form (M72/AS01$_E$) via intramuscular route. In result, the vaccine displayed 54% efficacy among HIV-negative latent TB individuals in phase IIb trials (7). In addition, the experiments using another two vaccine candidates consisted of the same Ags, MPL, and trehalose-6,6′-dibehenate (TDB) with different delivery systems (liposome or oil-in water) demonstrated that liposome delivery system was superior to oil-in water formulation in eliciting Th1 biased responses against an Mtb-infected murine model (10). These studies highlighted the importance of vaccine formulation in the efficacy of protection against Mtb. Therefore, adjuvant selection is a critical step for the better efficacy and development of effective subunit vaccine.

Thus, in current study, we assessed new subunit vaccine candidate formulated in different adjuvant systems and compared the immunogenicity among candidates for boosting the efficacy of BCG and surmounting the limitations of BCG itself. We previously developed the combination vaccine strategy of BCG with subunit vaccine composed of selected TB Ags, Rv0351 and Rv3628. These two Ags displayed novel TB vaccine potential against infection with hypervirulent clinical Mtb strain K (Mtb K) isolated from South Korea, which belongs to the Beijing family (16), by eliciting Th1-biased immune responses (17,18). The Rv0351/Rv3628 formulated with DMT adjuvant, which is conventionally used for TB vaccine testing (19,20) and composed of dimethyldioctadecylammonium bromide (DDA), MPL, and trehalose dicorynomycolate (TDM), also induced Ag-specific Th1 cytokine production *in vivo*. In addition, when BCG-primed mice were boosted by Rv0351/Rv3628-DMT, they elicited the more enhanced production of Th1 cytokines than mice with BCG-only vaccinated mice. Next, three different types of MPL-based adjuvants were formulated: liposome form (MPQ) containing MPL, Poly I:C, and QS21; another liposome form (MP) containing MPL and Poly I:C; emulsion form (MPS) composed of MPL, Poly I:C, and squalene. In result, MPQ and MPS displayed stronger ability in eliciting Rv0351/Rv2628-specific Th1 immune response to than MP did. Finally, BCG prime and Rv0351/Rv2628-MPS boost vaccine exhibited prolonged protection effect against Mtb K infection. Our results suggest that BCG prime and subunit boost vaccine with an appropriate formulation selection conferred improved protection against hypervirulent Mtb infection and should be considered to elicit strong immunogenicity and develop next-generation TB subunit vaccine.

MATERIALS AND METHODS

Mice
Specific pathogen-free female C57BL/6J at 6 wk of age were purchased from Japan SLC, Inc. (Shijuoka, Japan) and strictly maintained under barrier conditions in a ABSL-3 biohazard animal facility at the Yonsei University Medical Research Center. The animals were fed a sterile commercial mouse diet and provide with water *ad libitum*. The mice were monitored daily before and after immunization and Mtb infection, and none of the mice showed any clinical symptoms during the experiment. The experimental protocols used in this study were reviewed and approved by the Ethics Committee and Institutional Animal Care and Use Committee (Permit Number: 2015-0041) of the Laboratory Animal Research Center at Yonsei University College of Medicine (Seoul, Korea).

Preparations of Mycobacterial strains
Mtb strain K was acquired from the Korean Institute of Tuberculosis (Osong, Korea). *Mycobacterium bovis* BCG (Pasteur strain 1173P2) was generously provided by Dr. Brosch at the Pasteur Institute (Paris, France). These strains were cultured and prepared as previously described (21).

Preparation of MP and MPQ nanoliposome adjuvant
MP adjuvant and MPQ adjuvant were prepared by the thin-film hydration method. To prepare MP adjuvant platform, 0.2 mg of monophosphoryl lipid A (MPL) (Avanti Polar Lipid, Alabaster, AL, USA), 1 mg of 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Sigma Aldrich, St. Louis, MO, USA), and 0.25 mg of cholesterol (Sigma Aldrich), were dissolved in 1 ml of ethanol. To prepare MPQ adjuvant platform, 0.2 mg of QS21 (Desert King International, San Diego, CA, USA) was added into the MP adjuvant platform. Then, the organic solvent was removed with a rotary evaporator from both adjuvant platform solutions to generate the thin

film layer. The thin film was hydrated with 2 ml of PBS (Hyclone, Logan, UT, USA) at 60°C for 30 min, and the resulting solution was sonicated for 1 min with tip sonication under ice bath conditions. The resultant was rotated by tube revolver for 2 h. Finally, it was mixed with 2 mg of poly I:C (Invivogen, San Diego, CA, USA) dissolved in PBS at a 1:1 volume ratio, leading to the generation of MP or MPQ nanoliposome adjuvant.

Preparation of MPS nanoemulsion adjuvant

0.2 mg of MPL, 1 mg of DOPC, 1 mg of DDA (Sigma Aldrich), and 0.25 mg of cholesterol were dissolved in 1 ml of ethanol. The organic solvent was removed with a rotary evaporator to generate the thin film layer. The thin film was hydrated with 2 ml of PBS containing 2.5% v/v squalene (Sigma Aldrich) and 1% v/v tween-80 (Merck, Germany) at 60°C for 30 min. The resulting solution was sonicated for 1 min with tip sonication under ice bath conditions. Then, the resultant was rotated by tube revolver for 2 h. Finally, it was mixed with 2 mg of poly I:C dissolved in PBS at a 1:1 volume ratio.

Immunization protocol

Mice were immunized intramuscularly three times at 3 wk intervals with Rv0351 (0.5 or 1 μg) and Rv3628 (0.5 or 1 μg) formulated in following adjuvants; DMT (50 μg of DDA liposomes containing 5 μg of MPL and 5 μg of TDM) (Sigma-Aldrich); MP (liposome form containing 5 mg of MPL and 25 mg of Poly I:C); MPQ (liposome form containing 5 mg of MPL, 25 mg of Poly I:C, and 5 mg of QS21); and MPS (emulsion form containing 5 mg of MPL and 25 mg of Poly I:C in squalene). For BCG vaccinated group, BCG was subcutaneously or intramuscularly vaccinated once with 2×10^5 CFU, then mice were immunized with subunit vaccine candidates 10 wk or 24 wk after BCG vaccination. One week or four wk after final immunization, mice were sacrificed for immunological analysis or were challenged with bacteria.

Challenge protocol

For Mtb infection, mice were exposed to Mtb K in the calibrated inhalation chamber of an airborne infection apparatus for 60 min delivering a predetermined dose (Glas-Col, Terre Haute, IN, USA) including approximately 200 to 300 viable bacteria as previously described ([21]).

