**Mycobacterium abscessus** D-alanyl-D-alanine dipeptidase induces the maturation of dendritic cells and promotes Th1-biased immunity

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*MAB1843* encodes D-alanyl-D-alanine dipeptidase, which catalyzes the hydrolysis of D-alanyl-D-alanine dipeptide. We investigated whether MAB1843 is able to interact with DCs to enhance the effectiveness of the host’s immune response. MAB1843 was found to induce DC maturation via toll-like receptor 4 and its downstream signaling pathways, such as the mitogen-activated protein kinase and nuclear factor kappa B pathways. In addition, MAB1843-treated DCs stimulated the proliferation of T cells and promoted Th1 polarization. Our results indicate that MAB1843 could potentially regulate the immune response to *M. abscessus*, making it important in the development of an effective vaccine against this mycobacterium.

**INTRODUCTION**

*Mycobacterium abscessus* is the most well known rapidly growing mycobacterium (RGM), existing in natural habitats such as soil and water (1, 2) and usually found in intracellular compartments within an infected host. Infection by this mycobacterium can occur in various parts of the host’s body, regardless of their immunity. Approximately 80% of pulmonary RGM infections in the USA are attributed to *M. abscessus* (3). Unfortunately, the resistance of this species to antituberculosis drugs and antibiotics has become a major obstacle to its treatment in infected patients (4-6).

Recent studies have revealed that antigen-presenting cell (APC)-mediated innate and adaptive immunities play a pivotal role in combating *M. abscessus* infection (7-9). These studies also pointed out the importance of research on the pathogenicity of *M. abscessus* and relevant immunotherapies. In the *M. abscessus*-infected mouse model, the interferon-gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) secreted by various immune cells play a key role as regulators of the bacterial infection (10, 11). Furthermore, *M. abscessus* induces dendritic cell (DC) and macrophage secretion of TNF-α, interleukin-6 (IL-6), and IL-12p70 via the toll-like receptor 2 (TLR2) and TLR4 pathways (7, 8).

DCs are well known as the most powerful APCs. In peripheral tissues, immature DCs express low levels of costimulatory and major histocompatibility complex (MHC) molecules and exhibit a high level of endocytosis (12, 13). When DCs recognize pathogens via pattern recognition receptors, they start maturing, and in the process express high levels of costimulatory and MHC molecules, and pro-inflammatory cytokines, and their endocytosis capacity is downregulated. In addition, DC maturation occurs as the cells migrate towards the lymph nodes where they present antigens to naïve T cells, which induces T cell activation and
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RESULTS

MAB1843 induces dendritic cell maturation

First, we inspected the purity and toxicity of the recombinant MAB1843 before testing its physiological effect on DCs. The purity of MAB1843 was confirmed by Coomassie Brilliant Blue staining and western blot analysis using anti-His antibody (Ab). The apparent molecular size of the purified protein was approximately 24 kDa (Fig. 1A). In addition, the toxicity of MAB1843 on DCs was tested using Annexin V/propidium iodide (PI) staining and analyzed by fluorescence-activated cell sorting. Compared with the effect of a high dose of lipopolysaccharide (LPS; 1,000 ng/ml) as a positive control, MAB1843 was not cytotoxic to DCs (Fig. 1B). To exclude the effects of endotoxin contamination, we also checked for endotoxins in the purified MAB1843. Using the limulus amebocyte lysate (LAL) endotoxin assay kit (Lonza, Basel, Switzerland), we found the endotoxin levels in the purified MAB1843 to be < 0.1 EU/ml (Fig. 1C), indicating that certain

Fig. 1. Purification and cytotoxicity of recombinant MAB1843. (A) Recombinant MAB1843 was purified by affinity chromatography with Ni-NTA resin and then subjected to SDS-PAGE and staining with Coomassie Brilliant Blue (left panel), as well as analysis by western blotting using 1:1000 mouse anti-His tag antibodies (right panel). (B) Dendritic cells were treated with the indicated concentrations of MAB1843 and LPS for 24 h and then stained with Annexin V/PI. The results are representative of three independent experiments. (C) Endotoxin levels in the purified MAB1843 were analyzed by the LAL endotoxin assay kit.

