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Essential Engagement of Toll-Like Receptor 2 in Initiation of Early Protective Th1 Response against Rough Variants of *Mycobacterium abscessus*

Jong-Seok Kim,[a] Min-Jung Kang,[b] Woo Sik Kim,[a] Seung Jung Han,[a] Hong Min Kim,[a] Ho Won Kim,[a] Kee Woong Kwon,[a] So Jeong Kim,[a] Seung Bin Cha,[a] Seok-Yong Eum,[c] Won-Jung Koh,[d] Sang-Nae Cho,[a] Jong-Hwan Park,[b] Sung Jae Shin[a]

Department of Microbiology and Institute for Immunology and Immunological Diseases, Yonsei University College of Medicine, Seoul, South Korea[a]; Laboratory Animal Medicine, College of Veterinary Medicine, Chonnam National University, Gwangju, South Korea[b]; Division of Immunopathology and Cellular Immunology, International Tuberculosis Research Center, Changwon, South Korea[c]; Division of Pulmonary and Critical Care Medicine, Department of Medicine, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul, South Korea[d]

Although *Mycobacterium abscessus* (*M. abscessus*) is becoming more prevalent in patients without overt immunodeficiency, little is known about the factors that contribute to disease susceptibility. This study was undertaken to investigate how Toll-like receptor 2 (TLR2) functionally contributes to the generation of protective immunity against *M. abscessus* in a morphotype-specific manner. We found that *Tlr2*$^{-/-}$ mice were extremely susceptible to an intravenous (i.v.) model of infection by *M. abscessus* rough variants, displaying uncontrolled infection in the lungs and a significantly lower survival rate than with wild-type (WT) mice. This uncontrolled infection resulted from failures in the following processes: (i) production of the crucial cytokines gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin 12p70 (IL-12p70); (ii) early infiltration of neutrophils, monocytes, and dendritic cells (DCs) in the lungs of *Tlr2*$^{-/-}$ mice; (iii) rapid influx of CD4^{+} and CD8^{+} T cells; and (iv) the expansion of memory/effector T cells. Notably, systemic administration of *M. abscessus* culture filtrate-treated syngeneic DCs from WT mice greatly strengthened immune priming *in vivo*, resulting in a dramatic reduction in bacterial growth and improved long-term survival in *Tlr2*$^{-/-}$ mice, with a recovery of protective immunity. Our findings demonstrate that TLR2 is an essential contributor to instructive and effector immunity during *M. abscessus* infection in a morphotype-specific manner.

Mycobacterium abscessus (*sensu stricto*) is an emerging rapidly growing mycobacterium (RGM) that causes a wide spectrum of infections in humans, including lung, skin, soft tissue, and bone diseases (1). *M. abscessus* is the most drug-resistant mycobacterial pathogen, resulting in limited therapeutic options and a high rate of treatment failure (2).

Interestingly, the outcomes of mycobacterial infections can range from asymptomatic clearance to severe clinical disease (3). Different pathogen- and host-specific factors have been considered in determining the outcomes of these infections. However, it is clear that the interaction between mycobacteria and the innate and adaptive immune systems plays a central role in host defense and pathogenesis (3). In fact, the recognition of mycobacterial components by innate immune cells through various pattern recognition receptors (PRRs) induces a cytokine response that can promote early control of the infection (3, 4).

Toll-like receptor (TLR)-mediated immune activation occurs only in the presence of functional TLRs, and TLR variants may exhibit altered expression, function, and recognition of signaling mechanisms, thereby increasing disease susceptibility. Healthy individuals who develop nontuberculous mycobacterial (NTM) diseases, including those caused by *M. abscessus*, likely have specific susceptibility factors that make them vulnerable to these infections (5). Previous studies have demonstrated an association between human TLR2 polymorphisms and tuberculosis or *Mycobacterium avium* complex (MAC) lung disease (6–8). In addition, TLR1 is required for TLR2 signaling, and the TLR1 single nucleotide polymorphism (SNP) I602S has been found in a cystic fibrosis cell line, resulting in a lack of recognition of *M. abscessus* rough variants by TLR2.

Furthermore, an age-associated decrease in TLR function has been identified (9). The rate of *M. abscessus* infection has increased, predominantly in older women (5). Thus, delineating the molecular recognition pattern of *M. abscessus* is important for understanding how host resistance is induced, maintained, and regulated during infection. To date, a limited number of *in vivo* studies have investigated the roles of innate immunity and adaptive immunity in *M. abscessus* infections (10–13).

Based on colony morphology on solid growth medium, there are two major variants of *M. abscessus*: the smooth (S) and rough (R) morphotypes (12). The cell walls of the S variant of *M. abscessus* are enriched with glycopeptidolipids (GPLs), and this variant

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Address correspondence to Jong-Hwan Park, jonpark@jnu.ac.kr, or Sung Jae Shin, sjshin@yuhs.ac.

J.-S.K, M.-J.K., and W.S.K. contributed equally to this article.

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is thought to be the predominant morphotype in the environment; however, the R variant is more likely to cause invasive disease and is considered more pathogenic because of its association with excessive inflammation upon TLR2 ligation (14–16). Despite extensive research, no conclusive evidence of a link between innate and adaptive immunity to *M. abscessus* infections in a morphotype-dependent manner has been produced, especially *in vivo*. Therefore, an understanding of molecular immunity to *M. abscessus* infection is critical for the development of effective strategies to treat *M. abscessus* infection.

In the current study, we have found that TLR2 is essential for efficient control of hypervirulent *M. abscessus* R variant infection in mice and serves as an intermediary between the innate and adaptive immune responses. We also investigated the importance of dendritic cell (DC) activation through TLR2 signaling in the generation of early protective immunity, and we attempted DC therapy for *M. abscessus* infection for the first time.

MATERIALS AND METHODS

Bacterial strains, cultures, and preparation of single-cell suspensions. The type strain *M. abscessus* ATCC 19977^T was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All strains were initially cultured in 7H9 broth (Difco Laboratories, Detroit, MI, USA) supplemented with 10% (vol/vol) oleic acid-albumin-dextrose-catalase (OADC; Becton Dickinson, Sparks, MD, USA) for 7 days at 37°C. *M. abscessus* ATCC 19977^T originally showed a smooth morphotype (*M. abscessus* S), and an isogenic rough variant (*M. abscessus* R) was isolated from the lungs of C57BL/6J mice infected with the smooth morphotype of *M. abscessus* ATCC 19977^T during macrolide treatment. The smooth and rough isogenic strains of *M. abscessus* ATCC 19977^T were confirmed to have identical genotypes by variable-number tandem repeat (VNTR) analysis (17).

The morphotypes of these strains were stably maintained in *in vitro* culture for more than 20 passages. Additionally, infection experiments in mice using both variants resulted in no changes in the colony morphologies of the S and R variants of *M. abscessus* recovered from the lung. Single-cell suspensions of each strain were prepared as described previously, with slight modifications (16). Briefly, mycobacterial cells grown in OADC-supplemented 7H9 broth were harvested by centrifugation at 10,000 × *g* for 20 min and washed three times in 10 mM phosphate-buffered saline (PBS; pH 7.2). The pellets were homogenized using an overhead stirrer (Wheaton Instrument, Millville, NJ, USA) for 1 min on ice to minimize clumping of bacteria. The homogenized mycobacterial cells were passed through an 8-μm-pore-size filter (Millipore Corp., Bedford, MA, USA). The seed lots of each strain were then kept in small aliquots at −80°C until use. The number of CFU per 1 ml of the seed lots was measured by a viable counting assay on 7H10 agar plates.

Ethics statement. All animal experiments were performed in accordance with the guidelines of the Korean Food and Drug Administration (KFDA). The experimental protocols used in this study were reviewed and approved by the Ethics Committee and Institutional Animal Care and Use Committee (permit number 2012-010) of the Laboratory Animal Research Center at Yonsei University College of Medicine (Seoul, South Korea).

Animals and infection. Wild-type (WT), *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, OT-I, and OT-II T-cell receptor (TCR) transgenic mice (C57BL/6 background) were purchased from the Jackson Laboratory (Bar Harbor, ME). The animals were fed a sterile commercial mouse diet and were provided with water *ad libitum*. The *in vivo* virulence levels of the rough and smooth morphotype strains of *M. abscessus* were compared based on the survival rates of the mice and the numbers of bacterial CFU in the lungs in the initial animal infection study. Briefly, 6- to 7-week-old C57BL/6J, *Tlr2*$^{-/-}$, and *Tlr4*$^{-/-}$ mice were intravenously (i.v.) infected with low (1.4×10^7 CFU/mouse) or high (2.0×10^7 CFU/mouse) doses of *M. abscessus* R or S via tail vein injection. Some mice were intranasally (i.n.) infected with 9×10^6 CFU/mouse of *M. abscessus* R. At each postinfection time point, the mice were euthanized by CO_2 inhalation, and the lungs, livers, and spleens were aseptically collected for bacterial counts or immune cell analysis. The numbers of viable bacteria in the organs were determined by plating serial dilutions of organ homogenates onto 7H10-OADC agar plates. Colonies were counted after 5 days of incubation at 37°C. Differences between the groups are presented as the mean $\log_{10}$ CFU counts ± standard errors of the means (SEM) per group.

