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Research paper

Mycobacterium tuberculosis protein Rv2220 induces maturation and activation of dendritic cells

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ABSTRACT

Tuberculosis remains a serious health problem worldwide. Characterization of the dendritic cell (DC)-activating mycobacterial proteins has driven the development of effective TB vaccine candidates besides improving the understanding of immune responses. Some studies have emphasized the essential role of protein Rv2220 from *M. tuberculosis* in mycobacterial growth. Nonetheless, little is known about cellular immune responses to Rv2220. In this study, our aim was to test whether protein Rv2220 induces maturation and activation of DCs. Rv2220-activated DCs appeared to be in a mature state with elevated expression of relevant surface molecules and proinflammatory cytokines. DC maturation caused by Rv2220 was mediated by MAPK and NF-κB signaling pathways. Specifically, Rv2220-matured DCs induced the expansion of memory $CD62L^{low}CD44^{high}CD4^{+}$ T cells in the spleen of mycobacteria-infected mice. Our results suggest that Rv2220 regulates host immune responses through maturation of DCs, a finding that points to a new vaccine candidate against tuberculosis.

1. Introduction

Approximately 8 million people develop active tuberculosis (TB) every year, with 1.8 million dying from the disease. In addition to this already huge burden of disease, it is estimated that up to 2 billion people have been infected with the causative agent, *Mycobacterium tuberculosis* [1]. The only currently available TB vaccine, *Mycobacterium bovis* bacillus Calmette–Guérin (BCG), has been applied since the early 1920s but has variable effectiveness against pulmonary TB [2], particularly in adults. Therefore, the development of a new vaccine with improved protection or a BCG-booster subunit vaccine is needed to effectively control TB. The targets of an effective TB vaccine are based on its ability to activate a T helper 1 (Th1) immune response. Many mycobacterial proteins inducing a $CD4^{+}$ T-cell response have been reported, and only a few proteins have been tested in TB vaccines in clinical trials. Antigen-presenting cells like dendritic cells (DCs) play a critical role in determination of the polarization between Th1 and Th2 responses. It is well known that DCs orchestrate interactions between innate immunity and adaptive immunity against TB. For these reasons, the mycobacterial antigens that induce activation of DCs have been identified [3–5]. Consequentially, the functional characterization of DC-activating mycobacterial proteins has driven the development of effective vaccine candidates besides improving the understanding of host immune responses.

Activated DCs involved in a response to an infection show enhanced expression of major histocompatibility complex (MHC), adhesion, and costimulatory molecules [6]. Immature DCs readily take up mycobacteria and/or mycobacterial antigens for degradation and peptide loading onto MHC molecules [7]. The recognition and internalization of mycobacterial antigens induces the maturation of DCs, a process that is accompanied by their migration from peripheral tissues into the T-cell areas of draining lymph nodes [8]. In the course of maturation, DCs manifest marked upregulation of MHC class II. Expression of proinflammatory chemokine receptors, such as CCR1, CCR2, CCR5, and CCR6, and the responsiveness to their ligands decreases [9]. In addition, mature DCs express increased surface amounts of costimulatory and adhesion molecules such as CD80, CD86, and CD40 [10]. As a result of these multiple alterations, mature DCs acquire the distinct ability to trigger a primary T-cell response [9]. The DCs maturated by mycobacterial antigens also produce immunoregulatory cytokines such as IL-12, IL-1β, or IL-10, which may promote the activation of T cells [11]. DC-mediated IL-12 production within draining lymph nodes is necessary to initiate an optimal acquired-immunity interferon (IFN)-γ response from T cells, which is critical for control of the intracellular

Abbreviations: Ab, antibody; BMDCs, bone marrow–derived dendritic cells; DCs, dendritic cells; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte-macrophage colony-stimulating factor; GS, glutamine synthetase; HRP, horseradish peroxidase; IFN, interferon; LPS, lipopolysaccharide; mAb, monoclonal antibody; MHC, major histocompatibility complex; NTA, nitrilotriacetic acid; PI, propidium iodide; PE, phycoerythrin; PmB, polymyxin B; STS, staurosporine; TB, tuberculosis

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infection [12,13]. In contrast, IL-10 produced by DCs is an immunosuppressive cytokine essential for limiting the host immune response and related pathologies [14]. Many studies have indicated that the interplay of DCs with mycobacteria results in their maturation reflected by increased expression of surface molecules and secretion of elevated amounts of proinflammatory cytokines [9,15,16]. The maturation and activation of DCs enable these cells to effectively elicit an antigen-specific T-cell response.