Fig. 2. MAB1843 induces the maturation of dendritic cells (DCs). (A) Immature DCs were cultured with 0.5 or 1 μg/ml of MAB1843 or 50 ng/ml of LPS for 24 h and analyzed for the expression of surface markers by two-color flow cytometry. DCs were stained with FITC anti-CD11c antibodies and PE anti-CD86 antibodies, PE anti-MHC class II antibodies, or PE anti-MHC class I antibodies. *P < 0.05, **P < 0.01, and ***P < 0.001, when compared with DCs treated with MAB1843 only. (B and C) Immature DCs were cultured with 0.5 or 1 μg/ml of MAB1843 or 50 ng/ml of LPS for 24 h. The amounts of TNF-α, IL-1β, IL-6, IL-12p70, and IL-10 in the culture medium were measured by ELISA. The experiments were run at least in triplicates. *P < 0.05, when compared with DCs treated with MAB1843 only. (D) Mature DCs were generated by stimulating the immature cells with 50 ng/ml of LPS or 0.5 or 1 μg/ml of MAB1843 for 24 h. Endocytic activity at 37°C and at 4°C was assessed by flow cytometry, based on dextran-FITC uptake. The percentages of dextran-FITC⁺ CD11c⁺ cells are indicated. The results are representative of three independent experiments.

proliferation. In this way, DCs act as the link between innate immunity and adaptive immunity, and hence, they have gained much attention in cancer immunotherapy research (12-15).

Mycobacterial proteins, such as PE-RGRS, LprA, MAB2560, Rv0652, Rv0462, MAP1305, HspX, and HBHA, have been shown to induce DC maturation and T cell activation (8, 16-22). These proteins activate TLR2 or TLR4 signaling and can be used as adjuvants for DC maturation. We recently suggested MAB2560 as the first ligand isolated from M. abscessus that induces the activation of DCs via TLR4; however, little is known about how M. abscessus-derived ligands affect the immune system.

MAB1843, a 222-amino-acid D-alanyl-D-alanine dipeptidase (D-Ala-D-Ala dipeptidase; EC number: 3.4.13.22), is a hypothetical antigen of M. abscessus. However, the function of MAB1843 in M. abscessus and the manner in which it affects the immune system remain unknown.

In this study, we aimed to elucidate the effects of MAB1843 on the immune system, with a focus on the maturation of DCs as well as their endocytosis and cytokine-producing abilities. In particular, we examined whether MAB1843 affects DC maturation through TLRs and how the activated DCs affect T cells. Knowledge on these mechanisms could contribute to the development of vaccines against M. abscessus.
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amounts of MAB1843 are not cytotoxic to DCs. Next, to test the physiological effect of MAB1843 on DC maturation, we analyzed the expression of surface molecules and the secretion of cytokines in MAB1843- or LPS-induced (positive control) DCs. MAB1843 enhanced the expression of surface molecules such as CD80, CD86, and MHC class I and II (Fig. 2A), and promoted the secretion of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 (Fig. 2B), but His-tagged protein itself did not affect to the production of cytokines in DCs (Supplementary Fig. 1). Whereas LPS stimulated the production of both IL-12p70 (involved in Th1 polarization) and IL-10 (involved in Th2 polarization), MAB1843 specifically induced the secretion of IL-12p70 only (Fig. 2C), indicating that it may promote Th1 polarization. Furthermore, MAB1843 suppressed the capacity of dextran and LPS to induce endocytosis in DCs, indicating that it can functionally provoke the maturation of DCs towards promoting Th1 polarization.