Histology. Tissue samples collected for histopathology were preserved in 10% neutral buffered formalin, embedded in paraffin, cut into 2- to 3-μm sections, and stained with hematoxylin and eosin or acid-fast stain, as described previously (18, 19). Two independent pathologists performed blinded examinations of tissue sections from infected animals at 7 and 14 days postinfection. Each section was scored as absent (0), mild (1), moderate (2), severe (3), or extremely severe (4). The severity of the inflammatory response was scored on a scale of 0 to 4 based on lesion size and the number of lesions per field. Tissues with multiple large lesions in more than three fields were assigned a score of 4.

Antibodies and reagents. Recombinant mouse granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) were purchased from R&D Systems (Minneapolis, MN). The following antibodies were purchase from eBioscience (San Diego, CA, USA): fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody (MAb) for CD11b and CD62L; allophycocyanin (APC)-conjugated MAb for F4/80 and CD11c; eFluor450-conjugated MAb for CD3; peridinin chlorophyll protein (PerCP)-Cy5.5-conjugated MAb for CD4$^+$ and CD8$^+$; phycoerythrin (PE)-conjugated MAb for Gr-1, CD80, CD86, major histocompatibility complex class I (MHC-I), MHC-II, and CD44; PE-Cy5-conjugated MAb for CD4$^+$ and CD8$^+$; and PE-Cy7-conjugated MAb for CD11c.

Cytokine measurement. The levels of gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), IL-1β, IL-10, and IL-12p70 were determined by enzyme-linked immunosorbent assay (ELISA) in bronchoalveolar lavage (BAL) fluid and serum collected at 2, 5, 7 and 14 days postinfection, according to the manufacturer's instructions (eBioscience). BAL fluid samples were harvested by infusing 1 ml of ice-cold PBS through a 25-gauge blunt needle into the lungs via the trachea, followed by gentle aspiration of this fluid into a syringe at 2, 5, 7, and 14 days postinfection (20). BAL fluid and cells were separated by centrifugation at 13,000 × *g* for 10 min at 4°C. The supernatants were carefully collected for ELISA, and the cell pellets were collected for flow cytometry.

Flow cytometry. Cells were washed in ice-cold flow cytometry buffer (2%, vol/vol, bovine serum albumin and 2 mM EDTA in PBS, pH 7.5), incubated with each antibody for 15 min, and washed twice with flow cytometry buffer. Data were acquired on a FACSCanto II flow cytometer (BD Biosciences, San Diego, CA, USA) and analyzed with FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Preparation of CFAs. Culture filtrate antigens (CFAs) were prepared from cultures at the mid-log phase of growth after 2 weeks of incubation at 37°C, as described previously (21). Briefly, cells grown in modified Watson-Reid medium (mWR; pH 6.0) were removed by centrifugation at 30,000 × *g* for 30 min. After filtration through a 0.2-μm-pore-size filter (Millipore), the filtrate was concentrated 40- to 50-fold using a Centricon Plus-80 filter unit (5-kDa-molecular-weight cutoff; Amicon, Beverly, MA, USA) and dialyzed five times in 10 mM PBS using a Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL, USA).

Generation and culture of DCs. Immature mouse bone marrow-derived DCs (BM-DCs) were generated as described previously (22). Briefly, whole bone marrow cells isolated from C57BL/6, *Tlr2*$^{-/-}$, and *Tlr4*$^{-/-}$ mice were washed with serum-free RPMI 1640 medium. The cells were plated in petri dishes and cultured at 37°C in the presence of 5% CO_2 using RPMI 1640 medium supplemented with 100 units/ml penicillin-streptomycin (Lonza, Basel, Switzerland), 10% fetal bovine serum (Lonza, Basel, Switzerland), 50 μM mercaptoethanol (Lonza), 20 ng/ml

GM-CSF, and 5 ng/ml IL-4. On day 6, over 80% of the nonadherent cells expressed CD11c. To obtain highly purified populations for subsequent analyses, the DCs were labeled with bead-conjugated anti-CD11c MAb (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by positive selection on paramagnetic columns (LS columns; Miltenyi Biotec) according to the manufacturer's instructions. The purity of the cell fraction selected was >95%.

Mixed lymphocyte reaction (MLR). Responder T cells, which participate in allogeneic T-cell reactions, were isolated using a magnetic cell sorting (MACS) column (Miltenyi Biotec) from total mononuclear cells prepared from BALB/c mice. Ovalbumin (OVA)-specific $CD8^+$ and $CD4^+$ T cells (responders) were obtained from splenocytes of OT-1 and OT-2 mice, respectively. These T cells were stained with 1 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Carlsbad, CA, USA) as previously described (22). DCs treated with OVA peptide in the presence of 10 μg/ml *M. abscessus* CFA for 24 h were cocultured with CFSE-stained $CD8^+$ and $CD4^+$ T cells at a DC/T-cell ratio of 1:10. On day 2 or 3 of coculture, the T cells were stained with PerCP-Cy5.5-conjugated anti-$CD4^+$ MAb, PE-Cy5-conjugated anti-$CD4^+$ MAb, and PE-Cy5-conjugated anti-$CD8^+$ MAb prior to analysis by flow cytometry. The supernatants were harvested and evaluated to determine the production of IFN-γ, IL-2, and IL-4 by ELISA.

DC immunotherapy. Mice were infected i.v. with *M. abscessus* R (1.0×10^7 CFU/mouse) prior to the injection of immature DCs and *M. abscessus* CFA-treated BM-DCs (1.0×10^6) via the tail vein on postinfection day 2. At each postinfection time point, the mice were euthanized by CO_2 inhalation, and their lungs were aseptically collected for bacterial counts and histopathological analysis. The numbers of viable bacteria in the lungs were determined as described above. Each experiment was carried out twice.

Statistical analysis. All experiments were repeated at least three times with consistent results. Significant differences between samples were determined with Tukey's multiple-comparison tests using statistical software (GraphPad Prism software, version 4.03; GraphPad Software, San Diego, CA, USA). The data in the graphs are expressed as the means ± SEM, and *P* values of <0.05, <0.01, and <0.001 were considered statistically significant. Survival curves were analyzed using a Kaplan-Meyer log rank test.

RESULTS

TLR2-deficient mice are extremely susceptible to infection with *M. abscessus* R. To investigate the importance of TLR2 in the host defense against *M. abscessus* strains, we infected $Tlr2^{-/-}$, $Tlr4^{-/-}$, and WT mice i.v. with a low (1.4×10^7 CFU/mouse) or high (2×10^7 CFU/mouse) dose of isogenic *M. abscessus* R or *M. abscessus* S. We then examined survival rates and bacterial CFU counts in the lungs. The majority of the WT and $Tlr4^{-/-}$ mice infected with the low dose of *M. abscessus* R survived until day 25; however, mice lacking TLR2 had a significantly lower survival rate than WT and $Tlr4^{-/-}$ mice. All deaths occurred within 21 days of infection (Fig. 1A). In contrast, there was no significant difference in mortality rates among the three mouse strains infected with the low dose of *M. abscessus* S; all mice survived until day 15 (see Fig. S1A in the supplemental material). When the mice were infected with a high dose of *M. abscessus* R, WT and $Tlr4^{-/-}$ mice died within 12 days postinfection. Interestingly, all $Tlr2^{-/-}$ mice died within 5 days postinfection (Fig. 1B). We further assessed the growth of *M. abscessus* in the lungs, spleen, and liver after bacterial infection. When the mice were infected with a low dose of bacteria, bacterial loads in the lungs were slightly elevated at 7 days postinfection in all mouse strains, with no significant difference among the strains (Fig. 1C). The levels of bacteria in the lungs of WT and $Tlr4^{-/-}$ mice slowly decreased beginning at 7 days postinfection. In contrast, the bacteria in the lungs of $Tlr2^{-/-}$ mice grew in an unrestricted manner until 19 days postinfection (Fig. 1C). Similar results were obtained with the high-dose *M. abscessus* R infection (Fig. 1D). The numbers of bacteria in the spleen and liver of WT and $Tlr4^{-/-}$ mice decreased continuously by 19 days postinfection; however, bacterial numbers in the spleen and liver of $Tlr2^{-/-}$ mice increased or did not change (see Fig. S2A and B in the supplemental material). In contrast, bacterial CFU counts in the lungs decreased continuously in mice infected with a high dose of *M. abscessus* S, and the mice survived until the end of the experiment (see Fig. S1A and B). Histopathological analysis revealed well-defined granulomatous lesions in WT mice in the early stages of infection (day 7). In comparison, no definitive granuloma was observed in the lungs of $Tlr2^{-/-}$ mice. As the infection progressed in WT mice, the granulomatous lesions disappeared. In contrast, lesions with multifocal infiltration of most types of immune cells were found in the lungs of $Tlr2^{-/-}$ mice (Fig. 1E). The inflammation score reached 3 to 3.5 in the lungs of WT mice at 7 days postinfection. This score was significantly higher than that in $Tlr2^{-/-}$ mice. However, at 14 days postinfection, the inflammation score in the lungs was higher in $Tlr2^{-/-}$ mice than in WT mice (Fig. 1F).