Vaccine efficacy evaluation by bacterial counts and histopathological analysis

Mice were euthanized with CO_2, and lungs were homogenized. The number of viable bacteria was determined by plating serial dilutions of the organ homogenates onto Middlebrook 7H11 agar (Difco, Detroit, MI, USA) supplemented with 10% OADC (Difco) and amphotericin B (Sigma Aldrich). Colonies were counted after 4 wk of incubation at 37°C. For histopathological analysis, middle cross section from entire superior lobes of right lung were stained with H&E and assessed for the severity of inflammation. The level of inflammation in the lungs was evaluated using ImageJ (National Institutes of Health, Bethesda, MD, USA) program, as previously described ([22]).

Cell isolation and Abs

To perfuse the lungs, cold PBS containing heparin was injected into the heart until the lungs appeared white. Then, single-cell suspension of the lung and spleen was prepared as previously described ([23]). For phenotypic analysis of lymphocytes, single-cell suspensions were stained with the following Abs; BD Biosciences (San Jose, CA, USA): anti-CD4 (RM4-5)-PerCP-Cy5.5, anti-CD8a(53-6.7)-FITC; eBioscience (San Diego, CA, USA): anti-CD8a(53-6.7)-PerCPCy5.5; Biolegend (San Diego, CA, USA): anti-CD4(RM4-5)-BV605.

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Intracellular cytokine staining

Single cell suspensions from immunized or infected animals (1-2 × 10^6 cells) were stimulated with Rv0351 (4 µg/ml) or Rv3628 (4 µg /ml) for 12–16 h at 37°C in the presence of Golgi plug/Golgi stop (BD Biosciences). Cells were then stained with fixation, permeabilization and intracellular cytokine staining with anti-IFN-γ (XMG1.2) purchased from BD Biosciences, anti-TNF-α (MP6-XT22)-BV605 purchased from eBioscience, anti-IL-2(JES6-5H4)-PE purchased from BioLegend.

Quantification and statistical analysis

All stained samples were acquired on a FACS Canto II (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA). Statistical analysis was performed using Prism software version 7.0 (GraphPad, La Jolla, CA, USA). Comparison between multiple groups were done using one-way ANOVA with *post hoc*. The statistical details, including the statistical test, exact value of number, precison measure and statistical significance are reported in the Figures and Figure legends.

RESULTS

Mtb Ags, Rv0351 and Rv3628, were immunogenic when combined with DMT adjuvant in vivo

To validate the capacity of immunogenic function *in vivo* for the previously selected TB Ags, Rv0351 and Rv3628, mice were immunized with Rv0351/Rv3628 alongside MPL-based adjuvant, DMT (**Supplementary Fig. 1A**). To elucidate the Ag-specific CD4+ T cells producing IFN-γ, TNF-α, and IL-2, pulmonary lymphocytes of immunized mice were either stimulated with Rv0351 or Rv3628 followed by intracellular cytokine staining. When lymphocytes were *ex vivo* stimulated with Rv0351 and Rv3628 respectively, the number of Rv0351- or Rv3628-specific IFN-γ+, TNF-α+, and IL-2+ CD4+ T cells in the lung of Rv0351/Rv3628-DMT immunized group was more increased than those of PBS or Rv0351/Rv3628 only immunized group (**Fig. 1A and B**). Upon the assessment of humoral responses from the 3rd wk after the first immunization (**Supplementary Fig. 1A**), a marked enhancement of Rv0351- or Rv3628-specific IgG, IgG1, and IgG2c titers was observed in the mice that received DMT as an adjuvant (**Supplementary Fig. 1B**). Next, we validated the induction of multifunctional T cells which was well known as an important factor in elimination of intracellular pathogens (24). Pulmonary lymphocytes from the immunized mice were stimulated with Rv0351 or Rv3628 protein, the percentage of multifunctional CD4+ T cells that produced IFN-γ plus TNF-α and/or IL-2 was measured by multi-parameter flow cytometry. When stimulated with Rv0351 or Rv3628 protein, the immunized mice with Rv0351/Rv3628 and DMT exhibited enhanced levels of triple-positive (IFN-γ+TNF-α+IL-2+) and double-positive TNF-α+IL-2+ CD4+ T cells in the lung, while protein only immunized mice showed low frequency of these cell populations (**Fig. 1C**). In summary, Rv0351/Rv3628, the selected TB Ag candidates, were immunogenic to produce abundant Ag-specific CD4+ T cell responses along with elevated populations of multifunctional CD4+ T cells when combined with appropriate adjuvants like DMT.

Immunogenicity was evaluated in BCG-primed mice followed by Rv0351/Rv3628-DMT vaccination

Although muscle tissue is not expected to be an optimal site for immunization due to its low density of immune cells (25), Rv0351/Rv3628-DMT were immunogenic when immunized via intramuscular route. Thus, we further explored whether only two Rv0351/Rv3628-DMT

boosted immunizations could enhance Ag-specific immune responses at long intervals in intramuscularly BCG-primed mice. The mice were intramuscularly immunized with BCG and boosted with Rv0351/Rv3628 plus DMT at 24- and 27-wk post-BCG immunization (**Fig. 2A**). At 1 wk after the last protein immunization, pulmonary lymphocytes were isolated and stimulated with Rv0351 or Rv3628 *ex vivo*. The numbers of IFN-γ⁺, TNF-α⁺, and IL-2⁺ CD4⁺ T cells in the mice with BCG prime and Rv0351/Rv3628-DMT boost vaccine were more increased than in the nice with BCG vaccine or BCG prime and Rv0351/Rv3628 boost vaccine (**Fig. 2B and C**). Furthermore, in population of multifunctional CD4⁺ T cells, BCG prime and Rv0351/Rv3628-DMT boost vaccine showed increased frequencies of triple-positive and double-positive IFN-γ⁺ TNF-α⁺ CD4⁺ T cells in the lung (**Fig. 2D**). Taken together, Rv0351/Rv3628-DMT immunization efficiently elicited Rv0351- or Rv3628-specific CD4⁺ T cells that produce IFN-γ, TNF-α, and IL-2 with Ag-specific multifunctional properties in the lungs at long intervals in BCG-primed mice.

Two different adjuvant formulation, MPQ- and MPS-adjuvanted Rv0351/Rv3628 vaccination exhibited enhanced capacity of eliciting Ag-specific CD4⁺ Th1-biased responses