MAB1843 Induces DC Maturation via TLR4 Signaling

Toll-like receptors of immune cells, such as macrophages and DCs, act as initiators of innate immunity by recognizing various pathogen-associated molecular patterns (PAMPs) (23, 24). Apart from PAMPs, other M. abscessus antigens can activate DC maturation through TLR2 or TLR4 (7, 8). Thus, we tested whether MAB1843 can modulate DC maturation through TLRs as well. We analyzed the secretion of proinflammatory cytokines, such as TNF-α and IL-6, in wild type (WT), TLR2 knockout (KO), and TLR4 KO DCs treated with MAB1843, LPS (TLR4 agonist), Pam3CSK4 (TLR1/2 agonist), imiquimod (TLR7 agonist), ODN1826 (TLR9 agonist), or Poly I:C (TLR3 agonist). Whereas the secretion of TNF-α and IL-6 was observed in all three cell types stimulated by imiquimod, ODN1826, or Poly I:C, both cytokines were completely inhibited in Pam3CSK4-stimulated TLR2 KO DCs and in LPS-stimulated TLR4 KO DCs. The secretion of cytokines was completely blocked only in TLR4 KO DCs stimulated by MAB1843 or LPS (Fig. 3A), indicating that MAB1843 can modulate DC maturation through TLR4. As mitogen-activated protein kinases (MAPKs) and nuclear factor-kappa B (NF-κB) signaling are reportedly downstream mediators in TLR4 signaling (24), we investigated whether MAB1843 could enhance the activation of these two mediators. We measured the phosphorylation of MAPKs and the expression of NF-κB inhibitor-alpha (IκBα) as an upstream molecule of NF-κB in the cytosol. MAB1843 promoted the activation of MAPKs and NF-κB in DCs derived from WT and TLR2-knockout mouse but not TLR4-knockout mouse (Fig. 3B and Supplementary Fig. 2). In addition, to investigate MAB1843 how to activate TLR4 signaling, we measured the interaction between TLR4 and NF-κB signaling by direct binding with TLR4.

Fig. 3. MAB1843 induces TLR4 signaling during dendritic cell (DC) maturation. (A) DCs derived from wild type (WT), TLR2 knockout (KO), and TLR4 KO mice were treated with MAB1843 (1 μg/ml), LPS (50 ng/ml), Pam3CSK4 (Pam3) (10 μg/ml), imiquimod (Imiquimod) (1 μg/ml), ODN1826 (ODN) (1 μg/ml), or Poly I:C (10 μg/ml) for 24 h. The production of TNF-α and IL-6 in DCs was measured by ELISA. The experiments were run at least in triplicates. **P < 0.001. (B) DCs were treated with MAB1843 (1 μg/ml) or LPS (50 ng/ml) for 0, 5, 15, 30, and 60 min. Anti-p-ERK antibody, anti-p-JNK antibody, anti-ERK antibody, anti-JNK antibody, anti-IκBα antibody, and anti-p-actin antibody was added to the western blot membrane. The results are representative of three independent experiments.

Fig. 4. MAB1843 induces T cell proliferation and Th1 polarization. (A and B) Transgenic OVA-specific CD8+ T cells and transgenic OVA-specific CD4+ T cells were isolated from OT-I and OT-II mice, respectively. T cells were stained with carboxyflowrescein succinimidyl ester and co-cultured for 96 h with DCs treated with 1 μg/ml of MAB1843 or 50 ng/ml of LPS. The cells were then analyzed by flow cytometry. (C) Transgenic OVA-specific CD4+ T cells were co-cultured for 3 days with non-stimulated DCs, OVA257-264-pulsed DCs, OVA257-264-pulsed MAB1843-DCs, or OVA257-264-pulsed LPS-treated DCs. The CD4+ T cell expression of IFN-γ and IL-4 was detected by intracellular staining and flow cytometry. The results are representative of three independent experiments.
MAB1843 enhances CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation through DC maturation

Activated DCs mature during their migration to the lymph nodes. Once mature, they present antigens to naïve T cells and induce T cell proliferation (12). To characterize the effect of MAB1843 on DC and T cell interactions, we conducted a syngeneic mixed lymphocyte reaction assay using OT-I T cell receptor (TCR) transgenic CD8<sup>+</sup> T cells and OT-II TCR transgenic CD4<sup>+</sup> T cells (25). The carboxyfluorescein succinimidyl ester (CFSE)-conjugated ovalbumin (OVA)-specific CD8<sup>+</sup> and CD4<sup>+</sup> T cells were divided and co-cultured with DCs presenting OVA<sub>257-264</sub> (Fig. 4A) or OVA<sub>323-339</sub> (Fig. 4B), which were effectively activated by MAB1843 treatment and LPS stimulation. In addition, MAB1843 also potentiated the production of IFN-γ by the activated CD4<sup>+</sup> T cells, but not that of IL-4 (Fig. 4C). Considering these results and the fact that MAB1843 stimulated the DC production of IL-12p70 (Fig. 1C), we propose that this effect could be used in immunotherapy to polarize naïve CD4<sup>+</sup> and CD8<sup>+</sup> T cells towards an IFN-γ-producing Th1 phenotype.