To further investigate the importance of TLR2 in the relationship between the route of infection and susceptibility to *M. abscessus*, we challenged WT and $Tlr2^{-/-}$ mice i.n. with *M. abscessus* R and examined bacterial CFU counts and histological changes in the lungs over 20 days. At 10 days postinfection, bacterial loads in the lungs were significantly increased in $Tlr2^{-/-}$ mice compared with levels in WT mice (see Fig. S3A and B in the supplemental material). While WT mice eliminated *M. abscessus* R efficiently until 20 days postinfection, $Tlr2^{-/-}$ mice failed to do so (see Fig. S3A). Histopathological analysis revealed that most $Tlr2^{-/-}$ mice displayed a multifocal-to-coalescing well-delineated inflammatory lesion centered on the bronchioles and secondarily extending to the alveoli, leading to a complete loss of alveolar spaces. The alveolar spaces were filled by various immune cells, including lymphocytes, neutrophils, and macrophages, until 20 days postinfection. In contrast, WT mice displayed focal inflammatory lesions at 10 days postinfection, most of which had disappeared at 20 days (see Fig. S3C). As shown in Fig. S3D in the supplemental material, the inflammation score reached 2 to 2.5 in the lungs of $Tlr2^{-/-}$ mice at 10 and 20 days postinfection, which was significantly higher than that in the lungs of WT mice (score of 0 to 0.5). Accordingly, we concluded that TLR2 is crucial for protection against *M. abscessus* R and that $Tlr2^{-/-}$ mice are extremely susceptible to i.v. infection by *M. abscessus R*.

TLR2 participates in macrophage activation but not in intracellular control of *M. abscessus*. Although the above results indicated that TLR2 is a key factor for the regulation of *M. abscessus* R in mice, we could not exclude the possibility that TLR2 has a direct effect on the ability to control bacterial growth or cytokine production in the infected macrophages. To test this hypothesis, we infected WT, $Tlr2^{-/-}$, and $Tlr4^{-/-}$ bone marrow-derived macrophages (BMDMs) with *M. abscessus* S and *M. abscessus* R and measured intracellular growth for 72 h. The intracellular growth of S and R variants in $Tlr2^{-/-}$ BMDMs was similar to that in WT and $Tlr4^{-/-}$ cells (see Fig. S4A and B in the supplemental material); however, the production of TNF-α and IL-10 by $Tlr2^{-/-}$ BMDMs infected with R variants was significantly ($P < 0.001$) lower than that in WT and $Tlr4^{-/-}$ BMDMs. In contrast, no dif-

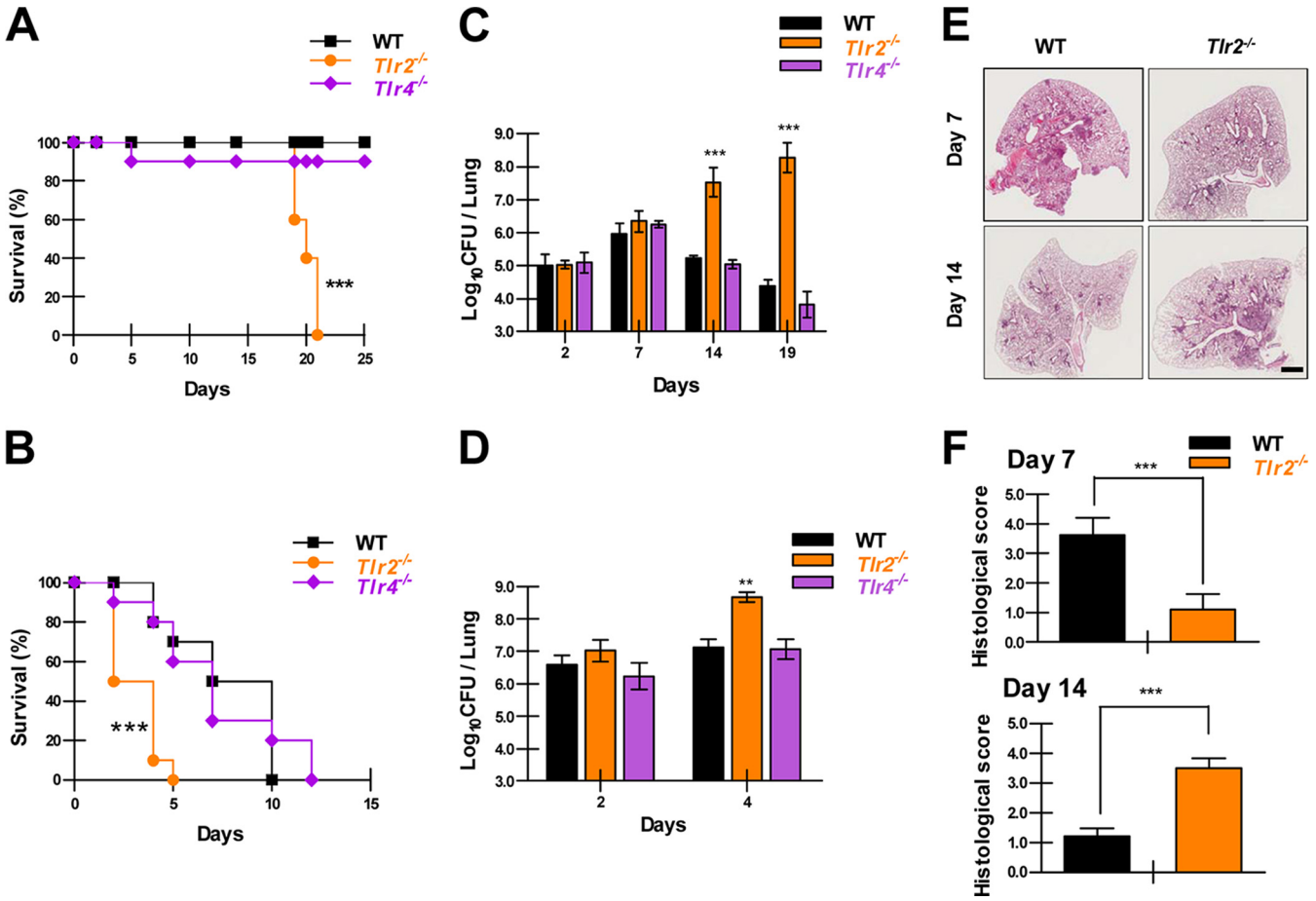

FIG 1 *Tlr2*$^{-/-}$ mice are highly susceptible to i.v. infection with *M. abscessus* R. WT, *Tlr2*$^{-/-}$, and *Tlr4*$^{-/-}$ mice were infected i.v. with 1.4×10^7 (A) or 2.0×10^7 (B) *M. abscessus* R CFU/mouse ($n = 30$ animals per group), and survival was monitored for the indicated number of days after infection. The results shown represent three independent experiments. (C and D) Three groups of mice were infected i.v. with 1.4×10^7 (C) or 2.0×10^7 (D) *M. abscessus* R CFU/mouse. The lungs were harvested at the indicated time points, and mycobacterial loads were determined using a standard plate count method. The results are expressed as the mean estimated number of CFU ± SEM from the lung samples of five to six mice in three independent experiments. Statistically significant differences in CFU are shown for *Tlr2*$^{-/-}$ compared with WT mice (**, $P < 0.01$; ***, $P < 0.001$). (E) Histopathology of lungs from WT and *Tlr2*$^{-/-}$ mice infected i.v. with 1.4×10^7 *M. abscessus* R CFU. Lung sections were stained with hematoxylin and eosin (magnification, ×4). (F) Inflammatory scores of hematoxylin and eosin-stained lung sections. The graph denotes the average inflammatory score observed in the lungs following infection with 1.4×10^7 CFU/mouse (five mice per group per time point). Each section was blindly evaluated and scored as absent (0), mild (1), moderate (2), severe (3), or extremely severe (4). Scale bar, 1 mm.

ference in the production of these cytokines was observed in cells infected with *M. abscessus* S (see Fig. S4C and D).

TLR2 is involved in cytokine production in the bronchoalveolar lavage fluid of mice infected with *M. abscessus* R. To investigate whether TLR2 is involved in the production of inflammatory cytokines following infection with R variants, BAL fluid was obtained from infected mice at each time point, and the levels of IFN-γ, TNF-α, IL-12p70, and IL-10 in the BAL fluid were measured by ELISA. The production of proinflammatory cytokines such as IFN-γ, TNF-α, and IL-12p70 in the BAL fluid of WT and *Tlr4*$^{-/-}$ mice increased significantly by 7 days postinfection and decreased to baseline levels after 14 days; however, significant changes in the levels of these cytokines were not observed until day 14 in *Tlr2*$^{-/-}$ mice (Fig. 2A). The production of the anti-inflammatory cytokine IL-10 gradually increased, but there was no significant difference in the BAL fluid of WT and *Tlr4*$^{-/-}$ mice until 14 days postinfection. In contrast, *Tlr2*$^{-/-}$ mice displayed a rapid and significant increase in IL-10 levels between day 7 and day 14 postinfection (Fig. 2A).