To further enhance the effect of Rv0351/Rv3628 immunization, our unique formulated adjuvant candidates were analyzed, instead of previously used DMT adjuvants. We also halved the dose of each Ag in this experiment to better compare adjuvant's ability to elicit Ag-specific immune responses. The newly formulated adjuvants to be tested are as follows; 1) MP composed of MPL and Poly I:C in liposome form, 2) MPQ composed of MPL, Poly I:C, and QS21 in liposome form, 3) MPS consisted of MPL and Poly I:C in squalene emulsion form (**Supplementary Fig. 2**). As a result, when pulmonary CD4⁺ T cells were stimulated with Rv0351 or Rv3628, the numbers of IFN-γ⁺, TNF-α⁺, and IL-2⁺ CD4⁺ T cells in the mice immunized with Rv0351/Rv3628 and, MPS or MPQ, were significantly increased than those in protein only immunized group or mice immunized with protein and DMT (**Fig. 3A and B**). Especially stimulated with Rv0351, CD4⁺ T cells in mice immunized with MPS or MPQ exhibited a better cytokine producing capacity than those in mice immunized with DMT (**Fig. 3B**). The similar patterns were observed in the spleen of the mice immunized with Rv0351/Rv3628 and MPS or MPQ, while the responses were not as strong as those observed in the lung (**Supplementary Fig. 3A and B**). Notably, there were no significant differences in the numbers of CD4⁺ T cells in the lung producing Th2-related cytokines, IL-5 and IL-13 among groups. However, immunization of Rv0351/Rv3628 adjuvanted with MPQ or MPS elicited Ag-specific Th1-biased responses by inducing higher Th1 cytokines compared to Th2 cytokines among all groups (**Fig. 3C** and **Supplementary Fig. 4A and B**). Unlike enhanced induction of Ag-specific CD4⁺ Th1 responses producing IFN-γ, TNF-α, or IL-2 in mice immunized with MPS- or MPQ-adjuvanted Rv0351/Rv3628 vaccine, Ag-specific IgG levels were similarly induced regardless of use of adjuvants (**Supplementary Fig. 5**). Criteria for vaccine formulation selection include not only the optimal immune responses but also ease and cost-effectiveness of manufacture with stability (12). In consideration of this criteria, we decided to create a new TB subunit vaccine candidate with an emulsion form of MPS adjuvant, and later experiments were conducted with MPS adjuvant.

Boosting with Rv0351/Rv3628 and MPS adjuvant in BCG-primed mice mediated protection against chronic infection with Mtb K

With Rv0351/Rv3628 combined with selected MPS adjuvant, we evaluated the protective efficacy of vaccine candidate in BCG-primed mice. To validate the protective efficacy, in terms of reduction in pulmonary inflammation and bacterial loads in mice, histopathological analysis for each group of mice was conducted at 16 wk after challenge with clinically isolated
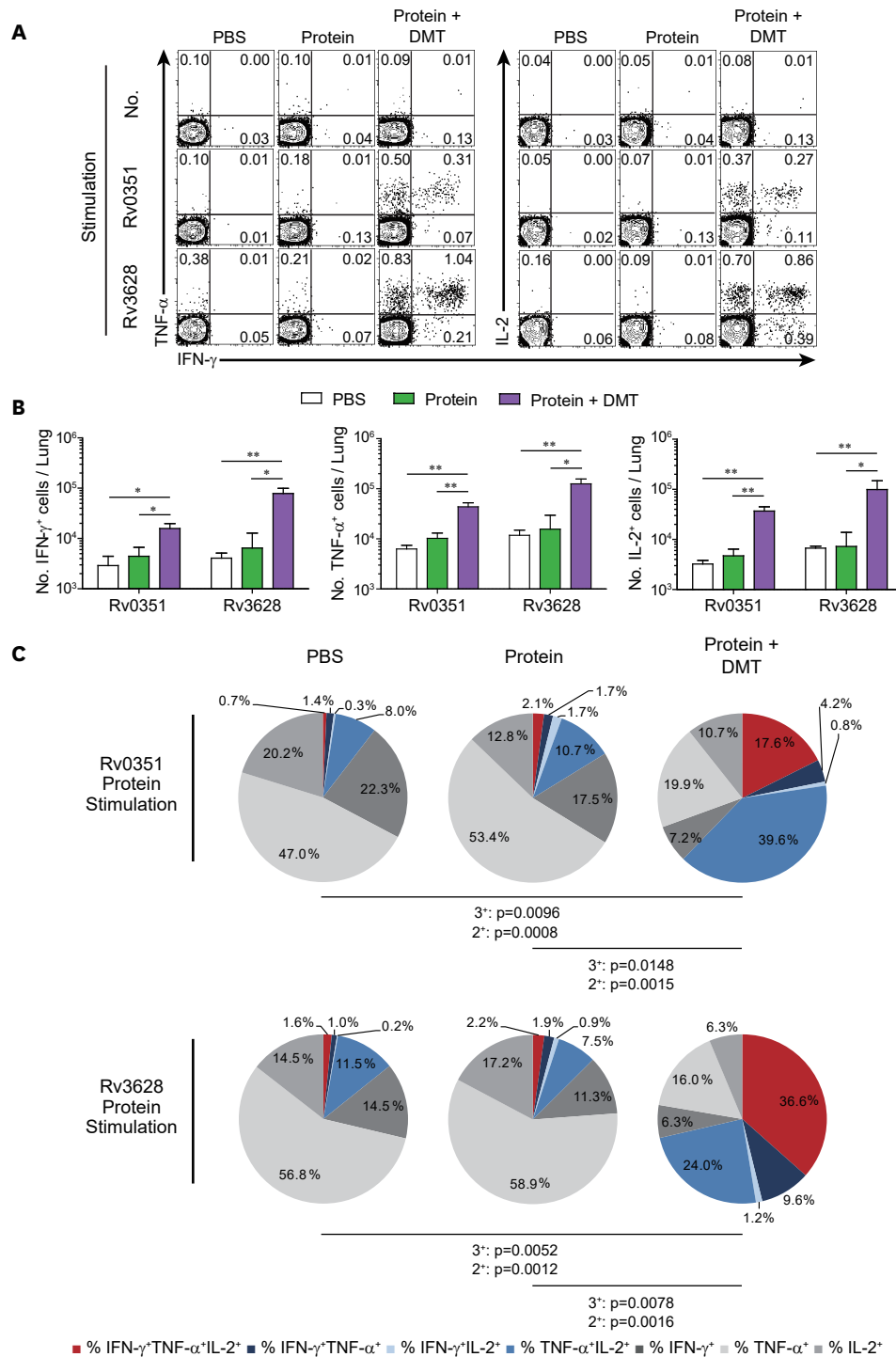
Fig. 1. Analysis of CD4+ T cell responses induced by Rv0351/Rv3628 immunization in the lung of immunized mice. Mice were immunized intramuscularly three times at 3-wk intervals with Rv0351 and Rv3628 without or with DMT. One week after final immunization, mice from each groups (n=5) were sacrificed, and lung cells were re-stimulated with Rv0351 or Rv3628. (A) Representative plots of IFN-γ+, TNF-α+, and IL-2+ in CD4+ T cells. The numbers in plots indicate the IFN-γ+TNF-α+, IFN-γ+TNF-α− and IFN-γ−TNF-α+ / IFN-γ+IL-2+, IFN-γ+ IL-2−+ and IFN-γ− IL-2+. (B) The number of IFN-γ+, TNF-α+, or IL-2+ CD4+ T cells in lungs were summarized in graph. (C) The pie charts summarized the fraction of triple, double, and single cytokine positive CD4+ T cell in each immunized group. Data were analyzed by one-way ANOVA with *post hoc* Tukey test. Graph shows mean ± SEM.
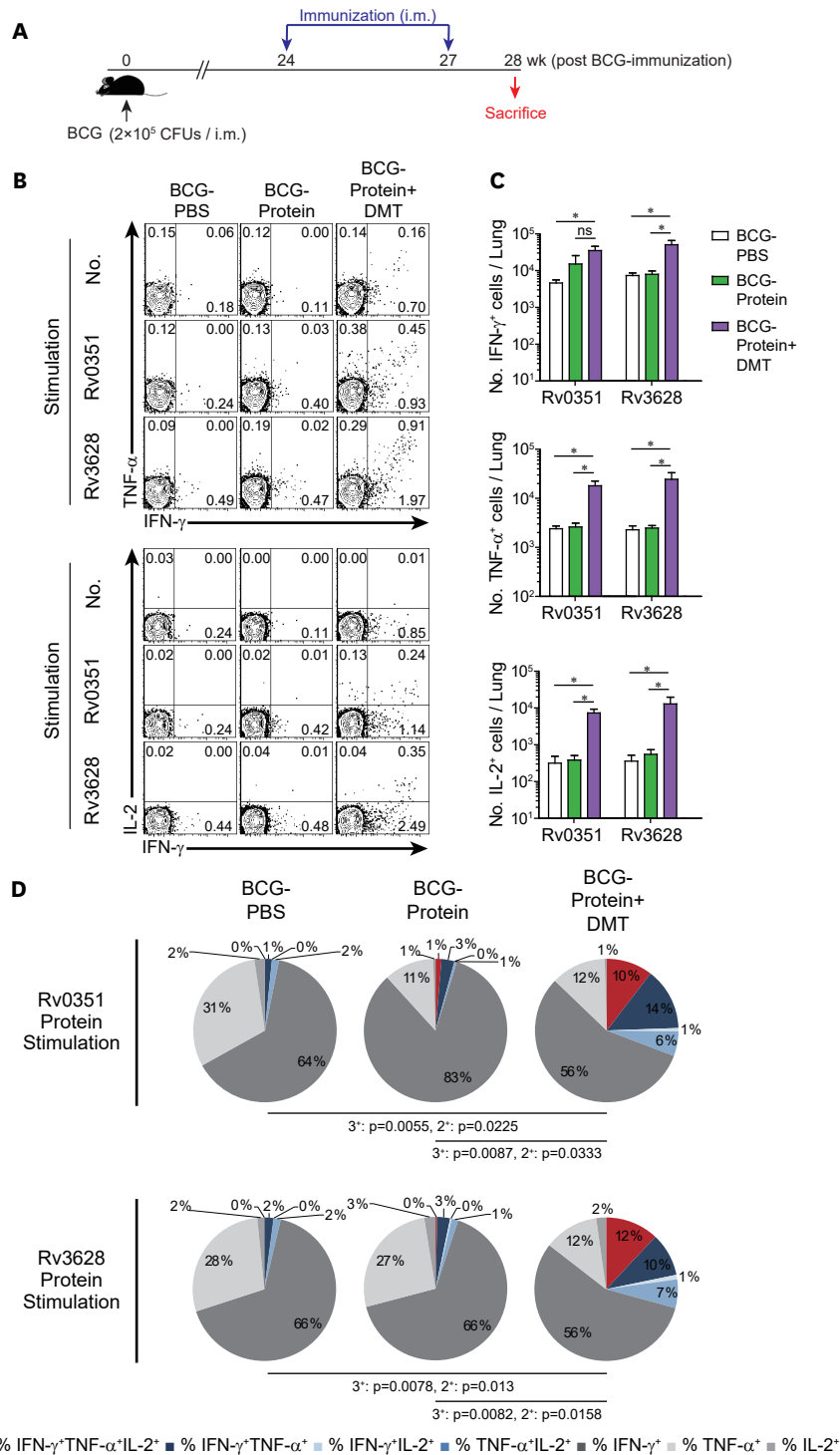*p<0.05; **p<0.01.