DISCUSSION

Several strains of non-tuberculous mycobacteria, such as Mycobacterium xenopi and Mycobacterium kansasi, have been known to cause pulmonary diseases (26, 27). Recent research has shown that M. abscessus induces an immune response through TLR2 and dectin-1 on host cells (7, 9), and MAB2560, the first purified antigen isolated from M. abscessus, activates DCs via TLR4 (8). It is essential to understand the relationship between M. abscessus and the immune system to develop a treatment strategy against infection by this species. Therefore, it was necessary to completely verify the immune response to M. abscessus. The identification of mycobacterial antigens that could be used as adjuvants to induce the activation of APCs may direct the development of immunotherapy in the future.

MATERIALS AND METHODS

Animals

Female 4-6-week-old C57BL/6 (H-2Kb and I-A<sup>b</sup>) mice were purchased from Orient Bio (Daejeon, South Korea). TLR2 and TLR4 KO mice, as well as transgenic OT-I and OT-II mice, were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All of the animals were housed in a specific pathogen-free facility and treated in accordance with the animal care guidelines (Institutional Animal Care and Use Committee (IACUC) number: KU14046).

Reagents and antibodies

Recombinant mouse granulocyte macrophage colony stimulating factor (rmGM-CSF) was purchased from BioLegend (San Diego, CA, USA). The Annexin V/PI apoptosis detection kit was purchased from BD Biosciences (East Rutherford, NJ, USA). Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated Abs—namely, FITC anti-mouse CD11c Abs (Clone: N418), PE anti-mouse CD80 Abs (Clone: 16-10A1), PE anti-mouse CD86 Abs (Clone: GL-1), PE anti-mouse H-2Kd/ H-2Dd Abs (Clone: 34-1-2S), PE anti-mouse I-A/I-E Abs (Clone: M5/114.15.2), FITC anti-mouse IFN-γ Abs (Clone: XMG1.2), and PE anti-mouse IL-4 Abs (Clone: BVD4-1D11) were purchased from BioLegend. TNF-α, IL-1β, IL-6, IL-10, and IL-12p70 ELISA kits were purchased from eBioscience (San Diego, CA, USA). FITC-conjugated dextran was purchased from Sigma-Aldrich (St. Louis, MO, USA). The TLR agonists (viz., LPS from Escherichia coli O111:B4, Pam3CSK4, imiquimod, Poly I:C, and ODN1826) were purchased from Invivogen (San Diego, CA, USA). Antibodies against phospho-ERK, phospho-p38, phospho-IκB, IκBα, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). CFSE was purchased from Life Technologies (Eugene, OR, USA).
Cloning of the MAB1843 gene

The MAB1843 gene was isolated from the full genome of *M. abscessus* for cloning into the *E. coli* BL21 strain. MAB1843 was amplified using a forward primer (5’-CATATGATGAA GCGTCTGATCCTCGGT-3’) and a reverse primer (5’-AACG TTGTGACGGGGCGGTACAGGA-3’). Thereafter, the PCR products were cleaved by NdeI and HindIII, and the cleaved genes were then inserted into the pET-22b(+) vector (Novagen, Madison, WI, USA).

Purification of MAB1843 from *E. coli* BL21

The transformed *E. coli* BL21 was incubated in Luria-Bertani broth containing 100 µg/ml of ampicillin. When the optical density at 600 nm reached 0.4-0.6, 1 mM of isopropylthio-β-D-galactoside was added to the *E. coli* BL21 culture medium, which was then incubated for 6 h. The *E. coli* BL21 cells were then harvested and lysed using lysozyme, dithiothreitol, phenylmethanesulfonyl fluoride (PMSF), and DNase I. The supernatant was analyzed by affinity chromatography with Ni-NTA agarose resin. The eluted solution was concentrated to a suitable volume, dialyzed against phosphate-buffered saline (PBS). Finally, endotoxins were removed by polymyxin B-agarose (Sigma-Aldrich), and the endotoxin level was measured using the LAL test kit (Lonza).

Generation of murine bone marrow dendritic cells

DCs were collected from the bone marrow of the tibia and femora of C57BL/6 mice. The progenitor cells were isolated from the bone marrow, and red blood cell lysis buffer was added (Sigma-Aldrich). These cells were then mixed with RPMI 1640 medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, and GM-CSF. The cells were seeded into 12-well cell culture plates and incubated at 37°C in a 5% CO₂ atmosphere. Fresh medium was added on the third day. On the sixth day, the cells were further treated or harvested.