We further investigated whether TLR2 is involved in the production of inflammatory cytokines in serum following *M. abscessus* R infection. Serum was collected from infected mice at each time point, and the levels of IFN-γ, TNF-α, IL-12p70, and IL-10 were measured by ELISA. The production of IFN-γ, TNF-α, and IL-12p70 in the serum of WT and *Tlr4*$^{-/-}$ mice increased by 2 days postinfection and returned almost to baseline after 7 days. In contrast, an increase in these cytokines was not observed until 14 days in *Tlr2*$^{-/-}$ mice (see Fig. S5A to C in the supplemental material). IL-10 gradually increased in the serum of all experimental groups until 14 days postinfection (see Fig. S5D). These results indicate that high levels of proinflammatory cytokine production may play an important role in the control of *M. abscessus* R variants in the early stages of infection and that TLR2 regulates the production of these cytokines.

TLR2 deficiency leads to decreased recruitment of inflammatory cells to the bronchoalveolar space in mice infected with *M. abscessus* R. To quantify inflammatory cell recruitment, BAL fluid was collected from WT, *Tlr2*$^{-/-}$, and *Tlr4*$^{-/-}$ mice at 7 and 14

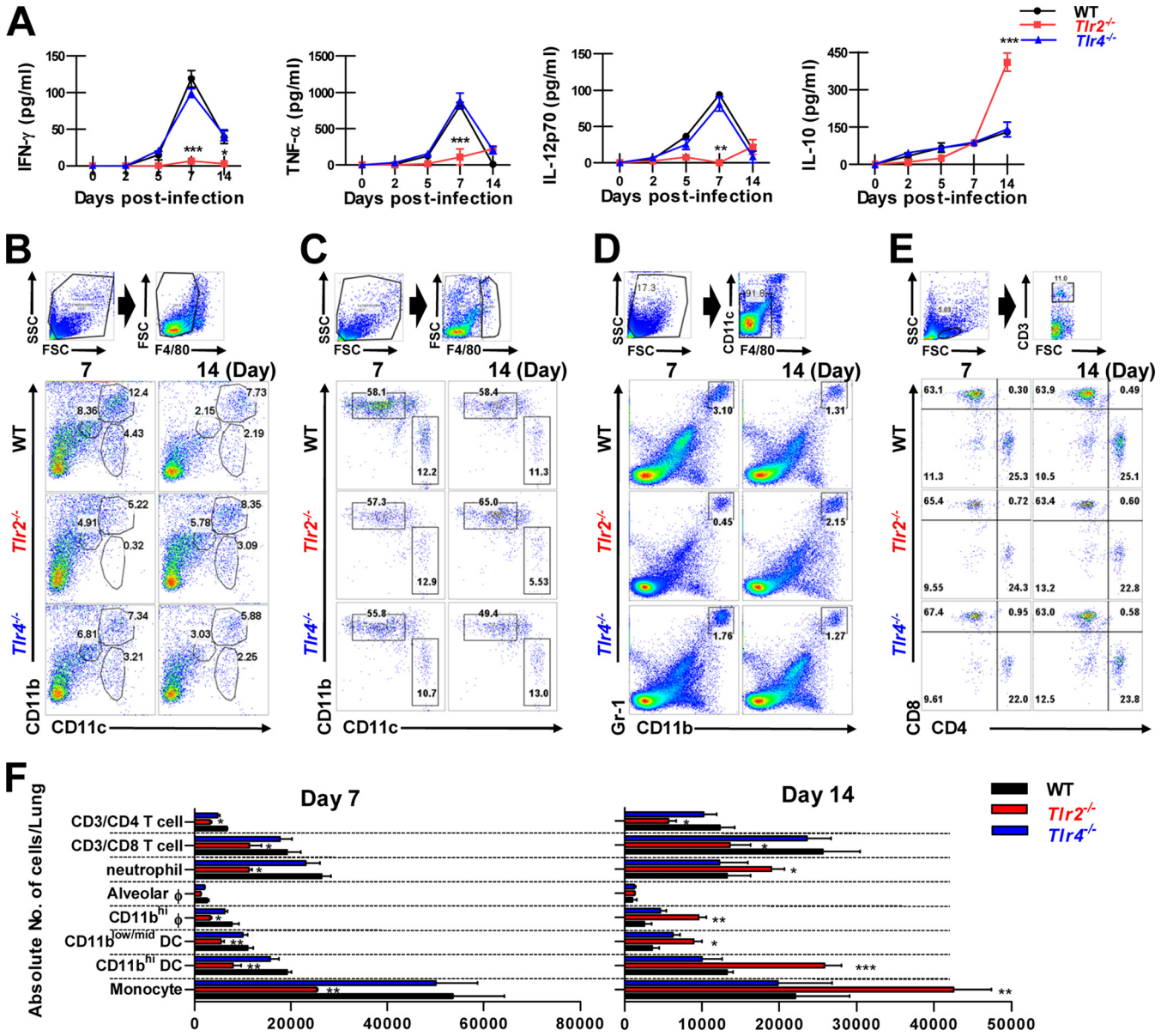

FIG 2 Cytokine production and cellular influx into the bronchoalveolar lavage fluid. WT, *Tlr2*$^{-/-}$, and *Tlr4*$^{-/-}$ mice were infected i.v. with 1.4×10^7 *M. abscessus* R CFU/mouse. (A) BAL fluid was harvested at the indicated time points, and the levels of IFN-γ, TNF-α, IL-12p70, and IL-10 were determined by ELISA. (B) Immune cells were assayed by flow cytometry on days 7 and 14 postinfection. A representative dot blot was primarily gated on FSC$^{mid/high}$/SSC$^{mid/high}$ (where FSC is forward scatter and SSC is side scatter) and secondarily gated on F4/80^{low}. F4/80^{low} CD11b^{mid} CD11c^{mid} cells were classified as monocytes and F4/80^{low} CD11b^{high} CD11c^{high} cells were classified as CD11b^{high} dendritic cells. F4/80^{low} CD11b^{low} CD11c^{high} cells were classified as CD11b^{low} dendritic cells. (C) A representative dot blot was primarily gated on FSC$^{mid/high}$/SSC$^{mid/high}$ and secondarily gated on F4/80^{high}. F4/80^{high} CD11b^{high} CD11c$^{low/mid}$ cells were classified as CD11b^{high} macrophages, and F4/80^{low} CD11b^{low} CD11c^{high} cells were classified as alveolar macrophages. (D) A representative dot blot was primarily gated on FSC$^{mid/high}$/SSC$^{mid/high}$ and secondarily gated on CD11c^{low} F4/80^{low}. F4/80^{low} CD11c^{low} CD11B^{high} Gr-1^{high} cells were classified as neutrophils. (E) CD4$^+$ and CD8$^+$ T-cell influx into the lungs. A representative dot blot of bronchoalveolar lavage cells was primarily gated on FSC^{mid}/SSC^{low} and secondarily gated on CD3^{high}. Numbers indicate the percentages of cells in each quadrant. (F) The bar graph represents the absolute number of cells in each quadrant or square on days 7 and 14 after the initial infection. The results are presented as the means ± SEM of each group (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

days after i.v. infection with *M. abscessus* R, and BAL cells retrieved from the BAL fluid were stained and counted to determine the types of cells recruited into the bronchoalveolar space. As shown in Fig. 2B to F, recruitment of F4/80$^-$ CD11b^{mid} CD11c^{mid} monocytes, F4/80$^-$ CD11b^{high} CD11c^{high} DCs, F4/80$^-$ CD11b^{mid} CD11c^{high} DCs, F4/80$^+$ CD11b^{high} CD11c^{low} macrophages, and F4/80$^-$ CD11c$^-$ CD11b$^+$ Gr-1$^+$ neutrophils (where low, intermediate [mid], and high levels of expression are indicated by superscripts) into the bronchoalveolar space was markedly reduced in *Tlr2*$^{-/-}$ mice compared with WT and *Tlr4*$^{-/-}$ mice at 7 days postinfection. Interestingly, recruitment of all inflammatory cells in the bronchoalveolar space was significantly reduced in WT and *Tlr4*$^{-/-}$ mice at 14 days postinfection, while recruitment of those cells was increased in *Tlr2*$^{-/-}$ mice. The recruitment of CD3$^+$