Fig. 2. Validation of CD4^{+} T cell responses induced by BCG and Rv0351/Rv3628 immunization in the lung of immunized mice. Mice were immunized with BCG, and then after 24 wk after BCG vaccination, mice were immunized with PBS, Rv0351/Rv3628-only or Rv0361/Rv3628-DMT. One week after final immunization, mice from each groups (n=5) were sacrificed, and lung cells were re-stimulated with Rv0351 or Rv3628. (A) Representative plots of IFN-γ^{+}, TNF-α^{+}, and IL-2^{+} in CD4^{+} T cells. The numbers in plots indicate the IFN-γ^{+}TNF-α^{+}, IFN-γ^{+}TNF-α^{-} and IFN-γ^{-}TNF-α^{+} / IFN-γ^{+}IL-2^{+}, IFN-γ^{+} IL-2^{-} and IFN-γ^{-} IL-2^{+}. (B) The number of IFN-γ^{+}, TNF-α^{+}, or IL-2^{+} CD4^{+} T cells in lungs were summarized in graphs. (C) The pie charts summarized the fraction of triple, double, and single cytokine positive CD4^{+} T cell in each immunized group. Data were analyzed by one-way ANOVA with *post hoc* Tukey test. Graph shows mean ± SEM. [*]p<0.05.
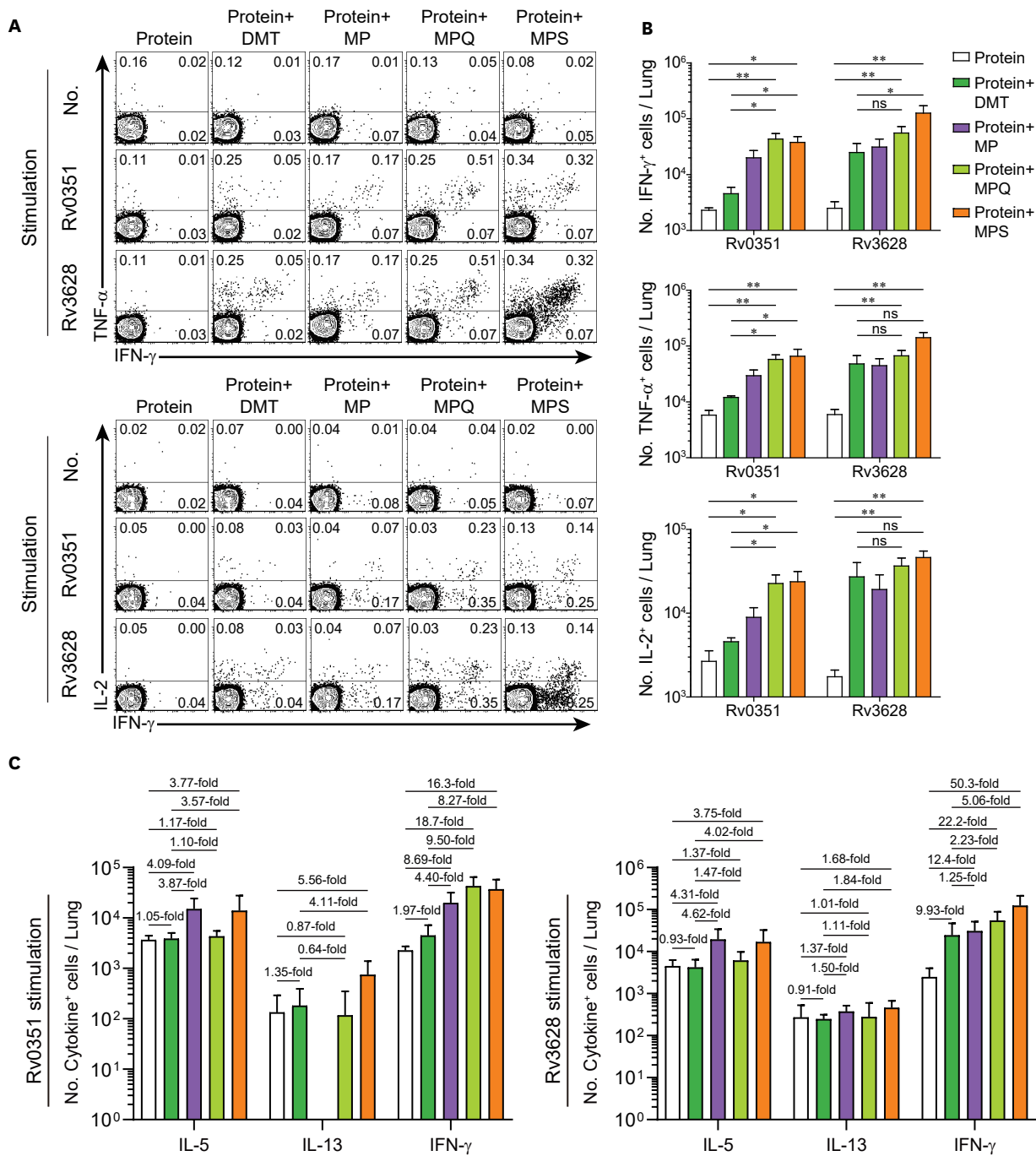
Fig. 3. Elucidation of CD4+ T cell responses induced by Rv0351/Rv3628 with diverse adjuvants. Mice were immunized with Rv0351/Rv3628 and adjuvants, and then after 1 wk final immunization, mice (n=4/group) were sacrificed. Harvested pulmonary lymphocytes were re-stimulated with Rv0351 or Rv3628. (A) Representative plots of IFN-γ+, TNF-α+, and IL-2+ in CD4+ T cells. The numbers in plots indicate the IFN-γ+TNF-α+, IFN-γ+TNF-α- and IFN-γ-TNF-α+ / IFN-γ+IL-2+, IFN-γ+ IL-2- and IFN-γ- IL-2+. (B) The number of IFN-γ+, TNF-α+, or IL-2+ CD4+ T cells in lungs were summarized in graphs. (C) The number of IL-5+, IL-13+, and IFN-γ+ CD4+ T cells in lungs were summarized in graphs with fold-change. Data were analyzed by one-way ANOVA with *post hoc* Tukey test. Graph shows mean ± SEM. *p<0.05; **p<0.01.