Previously we showed that culture filtrate proteins (CFPs) were fractionated using multistep chromatography via hydrophobic interaction, hydroxylapatite, and ion exchange [17,18]. To identify a DC-maturating mycobacterial protein in CFPs, we screened the ability of these Mtb-CFP fractions on the induction of proinflammatory cytokines in DCs. We found that an identified Rv2220 protein induces the maturation of DCs. In this study, our aim was to evaluate a possible immunostimulatory effect of the Rv2220 protein on DCs. *M. tuberculosis* protein Rv2220, glutamine synthetase type 1 (GlnA1), is an essential enzyme for the growth of *M. tuberculosis* both *in vitro* and *in vivo* [19]. Although the Rv2220 protein may have a crucial function in the virulence of *M. tuberculosis*, the cellular immune responses to this protein have not been elucidated. Here, we show that protein Rv2220 induces maturation and activation of DCs.

2. Materials and methods

2.1. Antibodies (Abs) and reagents

Recombinant mouse granulocyte-macrophage colony stimulating factor (GM-CSF), and interleukin 4 (IL-4) were purchased from CreaGene (Gyeonggi, Republic of Korea), and fluorescein isothiocyanate (FITC)-annexin V/propidium iodide (PI) kits from R&D Systems (Minneapolis, MN, USA). Lipopolysaccharide (LPS) from *Escherichia coli* O111:B4 was acquired from InvivoGen (San Diego, CA, USA), whereas the endotoxin filter (END-X) and endotoxin removal resin (END-X B15) from the Associates of Cape Cod (East Falmouth, MA, USA). The anti-phosphorylated (p)-ERK1/2 monoclonal Ab (mAb), anti-ERK1/2 monoclonal Ab, anti-phosphorylated p38 monoclonal Ab, anti-p38 monoclonal Ab, anti-p-IκB-α monoclonal Ab, anti-IκB-α monoclonal Ab, and anti-β-actin polyclonal Ab were acquired from Cell Signaling Technology (Danvers, MA, USA), and the horseradish peroxidase (HRP)-conjugated anti-mouse IgG Ab and HRP-conjugated anti-rabbit Ab from Calbiochem (San Diego, CA, USA). The FITC-conjugated mAbs against CD11c and the phycoerythrin (PE)-conjugated mAbs against CD80, CD86, MHC class I, and MHC class II were purchased from eBioscience (San Diego, CA, USA), whereas the PE-conjugated rat anti-IgG1, rat anti-IgG2a, and rat anti-IgG2b, allophycocyanin-conjugated rat anti-IgG2a and rat anti-IgG1, FITC-conjugated rat anti-IgG2b, and the PE-Cy7-conjugated mouse anti-IgG1 and rat anti-IgG2b Abs from eBioscience. These antibodies served as isotype controls. TNF-α, IL-1β, IL-10, and IL-12p70 ELISA kits were acquired from eBioscience.