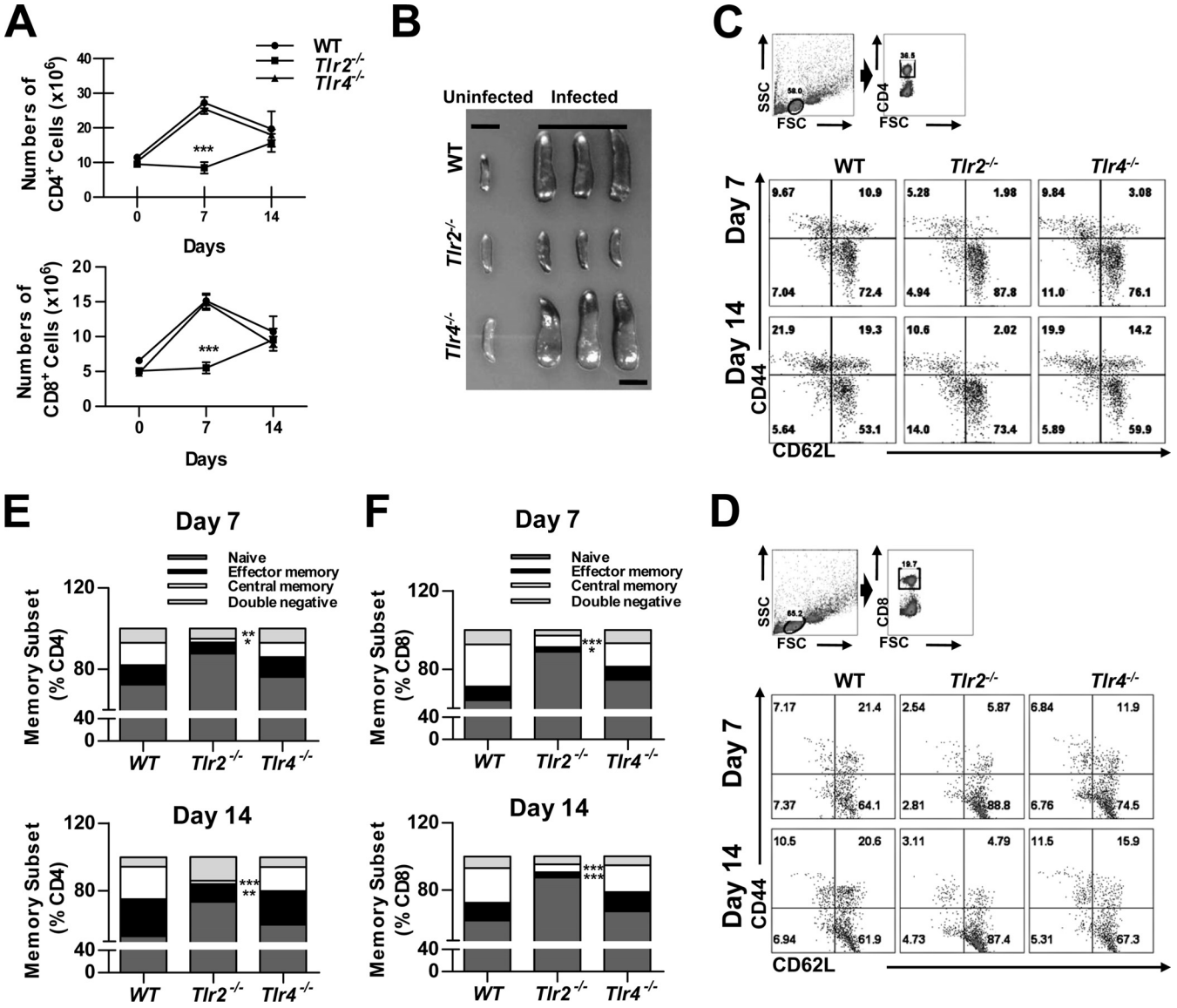

FIG 3 Comparative flow cytometric analysis of T-cell populations from the spleens of WT, *Tlr4*$^{-/-}$, and *Tlr2*$^{-/-}$ mice following infection with *M. abscessus* R. (A) Total numbers of CD4$^+$ and CD8$^+$ cells in the spleens of WT, *Tlr2*$^{-/-}$, and *Tlr4*$^{-/-}$ mice. Splenocytes from infected animals were harvested on days 0, 7, and 14 postinfection and stained with the indicated antibodies for each subset, as described in Materials and Methods. The results represent two to three independent experiments. (B) Spleen size was measured in WT, *Tlr4*$^{-/-}$, and *Tlr2*$^{-/-}$ mice at 7 days postinfection with *M. abscessus* R. (C and E) Population of memory/effector CD4$^+$ CD44^{high} CD62L^{low} T cells. (D and F) Population of memory/effector CD8$^+$ CD44^{high} CD62L^{low} T cells. Splenocytes from WT, *Tlr2*$^{-/-}$, and *Tlr4*$^{-/-}$ mice were harvested at the indicated time points, and CD4/CD44/CD62L (C) and CD8/CD44/CD62L (D) cells were stained as described in Materials and Methods. The bar graphs in panels E and F represent the percentages of cells in each quadrant. The results are presented as the means ± standard deviations of each group (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

CD4$^+$ and CD3$^+$ CD8$^+$ T cells in the bronchoalveolar space increased continuously from 7 to 14 days in WT and *Tlr4*$^{-/-}$ mice. In contrast, recruitment of these cells was not observed until 14 days in *Tlr2*$^{-/-}$ mice (Fig. 2E and F). These results indicate that TLR2 is essential for the recruitment of immune cells and the initiation of adaptive immunity against *M. abscessus* R.

TLR2 deficiency leads to decreased T-cell activation and IFN-γ production during *M. abscessus* R infection. TLR2 was found to play a major role in the secretion of cytokines and the recruitment of various immune cells in mice infected with *M. abscessus* R. The role of TLR2 was determined by analyzing the expansion of and phenotypic changes in splenic T cells. The numbers of CD4$^+$ and CD8$^+$ cells were significantly increased in the spleens of WT and TLR4$^{-/-}$ mice at 7 days, whereas these numbers were decreased by 14 days. In contrast, the percentages of CD4$^+$ and CD8$^+$ cells were not increased in *Tlr2*$^{-/-}$ mice at 7 days postinfection but were elevated at 14 days postinfection (Fig. 3A). These findings indicate that TLR2 is critical for inducing early-stage adaptive immunity. Similarly, the spleens of WT and *Tlr4*$^{-/-}$ mice were greatly enlarged at day 7 compared with the spleens of *Tlr2*$^{-/-}$ mice, which resembled those of uninfected mice (Fig. 3B). Changes in naive and effector/memory T cells were

determined by analyzing CD62L and CD44. $CD62L^{low}/CD44^{high}$ (effector and central memory) $CD4^+$ and $CD8^+$ T cells increased over time in WT and *Tlr4*$^{-/-}$ mice; however, more naive T cells were detected in *Tlr2*$^{-/-}$ mice (Fig. 3C to F). IFN-γ-producing T cells are essential for the control of *M. abscessus* infections in mice. To assess how the lack of TLR2 affected the development of this cell population, we measured the IFN-γ production capacity of all spleen cells obtained from infected animals following stimulation with *M. abscessus* R CFA. The secretion of IFN-γ also increased in WT and *Tlr4*$^{-/-}$ mice; however, the IFN-γ level was significantly lower in *Tlr2*$^{-/-}$ mice despite treatment with CFA (see Fig. S6A and B in the supplemental material).

TLR2 plays a key role in DC activation by *M. abscessus* R and in T-cell proliferation. The data above show that TLR2 plays an important role in the impairment of T-cell expansion and activation in *M. abscessus* R infection. Therefore, TLR2 serves to link the innate and adaptive immune responses in the regulation of *M. abscessus* infections. To test this hypothesis, the effects of TLR2 on the activation of DCs and on antigen presentation by DCs were investigated. First, BM-DCs were differentiated from WT, *Tlr2*$^{-/-}$, and *Tlr4*$^{-/-}$ mice using GM-CSF/IL-4. To test the ability of *M. abscessus* to activate BM-DCs via TLR2, we treated BM-DCs with *M. abscessus* CFA or performed direct infection with *M. abscessus* and measured the expression of surface molecules and proinflammatory cytokines. As shown in Fig. 4A and B, surface molecule expression and proinflammatory cytokine secretion were enhanced in WT and *Tlr4*$^{-/-}$ BM-DCs following treatment with *M. abscessus* CFA or direct infection with *M. abscessus* but were strongly diminished in *Tlr2*$^{-/-}$ BM-DCs. To verify the effects of TLR2 on T-cell proliferation, we performed a syngeneic MLR assay using OT-I T-cell receptor (TCR) transgenic $CD8^+$ T cells and OT-II TCR transgenic $CD4^+$ T cells. Transgenic CFSE-labeled OVA-specific $CD4^+$ and $CD8^+$ T cells were cocultured with *M. abscessus* CFA-treated BM-DCs isolated from WT, *Tlr2*$^{-/-}$, and *Tlr4*$^{-/-}$ mice. The results showed that BM-DCs from WT and *Tlr4*$^{-/-}$ mice induced significantly greater $CD4^+$ and $CD8^+$ T-cell proliferation than *Tlr2*$^{-/-}$ BM-DCs pulsed with *M. abscessus* CFA (Fig. 5A and B). In addition, syngeneic $CD4^+$ and $CD8^+$ T cells primed with *M. abscessus* CFA-treated BM-DCs isolated from WT and *Tlr4*$^{-/-}$ mice produced significantly ($P < 0.001$) higher levels of IFN-γ and IL-2 than those primed with *Tlr2*$^{-/-}$ BM-DCs (Fig. 5C and D); however, the differences in IL-4 secretion levels between BM-DCs from WT, *Tlr2*$^{-/-}$, and *Tlr4*$^{-/-}$ mice were barely detectable. These results support the hypothesis that TLR2 plays a key role in T-cell proliferation through the presentation of *M. abscessus* antigens and the conversion from innate to adaptive immunity.