strain, Mtb K (**Fig. 4A**). Given that vaccine-induced CD4+ T cell responses were evaluated at 1 wk after the last immunization, we further investigated the immune responses prior to Mtb K challenge to consider the duration of vaccine-induced responses. In line with previous data, BCG prime and Rv0351/Rv3628-MPS boost vaccine enhanced Ag-specific Th1 responses
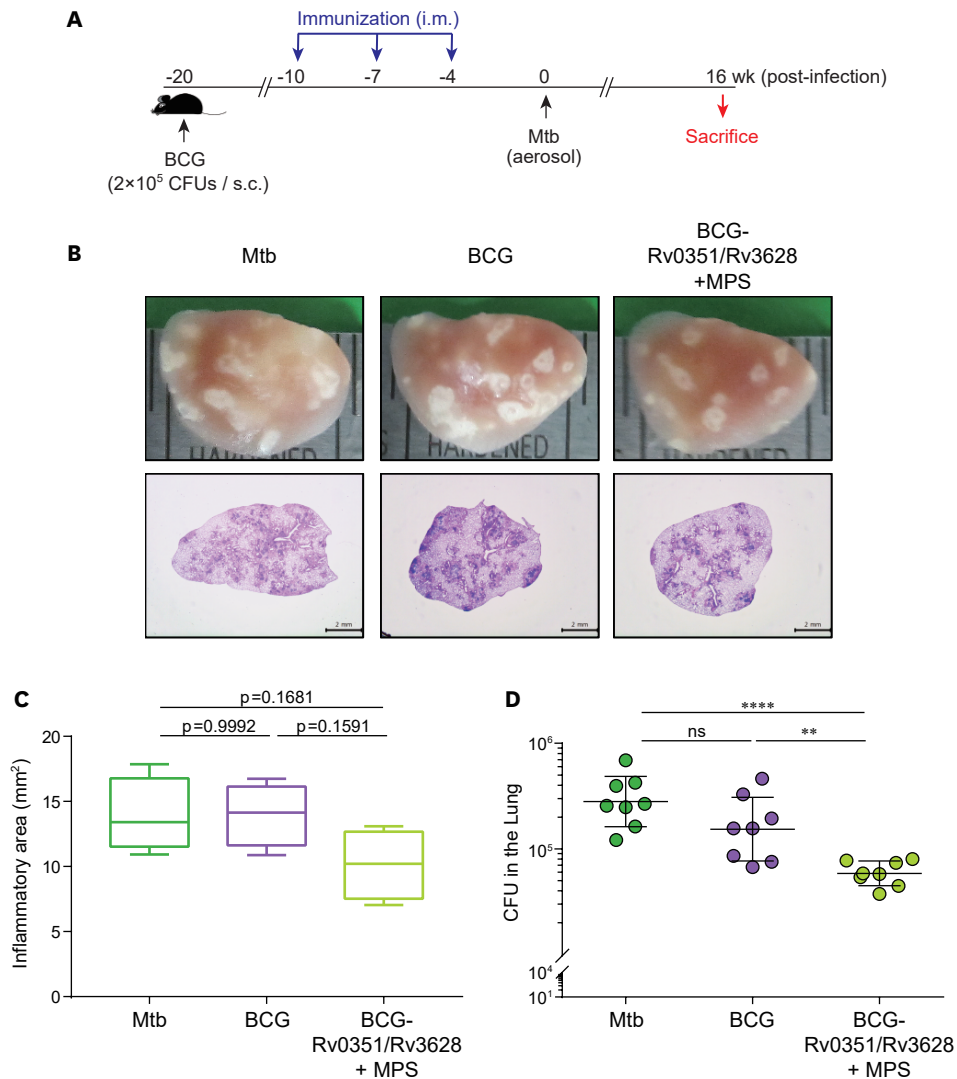
Fig. 4. Protective efficacy against the hypervirulent Mtb K strain in BCG-primed Rv0351/Rv3628-MPS boosted mice. Mice (n=12/group) was immunized with BCG, and then after 10 wk after BCG immunization, mice were boosted with Rv0351/3628-MPS immunization. Four weeks after the final Rv0351/Rv3628 immunization, mice (n=8/group) were infected with Mtb K, and then after 16 wk post Mtb K infection, mice were sacrificed. (A) Experimental scheme of analyzing vaccine efficacy. (B) Gross pathology and H&E staining of lungs of each group. (C) Data represent the area of the superior lobe of lung displaying inflammation and are shown as box-and-whisker plots showing the minimum and maximum values. (D) Bacterial loads in lungs of each group at 16 wk post Mtb K infection. Data were analyzed by one-way ANOVA with *post hoc* Tukey test. Graph shows mean ± SEM.
**p<0.01; ****p<0.0001.