Western blot analysis

The harvested cells were washed with PBS and lysed using a lysis buffer (containing 0.5% NP-40, 1 mM EDTA, 50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 0.5 mM PMSF, 0.5 M NaF, and 0.01% protease inhibitor cocktail (Biobasic, Amherst, NY, USA)). SDS-PAGE was conducted using a 10% polyacrylamide gel, following which the protein bands were transferred to a polyvinylidene difluoride membrane. After blocking the membrane with 5% skimmed milk, primary Abs (anti-α-actin, anti-β-actin, anti-p-ERK, anti-p-JNK, anti-IκBα, and anti-β-actin) were added to the membrane and incubation was carried out overnight at 4°C. On the next day, after rinsing with Tris-buffered saline and Tween 20, the anti-mouse and anti-rabbit secondary Abs were added to the membrane and a further incubation for 1 h at room temperature (RT) was carried out. After rinsing the membrane, enhanced chemiluminescence solution was added, and target bands were identified using the LAS-4000 imaging system (Fuji Film, Tokyo, Japan). To enable interaction with other antibodies, stripping buffer (Thermo Scientific, Waltham, MA, USA) was added to the membrane, following which the blocking step was repeated, and new antibodies were added.

Cytotoxicity analysis

MAB1843- or LPS-treated DCs were harvested and rinsed with PBS. Annexin V/PI staining was performed on these cells for 15 min at RT. The cytotoxicity of MAB1843 on DCs was analyzed by flow cytometry using the FACS Calibur instrument (BD Biosciences).

Endocytosis ability analysis

MAB1843- and LPS-treated DCs were treated with FITC-conjugated dextran and incubated for 45 min at 37°C and 4°C, respectively. The DCs were then harvested and rinsed with PBS. Thereafter, the DCs were stained using the PE-conjugated anti-CD11c Ab and analyzed by flow cytometry.

Mixed Lymphocyte Reaction (MLR)

MAB1843- or LPS-treated DCs were incubated with OVA257-264 or OVA323-339. Then, 1 × 10⁴ DCs and 1 × 10⁵ CFSE-labeled T cells were co-cultured in a 96-well cell culture plate. These transgenic OVA-specific CD8⁺ and CD4⁺ T cells were isolated from splenocytes of OT-I and OT-II mice, respectively. After 96 h, the cells were harvested and rinsed with PBS. Then, the cells were stained with Cy5 anti-CD8 or Cy5 anti-CD4 Abs and further analyzed by flow cytometry.

Intracellular staining

The harvested and rinsed cells were fixed in 4% paraformaldehyde and then permeabilized using saponin buffer. The cells were stained with anti-IFN-γ and anti-IL-4 Abs for 20 min.

Enzyme-Linked immunosorben assay

The levels of IL-12p70, IL-10, IL-1α, TNF-α, IL-6, and IFN-γ were measured by ELISA (eBioscience; R&D Systems, Minneapolis, MN, USA).

Blitz assay

The direct binding between rTLR4/MD2 and MAB1843 was performed using the BLItz system (Fortebio, Menlo Park, CA). Human rTLR4/MD2 tagged with anti-penta-His (HIS) was purchased from R&D Systems (Minneapolis, MN). HIS biosensors (catalog no. 18-5078, Fortebio) were hydrated for 10 min prior to the experiment. The concentration of HIS-tagged rTLR4/MD2 was 0.1 mg/ml, and that of purified MAB1843 was 0.1, 0.5, and 1 mg/ml and BSA was 1 mg/ml. The setting was as follows: initial baseline for 30 s, loading for 300 s, baseline for 0.1, 0.5, and 1 mg/ml and 0.1, 0.5, and 1 mg/ml and BSA was 1 mg/ml. The setting was as follows: initial baseline for 30 s, loading for 300 s, baseline for 60 s, association for 120 s, and dissociation for 120 s.

Statistical analysis

All experiments were repeated at least three times, with consistent results. Unless otherwise stated, data are expressed as the mean ± SEM. Variance analysis was used to compare
experimental groups with control values, whereas comparisons between multiple groups were made using Tukey’s multiple comparison tests (Prism 3.0; GraphPad software). A P value of less than 0.05 was considered to indicate statistical significance.

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