Therapeutic intervention via activated dendritic cells. An analysis of the *Tlr2*$^{-/-}$ mice infected with *M. abscessus* R suggests that TLR2 is part of an effective immune response and that TLR2 plays a key role in DCs in the conversion from innate immunity to adaptive immunity against *M. abscessus* R. To test this hypothesis, we transferred *M. abscessus* CFA-treated mature BM-DCs from WT mice to *Tlr2*$^{-/-}$ mice infected with *M. abscessus* R. Unstimulated immature BM-DCs from WT mice were injected into *Tlr2*$^{-/-}$ mice as a control, and the survival rates and bacterial CFU counts were monitored for 30 days (Fig. 6A). While all *M. abscessus* R-infected *Tlr2*$^{-/-}$ mice died within 25 days of infection, the recipient mice were almost completely protected against *M. abscessus* R (Fig. 6B). In addition, bacterial CFU counts in the lungs were significantly reduced in the *Tlr2*$^{-/-}$ mice receiving mature BM-DCs (Fig. 6C). We further investigated the effect of CFA-treated mature BM-DCs from WT mice on the recruitment of inflammatory cells. As shown in Fig. 6D, the recruitment of monocytes, DCs, $CD11b^{mid}$ DCs, $CD11b^{high}$ macrophages, neutrophils, and $CD4^+$ T and $CD8^+$ T lymphocytes into the bronchoalveolar space was markedly increased in the recipient mice compared with levels in *Tlr2*$^{-/-}$ mice at 7 days postinfection. The recipient mice also showed significantly increased cytokine responses, which reached levels comparable to those observed in *Tlr2*$^{-/-}$ mice (Fig. 6E). Finally, to determine whether CFA-treated mature BM-DCs could enhance adaptive immunity in *Tlr2*$^{-/-}$ mice, we isolated splenocytes from each group of mice and analyzed the changes in spleen size, CD4 T cells, and the CD8 effector/memory T lymphocyte ratio. As shown in Fig. 6F, the spleens were substantially larger in the recipient mice than in *Tlr2*$^{-/-}$ mice at day 7. Levels of $CD62L^{low}/CD44^{high}$ (effector and central memory) $CD4^+$ and $CD8^+$ T cells were markedly increased in the recipient mice compared with those in *Tlr2*$^{-/-}$ mice (Fig. 6G). These results strongly suggest that TLR2 from DCs plays a key role in the conversion from innate immunity to adaptive immunity during infection with *M. abscessus* R. DC therapy is likely to be an excellent approach for the treatment of *M. abscessus*.

DISCUSSION

In this study, we investigated the functional role of TLR2 in the generation of early protective immunity against *M. abscessus*. We found that TLR2 contributes to the initiation of Th1-type T-cell immunity rather than to the innate killing of mycobacteria. Macrophage infection with an *M. abscessus* S or R variant did not differ among WT, *Tlr2*$^{-/-}$, and *Tlr4*$^{-/-}$ mice in terms of intracellular CFU counts (see Fig. S4 in the supplemental material). Nevertheless, the recognition of *M. abscessus* R variants by TLR2 is crucial for the production of TNF-α and IL-12p70 from both macrophages and DCs. Interestingly, TLR2 did not affect cytokine production in response to *M. abscessus* S. This result was consistent with a previous study showing that GPL in the outermost layer of the *M. abscessus* cell wall masks the role of underlying cell wall lipids that are involved in stimulating the innate immune response through TLR2 (23). In addition, our results suggest that a particular mycobacterial morphotype is associated with a specific disease phenotype and that the effective control of this morphotype requires a more efficient immune response (23, 24).

Most innate immune cells such as neutrophils and macrophages are rapidly recruited to the site of a mycobacterial infection, where they recognize the pathogens and initiate and regulate immune responses. The ability of phagocytes to kill mycobacteria *in vivo* most likely depends on the tissue microenvironment, stage of infection, individual host, and infecting organism. The interaction of phagocytes with other cells and the downstream effects on T-cell activity could result in a range of outcomes, from early clearance of infection to the dissemination of viable bacteria and an attenuated acquired immune response. In *Tlr2*$^{-/-}$ mice with established disease, neutrophils, monocytes, DCs, and macrophages accumulate under conditions of high pathogen load or immunological dysfunction in the later stages of infection. These cells are likely to contribute to pathology.

The development and progression of *M. abscessus*-related lung disease may depend on the balance between bacterial virulence and host defense. Mycobacterial virulence and host predisposition

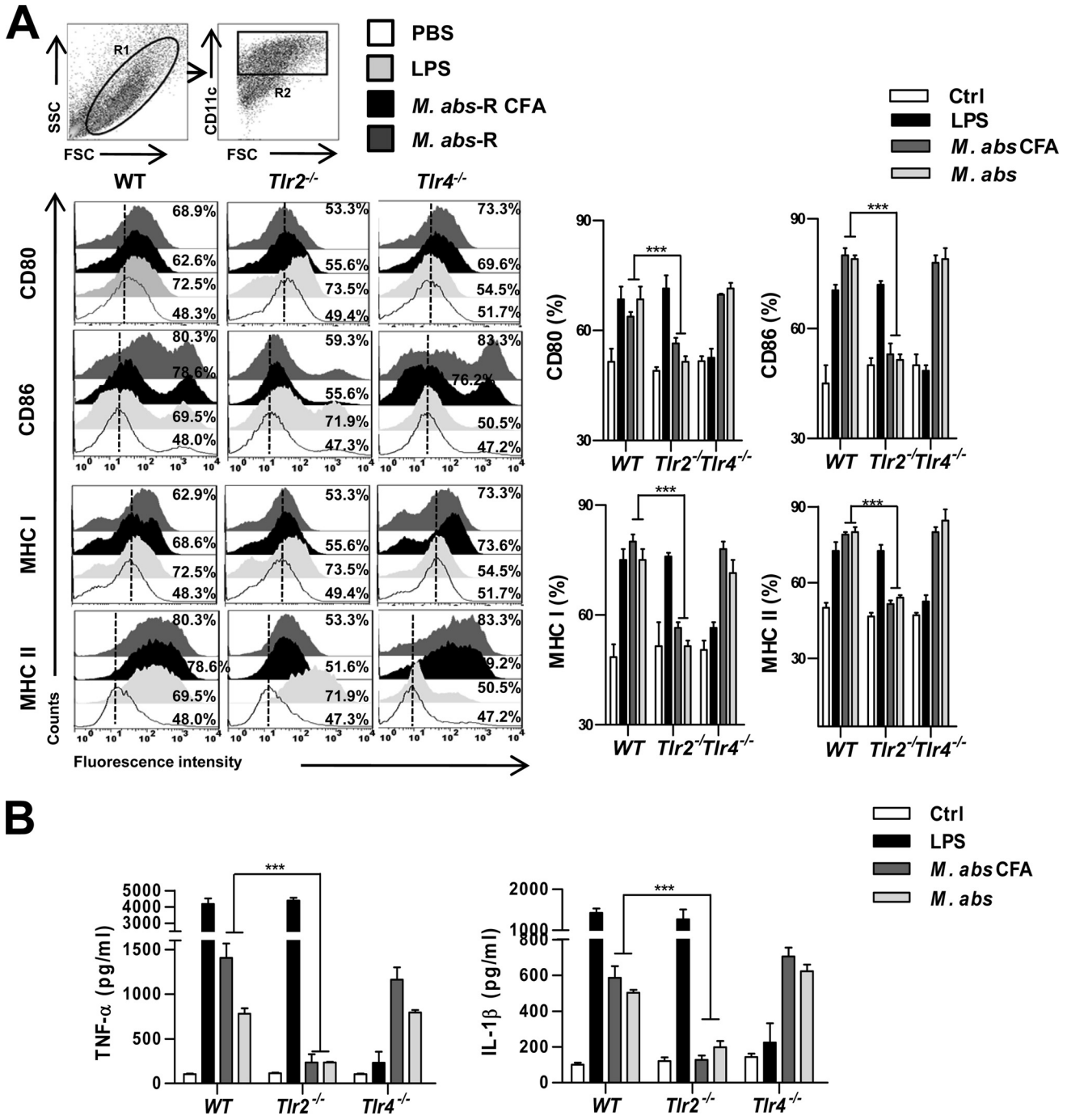

FIG 4 Maturation of bone marrow-derived dendritic cells (BM-DCs) via the direct engagement of TLR2 in response to *M. abscessus* R or its culture filtrate antigen. (A) A representative dot plot of dendritic cells derived from WT, *Tlr2*$^{-/-}$, and *Tlr4*$^{-/-}$ mice primarily gated on FSC and SSC (R1) and secondarily gated (R2) according to the expression of CD11c. Histograms for CD80, CD86, MHC-I, and MHC-II expression by *M. abscessus* R-infected (MOI of 1) or *M. abscessus* culture filtrate antigen (10 μg/ml)-treated CD11c^{+}-gated DCs derived from WT, *Tlr2*$^{-/-}$, and *Tlr4*$^{-/-}$ mice. The percentage of positive cells is shown for each panel. Bar graphs show the means ± SEM of the percentages for each surface molecule on CD11c^{+} cells in three independent experiments. (B) DCs derived from WT, *Tlr2*$^{-/-}$, and *Tlr4*$^{-/-}$ mice were treated or infected as described in panel A. TNF-α and IL-1β production in DCs derived from WT, *Tlr2*$^{-/-}$, and *Tlr4*$^{-/-}$ mice was measured by ELISA in response to *M. abscessus* R or its culture filtrate. All data are expressed as the means ± SEM ($n = 3$), and statistical significance (***, $P < 0.001$) is shown for BM-DCs from *Tlr2*$^{-/-}$ DCs compared to those from WT and *Tlr4*$^{-/-}$ DCs.