but did not Th2 responses in the lungs at 4 wk after the last immunization (**Supplementary Figs. 6** and **7**). In addition, the numbers of IFN-γ⁺CD4⁺ T cells in the spleen of BCG prime and subunit boost vaccinated mice were increased compared to those of non-immunized mice (**Supplementary Fig. 8**). Upon Mtb K challenge, compared to the non- and BCG-immunized mice, the mice that received BCG prime and Rv0351/Rv3628-MPS boost vaccine had tendency to decreased pulmonary inflammation (p=0.1681, versus non-immunized mice; p=0.1591, versus BCG-only vaccinated mice) (**Fig. 4B and C**). In addition, bacterial loads between non-immunized and BCG-only vaccinated mice displayed no significant difference at 16 wk post Mtb K infection. However, BCG prime and Rv0351/Rv3628-MPS boost vaccine significantly diminished bacterial loads in the lung compared to other groups (**Fig. 4D**).

Previously, our results suggested that Rv0351/Rv3628 with adjuvant exhibited enhanced cellular immune response *in vivo* (17,18). Next, to distinguish the Rv0351/Rv3628-MPS vaccine-induced immune responses from those by Mtb infections in aspect of multifunctionality of T cells, lymphocytes were isolated, and *ex vivo* stimulated with Rv0351 or Rv3628 protein for intracellular cytokine analysis at 16 wk post-challenge. The frequency and number of multifunctional CD4^{+} T cells producing more than two different effector cytokines in Rv0351/Rv3628-MPS immunized mice were similar with those of non-immunized group or BCG-only immunized control group (**Supplementary Fig. 9A-C**). However, the frequency of IFN-γ single-positive CD4^{+} T cells was decreased in Rv0351/Rv3628-MPS vaccinated group than the other groups (**Supplementary Fig. 9B and C**). Although there were no significant differences in the frequency and number of multifunctional CD4^{+} T cells between Rv0351/Rv3628 vaccinated group and other control groups, bacterial loads were clearly decreased in Rv0351/Rv3628 immunized mice, suggesting that cytokine-producing CD4^{+} T cell responses after Mtb challenge reflect the results from the remaining bacteria-induced response as well as the vaccine-induced response. Collectively, these data suggested that BCG prime and Rv0351/Rv3628-MPS boost vaccine mediated protection against chronic infection with Mtb K especially in aspects of bacterial reduction.

DISCUSSION

BCG, the only licensed TB vaccine is ineffective to protect from the most prevalent pulmonary form of TB disease, and then it enforces us to develop new TB vaccines for alleviation of TB prevalence. Here, we evaluated and validated the effect of a new subunit vaccine candidate to enhance the immunogenicity conferred by BCG vaccination against Mtb infection. To overcome limitations of BCG vaccine, we selected Rv0351/Rv3628, which were previously defined as immunogenic components (17,18) of TB vaccine, as subunit booster in BCG-primed mice. We also conducted tests to select the proper adjuvant formulation that can induce the most effective Th1 immune response when used with the subunit booster. The immunogenic Rv0351/Rv3628 protein elicited robust Th1 immune responses *in vivo* regardless of the type of adjuvants. Moreover, the more multifunctional Th1 responses were induced when mice were immunized with MPQ- or MPS-adjuvanted Rv0351/Rv3628 than immunized with DMT- or MP-adjuvanted Rv0351/Rv3628. Furthermore, the mice that received BCG prime and Rv0351/Rv3628-MPS boost vaccine conferred more enhanced prolonged protection against hypervirulent Mtb K infection than BCG-only or non-immunized control group. Overall, our data highlight the importance of adjuvant formulation to induce an optimal protective immune response in development of TB subunit vaccines.

One of the most advanced TB vaccine in clinical trials is protein-based subunit vaccine with the recent success of M72/AS01_E by providing approximately 50% protection against active TB progression for 3 years without any safety concerns (26). Ags commonly used in TB subunit vaccine development mostly have focused on the use of early-secreted or conserved Ags highly associated with active bacterial replication such as ESAT-6, CFP-10, and Ag85 family, all of which have been proved to be immunogenic resulting in protection in animal models, although this is not always the case (27-29). To satisfy this trend, in our study, we selected Rv0351 and Rv3628 as our vaccine Ag targets for TB subunit vaccine. GrpE, encoded by *rv0351*, is a cofactor of heat-shock protein 70 (HSP70) in the DnaK operon and is one of stress response-associated Mtb Ags represented by rapid expression during Mtb infection (17,30,31). In addition, Rv3628 as a unique soluble inorganic pyrophosphatase (PPase) has

been reported to be constitutively expressed and up-regulated in a dormant phase of TB, and essential for the survival of Mtb (32-34), indicating this protein is obligated to express for Mtb's survival. Furthermore, in our previous studies, both individual Ags displayed vaccine potential by conferring protection against Mtb infection accompanied with the durable induction of Ag-specific Th1-biased responses (17,18). In line with this, it was also confirmed in this study that Rv0351/Rv3628 were immunogenic to each Ag stimulation by inducing Ag-specific Th1-biased responses in aspects of quality and quantity in mice when received these two Ags altogether.

Considering that Ag alone, in general, is poorly immunogenic, the second key component is adjuvant system which is essentially required for subunit vaccine to shape the desired immune responses. Adjuvant selection is indispensable for vaccine success and different adjuvants can affect the immune responses and ultimately vaccine efficacy (11,35). We compared the immunogenicity by employing 4 different MPL-based adjuvants: DMT, MP, MPQ, and MPS for eliciting desired Ag-specific immune responses. Among them, MPQ- and MPS-adjuvanted Rv0351/Rv3628 immunization ranked at the top two in aspects of enhanced induction of Th1-biased responses in both quality and quantity manner. In DMT adjuvant, TDM is included as component and have been reported to be recognized by macrophage-inducible C-type lectin (Mincle) which belongs to the C-type lectin receptors of host (36). As one of TB vaccine adjuvants, CAF01 has demonstrated efficacy in preclinical models by inducing Th17-polarized responses and the main component of CAF01 is TDB, synthetic analog of TDM, responsible for Th17 polarization by activating Mincle (19,37). It may be conceivable that Mincle-activating property of TDM may make an intervention on T cell polarization by relatively waning Th1 responses, although we have not evaluated this. On the other hand, Poly I:C was incorporated to rest of adjuvants given that Poly I:C and its derivatives have been reported to be necessary for eliciting Th1/CTL immune responses against various pathogens (38). QS21 which is constituents of MPQ adjuvant can cause lysosomal disruption resulting in Syk- and NLRP3 inflammasome-activation, which is responsible for promoting production of inflammatory cytokines and enhancing cross-presentation with CD8$^+$ T cells (35,39). This property of QS21 acted in synergy with MPL, resulting in Th1-polarizing effects of AS01 adjuvant (40), as superior induction of Ag-specific Th1 responses was observed in the mice that received Rv0351/Rv3628-MPQ. Meanwhile, there was only difference in formulation between MP and MPS as liposome form or emulsion form, respectively. Intriguingly, the same Ags can display differential immunogenicity profile and protective efficacy depending on the adjuvant formulation type, liposome or emulsion (10,12). Furthermore, many TLR 4 agonists including MPL are reported to be likely aggregated without proper formulation, which can influence vaccine stability (12). This might contribute to differential immunogenicity observed in MP- and MPS-adjuvanted Rv0351/Rv3628 vaccinated mice. In addition, the manufacturing vaccine-grade liposomes is not cost-effective (41). Considering these, we selected Rv0351/Rv3628-MPS as our vaccine candidate for final validation.

Then, our new subunit TB vaccine was evaluated as an effective booster to BCG vaccine, as BCG will not be completely replaced due to its nonspecific mechanisms-derived general efficacy in childhood (42,43). Given that primary BCG vaccination may mediate limited protections based on observational studies (44-46), BCG revaccination together with H4:IC31, a subunit vaccine candidate, was evaluated in phase 2 clinical trial, resulting in reduction of the rate of sustained QuantiFERON-TB Gold In-tube assay (OFT) conversion (6). During Mtb infection, the induction of multifunctional Ag-specific T cells by vaccination

is a principal purpose for protection against Mtb infection based on clinical study results so far (47,48). In our data, quantity of Ag-specific CD4+ T cells were enhanced along with multifunctional Ag-specific CD4+ T cells when naïve mice were immunized with combination of Rv0351/Rv3628-DMT and BCG compared to control groups. However, when the mice immunized with BCG prime and Rv0351/Rv3628-MPS boost vaccine were challenged with Mtb K, they exhibited similar number of multifunctional Ag-specific CD4+ T cells and displayed decreased number of IFN-γ single-positive CD4+ T cells at 16 wk post-infection compared with other control groups. In previous research, elevated levels of PD-1 were reported in IFN-γ single-positive Mtb-specific CD4+ T cells (49). Furthermore, this IFN-γ single-positive Mtb-specific CD4+ T cells also reflected the loads of Mtb by acting as poor correlates of protection (50). Therefore, it is conceivable that increased frequency of single cytokine positive Ag-specific CD4+ T cells might be positively correlated with T cell exhaustion or bacterial loads during chronic bacterial infection.

Our current study has certain limitations that deserve further consideration. First, we employed two different prime-boost regimens regarding BCG routes, intramuscular and subcutaneous, respectively. Muscle tissue is not regarded as an optimal site for immunization due to the low density of immune cells (25). In this study, Rv0351/Rv3628 adjuvanted with DMT and MPS elicited Ag-specific Th1 responses regardless of BCG-priming routes. However, protective efficacy against Mtb infection was only evaluated with the mice that received subcutaneous BCG vaccine followed by subunit boost vaccine (21,51). For the development of improved TB subunit vaccines, the effect of the vaccine routes and the gap in Ag-specific Th1 responses accompanied with protective efficacy should be further considered. Second, our vaccine candidate, Rv0351/Rv3628 combined with adjuvants elicited Ag-specific immune responses in the lung when immunized via intramuscular route. On the one hand, intramuscular vaccination of some vaccines using adjuvants with either glycoprotein B or D in the herpes simplex virus type 2 (HSV-2) vaccine trials did not mediate genital protection against HSV-2 infection (52,53). On the other hand, it was reported that intramuscular injection of vaccines such as a replication-defective adenovirus vector and inactivated *Mycoplasma hyopneumoniae* can elicit the mucosal immune responses in the lungs or intestines (54,55). In addition, mice immunized with TB subunit vaccine ID93 formulated with GLA-LSQ adjuvant displayed enhanced resident memory CD4 T cells in the lung (56) although this vaccine was evaluated as therapeutic approach. Furthermore, in our data, Ag-specific splenic CD4+ T cell responses were induced at 1 and 4 wk after the last immunization, while the responses were not as strong as those observed in the lung. However, the possibility that Ag-specific T cells that we determined in the lung can be derived from central memory T cells might not be completely excluded. Additional approach such as treatment of FTY720 which inhibits cell recruitment from secondary lymphoid organs to inflamed sites (57) would be required for assessing mucosal immune responses represented by vaccination-induced resident memory CD4 T cells in the lung, and their protective function against Mtb infection as our further study.

In summary, our data suggest that the optimal Ag-specific immune responses can be achieved by proper selection of the adjuvant formulation. In addition, Rv0351/Rv3628-MPS subunit vaccine have substantial capacity to boost the efficacy of BCG by mediating Th1-mediated protection against chronic infection with hypervirulent Mtb K strain, which warrants for further preclinical research to pave the way for the development of improved TB subunit vaccine.

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SUPPLEMENTARY MATERIALS

Supplementary Fig. 1

Humoral responses elicited by Rv0351/Rv3628-DMT vaccination. (A) Experimental scheme of the *in vivo* experiment. Naïve C57BL/6J mice were immunized intramuscularly three times at 3-wk intervals with Rv0351 (1 μg) and Rv3628 (1 μg) formulated in DMT adjuvant (50 μg/injection of DDA liposomes containing 5 μg/injection of MPL and 5 μg/injection of TDM). Then, 7 wk after the first immunization, lung cells harvested from each group (n=5) were analyzed. (B) Individual mouse sera were isolated at the indicated time points after the first immunization, and Rv0351- or Rv3628-specific IgG, IgG1, and IgG2c levels were measured with an ELISA. Data were analyzed by one-way ANOVA with *post hoc* Tukey test. Graph shows mean ± SEM.

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Supplementary Fig. 2

Experimental scheme of the *in vivo* experiment. Naïve C57BL/6J mice were immunized intramuscularly three times at 3-wk intervals with Rv0351 (1 μg) and Rv3628 (1 μg) formulated in MP, MPQ or MPS. Then, 7 wk after the first immunization, lung cells harvested from each group (n=4) were analyzed.