influence the progression of lung disease mediated by nontuberculous mycobacteria, including *M. abscessus*. Nevertheless, many reports have revealed that rough variants tend to be more virulent in experimental infections. Previous studies have suggested that the hyperlethality of rough *M. abscessus* strains may be related to mediators of the host innate response via TNF-α (23). In addition, overproduction of TNF-α may directly participate in immunopathological reactions or reflect a dysregulated immune response;

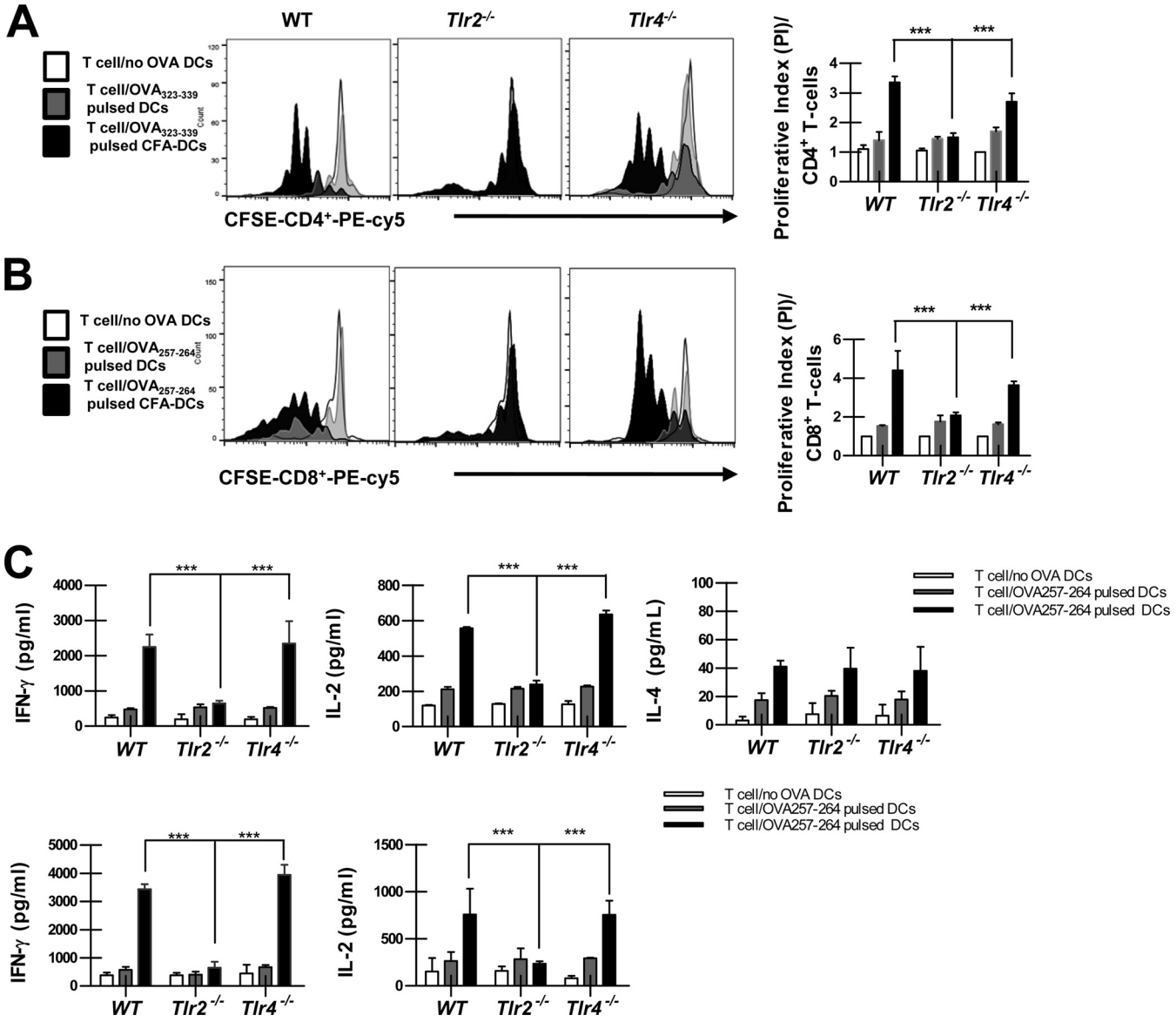

FIG 5 Proliferation and polarization of naive $CD4^+$ and $CD8^+$ T cells after coculture with *M. abscessus* R CFA-treated mouse BM-DCs generated from WT, *Tlr2*$^{-/-}$, and *Tlr4*$^{-/-}$ mice. Transgenic OVA-specific $CD4^+$ T cells and transgenic OVA-specific $CD8^+$ T cells were isolated, stained with CFSE, and cocultured for 96 h with DCs treated with *M. abscessus* CFA (10 μg/ml). OVA-specific $CD4^+$ T cells were then pulsed with an OVA peptide consisting of residues 323 to 339 ($OVA_{323–339}$; 1 μg/ml) (A), and OVA-specific $CD8^+$ T cells were pulsed with $OVA_{257–264}$ (1 μg/ml) (B). T cells alone and T cells cocultured with untreated DCs served as controls. The proliferation of OT-II^+ (A) and OT-I^+ (B) T cells was then assessed by flow cytometry. (C) The culture supernatants obtained from the conditions described in panel A were harvested after 24 h, and IFN-γ, IL-2, and IL-4 were measured by ELISA. The mean ± SEM is shown for three independent experiments (***, $P < 0.001$). (D) The culture supernatants obtained from the conditions described in panel B were harvested after 24 h, and IFN-γ and IL-2 were measured by ELISA. The mean ± SEM is shown for three independent experiments (***, $P < 0.001$).

however, the production of TNF-α in response to mycobacterial infection orchestrates the early induction of chemokines and is required for the coordination of leukocytes recruited into granulomas (25). It is obvious that TLR2 contributes to the host defense mechanisms that control *M. abscessus* infection for several reasons. First, *Tlr2*$^{-/-}$ mice were extremely susceptible to infection by the *M. abscessus* rough strain. Second, *Tlr2*$^{-/-}$ mice infected with *M. abscessus* rough strains displayed uncontrolled bacterial burdens. Finally, the reconstitution of TLR2 WT DCs enabled *Tlr2*$^{-/-}$ mice to control infection by a hypervirulent rough morphotype of *M. abscessus*. Thus, TLR2-mediated immune responses are protective rather than pathological host responses.

The uncontrolled infection in *Tlr2*$^{-/-}$ mice resulted from the noninduction of immune effectors. First, *Tlr2*$^{-/-}$ mice systemically and locally failed to produce crucial cytokines (IFN-γ, TNF-α, and IL-12p70) to generate Th1-polarized immunity. Impaired TLR2 expression resulted in decreased IL-12 and TNF-α production in NTM lung disease, which contributed to host susceptibility (26, 27). In addition, researchers using the same *M. abscessus* infection model (IFN-γ receptor-1 KO mice and TNF-α KO mice) found that mice with either genetic disruption had significantly more *M. abscessus* bacteria in their livers and spleens than WT mice (11, 13). TNF-α and IFN-γ were thus shown to be essential in the host defense against *M. abscessus* in mice. Second,

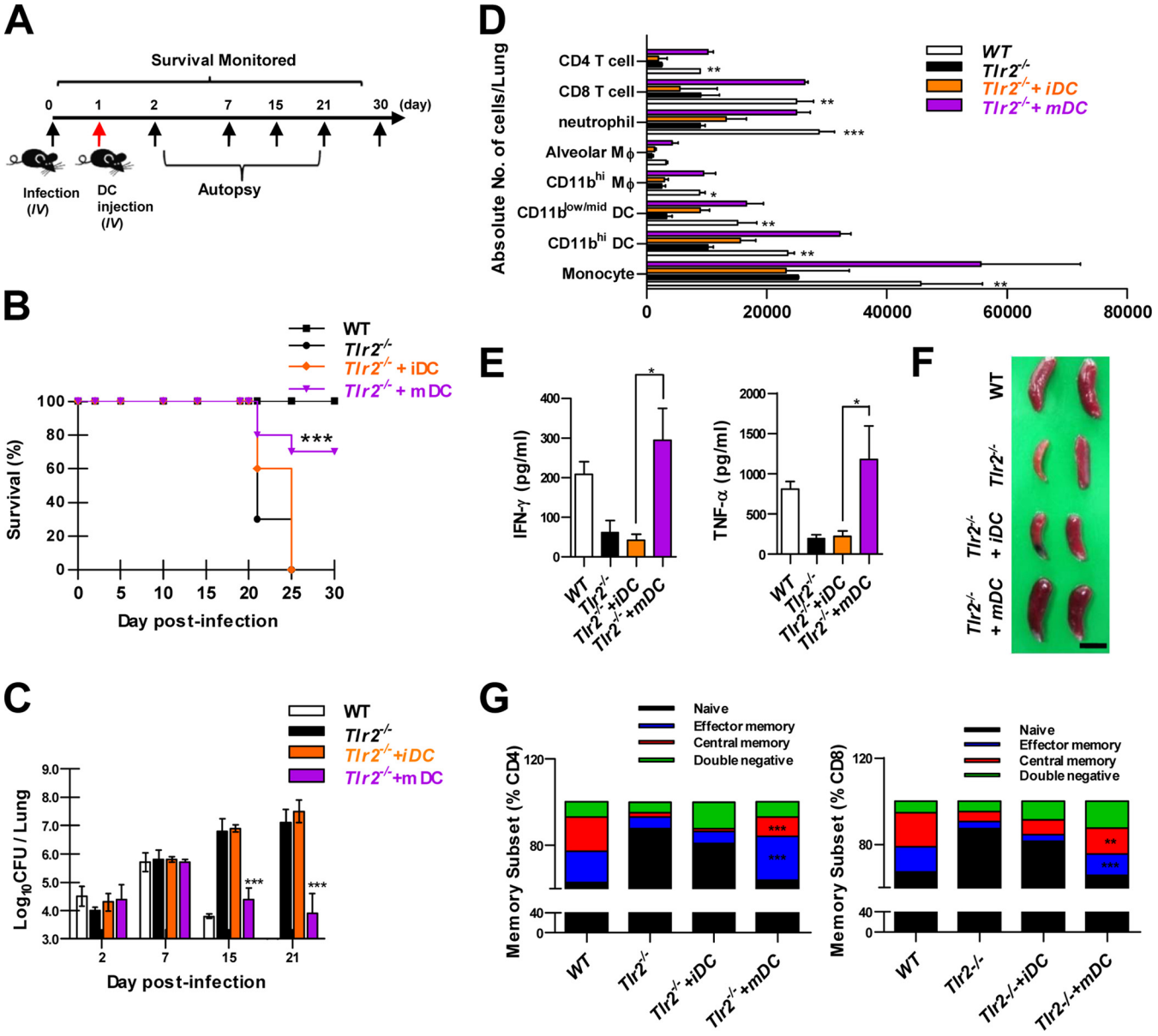

FIG 6 Immunotherapeutic intervention using *M. abscessus* CFA-treated BM-DCs from WT mice on $Tlr2^{-/-}$ mice. (A) Schematic diagram of the experiment. (B and C) WT and $Tlr2^{-/-}$ mice were infected i.v. with 1.4×10^7 CFU/mouse ($n = 30$ animals per group), and the *M. abscessus* culture filtrate antigen (CFA)-treated DCs (1.0×10^6) were injected via the tail vein on day 1 postinfection. Mouse survival and CFU counts were monitored for 30 days postinfection. The results are expressed as the mean estimated numbers of CFU ± SEM from the lung samples of five to six mice in two independent experiments (***, $P < 0.001$). iDCs, immature dendritic cells; mDCs, CFA-treated DCs. (D) The bar graph represents the absolute number of cells in the quadrants or squares on day 7 of the initial infection. The results are presented as the means ± standard deviations of each group (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). (E) Comparison of cytokine production in bronchoalveolar lavage fluid. Bronchoalveolar lavage fluid from infected animals was harvested at day 7 postinfection, and IFN-γ and TNF-α levels were determined by ELISA. The results are expressed as the means ± SEM of measurements from five to six animals, and statistical significance is indicated (*, $P < 0.05$). (F and G) Measurement of spleen size and analysis of memory/effector $CD4^+$ $CD44^{high}$ $CD62L^{low}$ and $CD8^+$ $CD44^{high}$ $CD62L^{low}$ T cells. The bar graph represents the percentages of cells in the quadrants. The results are presented as the means ± standard deviations of each group (**, $P < 0.01$; ***, $P < 0.001$).

early recruitment of neutrophils, macrophages, and DCs into the lungs failed in $Tlr2^{-/-}$ mice infected with the *M. abscessus* rough variant. Interestingly, in a murine model of infection with *Mycobacterium fortuitum*, which is similar to *M. abscessus*, the early neutrophilic response was an important contributor to infection control (28). Although increased neutrophilic infiltration in the lungs was observed in leptin-deficient *ob*/*ob* mice, which were more susceptible to infection with aerosolized *M. abscessus* (11), whether neutrophils were important in the host defense or the pathogenesis of *M. abscessus* lung infection was not specifically addressed; however, the delayed influx of neutrophils, macrophages, and DCs into the lungs of $Tlr2^{-/-}$ mice clearly indicates that these immune cells are more likely related to host defense. Notably, immune cells involved in the innate immune response

may not be directly involved in regulating bacterial infections as no differences were noted in bacterial counts among WT, *Tlr2*$^{-/-}$, and *Tlr4*$^{-/-}$ mice by day 7. Rather, the cells that regulate innate immune responses may suppress early bacterial dissemination and help to stimulate the adaptive immune response. Because *M. abscessus* growth is inhibited when the adaptive immune response is activated, cells responsible for adaptive immunity play a major role in the regulation of *M. abscessus* infection. In the absence of TLR2, the conversion from the innate to the adaptive immune response was inhibited, potentially leading to a failure to regulate *M. abscessus* infection. Additionally, the rapid influx of CD4$^+$ and CD8$^+$ T cells into the lungs failed in *Tlr2*$^{-/-}$ mice infected with the *M. abscessus* rough variant, indicating that T cells are likely an essential component of the protective immune response against *M. abscessus*. Both the number and functionality of T cells are crucial for protective immunity against mycobacterial infections. For example, the increased susceptibility to disseminated *M. tuberculosis* in mice with genetic disruption of IFN-γ or CD4$^+$ demonstrates the importance of these host immune components in pulmonary tuberculosis (29). Our data show that fewer splenic CD4$^+$ and CD8$^+$ cells were detected in *Tlr2*$^{-/-}$ mice than in WT and *Tlr4*$^{-/-}$ mice. Lastly, the expansion of memory/effector CD4$^+$/CD8$^+$ CD44^{high} CD62L^{low} T cells failed in *Tlr2*$^{-/-}$ mice infected with the *M. abscessus* rough variant. A population of CD44^{high} CD62L^{low} CD4$^+$/CD8$^+$ effectors was specifically generated from splenic T cells of *M. abscessus*-infected mice in response to CFA-treated DCs, indicating that TLR2 generates effector/memory T cells against *M. abscessus* infection. Protection against mycobacterial infection depends upon the rapid generation of effector memory T cells capable of producing IFN-γ (30). Thus, we concluded that the absence of TLR2 signaling in *M. abscessus*-infected DCs leads to the failure to initiate T-cell immune responses. Less IFN-γ is thus released, resulting in a failure to activate mycobacterium-containing macrophages to kill mycobacteria.

Notably, systemic administration of CFA-treated syngeneic DCs from WT mice *ex vivo* greatly strengthened immune priming *in vivo*, inducing a dramatic reduction in bacterial CFU and enhancing long-term survival in *Tlr2*$^{-/-}$ mice infected with the hypervirulent *M. abscessus* rough variant. Our findings highlight the essential contribution of TLR2 to protective Th1-type immunity during *M. abscessus* infection in a morphotype-dependent manner. Importantly, injecting DCs into WT mice did not accelerate the generation of protective immunity against a rough variant of *M. abscessus* in terms of bacterial CFU counts as normal host defense mechanisms were sufficient to prevent infection. Thus, activated DCs are not necessary for controlling mycobacteria because host immune responses can adequately contain mycobacterial infection in WT mice. Alternative immunotherapeutics using activated DCs may therefore be warranted for *M. abscessus* treatment if TLR2 variants are detected. The high therapeutic response rates attained with *M. abscessus*-targeted Th1-type T-cell immunity as a result of CFA-stimulated DCs in a model of severe infection suggest that the effectiveness and specificity of DCs can be harnessed to treat *M. abscessus*. Immunotherapy may be of clinical importance in the development of new treatments and targeted interventions for difficult to treat pathogens. Thus, optimal conditions for generating potent immunostimulatory DCs that overcome antibiotic resistance and immune suppression are key factors in DC-based *M. abscessus* immunotherapy. DC-dependent aspects of the optimal immune response contribute to resolving infections and could also represent a compensatory host mechanism in the face of uncontrolled bacterial replication. The ideas presented in this study might ultimately be of importance for patients. Their early defensive function and potential contribution to disease progression may make DCs especially significant.

In conclusion, this study provides essential evidence that TLR2 plays a crucial role in the initiation of protective immune responses against a rough morphotype of *M. abscessus* by activating innate immune cells and polarizing the development of T cells to generate a Th1 response. The development of specific agents that compensate for the TLR2 signaling pathway may be useful in the design of new strategies to prevent chronic diseases caused by rough morphotypes of *M. abscessus*. The successful use of DC immunotherapy in the treatment of *M. abscessus* will open avenues for the development of new immunotherapeutic strategies and better clinical outcomes.

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