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Supplementary Fig. 3

Elucidation of CD4+ T cell responses in the spleen induced by Rv0351/Rv3628 with diverse adjuvants. Mice were immunized with Rv0351/Rv3628 and adjuvants, and then after 1 wk final immunization, mice (n=4/group) were sacrificed. Harvested lymphocytes were re-stimulated with Rv0351 or Rv3628. (A) Representative plots of IFN-γ+, TNF-α+, and IL-2+ in CD4+ T cells. The numbers in plots indicate the IFN-γ+TNF-α+, IFN-γ+TNF-α− and IFN-γ−TNF-α+ / IFN-γ+IL-2+, IFN-γ+ IL-2− and IFN-γ− IL-2+. (B) The number of IFN-γ+, TNF-α+, or IL-2+ CD4+ T cells in spleen were summarized in graphs. Data were analyzed by one-way ANOVA with *post hoc* Tukey test. Graph shows mean ± SEM.

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Supplementary Fig. 4

Analysis of CD4+ Th2 responses in the lung induced by Rv0351/Rv3628 with diverse adjuvants. Mice were immunized with Rv0351/Rv3628 and adjuvants, and then after 1 wk final immunization, mice (n=4/group) were sacrificed. Harvested lymphocytes were re-stimulated with Rv0351 or Rv3628. (A) Representative plots of IL-5+ and IL-13+ in CD4+ T cells. The

numbers in plots indicate the frequency of IL-5$^+$IL-13$^+$, IL-5$^+$IL-13$^-$ and IL-5$^-$IL-13$^+$. (B) The number of IL-5$^+$ CD4$^+$ and IL-13$^+$ CD4$^+$ T cells in the lung were summarized in graphs. Data were analyzed by one-way ANOVA with *post hoc* Tukey test. Graph shows mean ± SEM.

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Supplementary Fig. 5

Humoral responses elicited by Rv0351/Rv3628 vaccination with different formulation. Individual mouse sera were isolated at the indicated time points after the first immunization, and Rv0351- or Rv3628-specific IgG, IgG1, and IgG2c levels were measured with an ELISA. Data were analyzed by one-way ANOVA with *post hoc* Tukey test. Graph shows mean ± SEM.

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Supplementary Fig. 6

Analysis of the Ag-specific CD4$^+$ T cell responses in the lungs of BCG-primed and Rv0351/Rv3628-boosted mice. The same prime boost schedule used in **Figure 4A** was applied. The pulmonary lymphocytes were isolated from each group of mice (n=4) at 4 wk after the last immunization, and lymphocytes were stimulated with Rv0351 or Rv3628 *ex vivo*. (A) Representative plots of IFN-γ$^+$, TNF-α$^+$, and IL-2$^+$ in CD4$^+$ T cells. The numbers in plots indicate the IFN-γ$^+$TNF-α$^+$, IFN-γ$^+$TNF-α$^-$ and IFN-γ$^-$TNF-α$^+$ / IFN-γ$^+$IL-2$^+$, IFN-γ$^+$ IL-2$^-$ and IFN-γ$^-$ IL-2$^+$. (B) The number of IFN-γ$^+$, TNF-α$^+$, or IL-2$^+$ CD4$^+$ T cells in lungs were summarized in graphs. (C) The number of IL-5$^+$, IL-13$^+$, and IFN-γ$^+$ CD4$^+$ T cells in lungs were summarized in graphs with fold-change. Data were analyzed by one-way ANOVA with *post hoc* Tukey test. Graph shows mean ± SEM.

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Supplementary Fig. 7

Analysis of CD4$^+$ Th2 responses in the lungs of BCG-primed and Rv0351/Rv3628-boosted mice. The same prime boost schedule used in **Fig. 4A** was applied. The pulmonary lymphocytes were isolated from each group of mice (n=4) at 4 wk after the last immunization, and lymphocytes were stimulated with Rv0351 or Rv3628 *ex vivo*. (A) Representative plots of IL-5$^+$ and IL-13$^+$ in CD4$^+$ T cells. The numbers in plots indicate the frequency of IL-5$^+$IL-13$^+$, IL-5$^+$IL-13$^-$ and IL-5$^-$IL-13$^+$. (B) The number of IL-5$^+$ CD4$^+$ and IL-13$^+$ CD4$^+$ T cells in the lung were summarized in graphs. Data were analyzed by one-way ANOVA with *post hoc* Tukey test. Graph shows mean ± SEM.

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Supplementary Fig. 8

Analysis of the Ag-specific CD4$^+$ T cell responses in the spleen of BCG-primed and Rv0351/Rv3628-boosted mice. The same prime boost schedule used in **Figure 4A** was applied. The lymphocytes were isolated from the spleen of each group of mice (n=4) at 4 wk after the last immunization, and lymphocytes were stimulated with Rv0351 or Rv3628 *ex vivo*. (A) Representative plots of IFN-γ$^+$, TNF-α$^+$, and IL-2$^+$ in CD4$^+$ T cells. The numbers in plots indicate the IFN-γ$^+$TNF-α$^+$, IFN-γ$^+$TNF-α$^-$ and IFN-γ$^-$TNF-α$^+$ / IFN-γ$^+$IL-2$^+$, IFN-γ$^+$ IL-2$^-$ and IFN-γ$^-$ IL-2$^+$. (B) The number of IFN-γ$^+$, TNF-α$^+$, or IL-2$^+$ CD4$^+$ T cells in lungs were summarized in graphs. Data were analyzed by one-way ANOVA with *post hoc* Tukey test. Graph shows mean ± SEM.

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Supplementary Fig. 9

Analysis of the Ag-specific CD4$^+$ T cell responses in the lungs of BCG-primed Rv0351/Rv3628 boosted mice following with the Mtb K infection. The pulmonary lymphocytes were isolated from each group of mice (n=8) at 16 wk post Mtb K infection, and lymphocytes were stimulated with Rv0351 or Rv3628 *ex vivo*. (A) Representative plots of IFN-γ$^+$, TNF-α$^+$, and IL-2$^+$ in CD4$^+$ T cells. The numbers in plots indicate the IFN-γ$^+$TNF-α$^+$, IFN-γ$^+$TNF-α$^-$, and IFN-γ$^-$ TNF-α$^+$ / IFN-γ$^+$IL-2$^+$, IFN-γ$^+$ IL-2$^-$, and IFN-γ$^-$ IL-2$^+$. (B) The number and frequency of Rv0351 or Rv3628 stimulated CD4$^+$ T cells were summarized with different patterns of cytokine production. (C) The pie charts summarized the fraction of triple, double, and single cytokine positive CD4$^+$ T cells in each immunized group. Data were analyzed by one-way ANOVA with *post hoc* Tukey test. Graph shows mean ± SEM.

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