2.2. Purification of the recombinant Rv2220 protein

To produce a recombinant Rv2220 protein, the corresponding gene was amplified by PCR from *M. tuberculosis* H37Rv ATCC27294 genomic DNA as a template with the following primers: Rv2220 forward, 5′-CATATGACGGAAAAGACGCCC-3′, and reverse, 5′-AAGCTTAACGTC GTAGTACAGCGC-3′. The PCR product of *Rv2220* was digested with *NdeI* and *EcoRI*. The amplicon was inserted into the pET22b (+) vector (Novagen, Madison, WI, USA). The His-tagged recombinant plasmids were transfected into *E. coli* BL21 cells by heat shock for 1 min at 42 °C. The recombinant protein was prepared as previously described [20]. Briefly, *E. coli* BL21 cells carrying the plasmid were induced with isopropyl b-D-thiogalactopyranoside, and the His-tagged recombinant protein was purified on Ni-nitrilotriacetic acid (Ni-NTA) columns (Qiagen). The amount of residual LPS in the Rv2220 preparation was evaluated using the Limulus Amoebocyte Lysate (LAL) Test Kit (Lonza, Basel, Switzerland). The purity of Rv2220 was evaluated by CB staining and western blotting analysis with an anti-histidine antibody.

2.3. Generation and culture of mouse DCs

Murine bone marrow–derived DCs (BMDCs) were isolated, cultured, and purified as described recently [20]. Briefly, bone marrow cells isolated from 5- to 6-week-old C57BL/6 mice were disrupted with red blood cell (RBC)-lysing buffer. The obtained cells were cultured at 37 °C in the presence of 5% CO_2 in the RPMI 1640 medium supplemented with 100 μg/mL penicillin and 100 μg/mL streptomycin (Welgene, Daegu, Korea), 10% of fetal bovine serum (Welgene), 50 μM mercaptoethanol (Sigma, St. Louis, MO, USA), 0.1 mM nonessential amino acids (Welgene), 1 mM sodium pyruvate (Sigma), 20 ng/mL GM-CSF, and 10 ng/mL IL-4.

2.4. Cytotoxicity analysis

This analysis was conducted using an Annexin V/PI staining kit (BD Biosciences). The cells were stained with FITC-conjugated with annexin V and PI. Assessment of the stained cells was performed on a FACSCanto II with FACSDiva, and the results were analyzed in the FlowJo software (Tree Star, Ashland, OR, USA).

2.5. Analysis of the expression of surface molecules by flow cytometry

On day 7, BMDCs were harvested, washed with PBS, and resuspended in FACS washing buffer (2% FBS and 0.1% sodium azide in PBS). The cells were stained with PE-conjugated anti-H-2 Kb (MHC class I), anti-I-Ab (MHC class II), anti-CD80, and anti-CD86 Abs along with FITC-conjugated anti-CD11c Abs for 30 min at 4 °C. The cells were washed three times with PBS and resuspended in PBS. The fluorescence was measured by flow cytometry.

2.6. Confirmation of LPS decontamination of Rv2220

To confirm that the maturation of DCs induced by Rv2220 was not due to contaminating endotoxins such as LPS in the protein preparations, a pretreatment with polymyxin B (PmB; Sigma), heat denaturation, and digestion with proteinase K (Sigma) were carried out. DCs were preincubated with 50 μg/mL PmB for 1 h at room temperature prior to treatment with 100 ng/mL LPS and 10 μg/mL Rv2220. For heat denaturation, LPS or Rv2220 was incubated at 100 °C for 1 h. For digestion with proteinase K, LPS or Rv2220 was incubated for 1 h at 37 °C with soluble proteinase K at the concentration of 10 μg/mL followed by heating for 15 min at 100 °C to deactivate the enzyme, and subsequently added to BMDC cultures. After 24 h, cytokine levels in the culture supernatant of BMDCs were analyzed by an enzyme-linked immunosorbent assay (ELISA).

2.7. Confocal laser scanning microscopy

DCs were plated overnight on poly-L-lysine-coated glass coverslips. After treatment with Rv2220, the cells were fixed in 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, and then blocked with 2% bovine serum albumin (BSA) in PBS containing 0.1% Tween 20 (PBS/T) for 2 h before incubation with 2% BSA in PBS/T containing an anti-p65 Ab for 2 h at room temperature. After a wash with PBS/T, the cells were incubated with an Alexa 488–conjugated Ab (Abcam, Cambridge, UK) (secondary Ab) in a dark room for 1 h, and then were stained with 1 μg/mL DAPI for 10 min at room temperature. Cell morphology and fluorescence intensity were examined under a confocal laser scanning microscope (Leica, Milton Keynes, UK). Images were captured by means of the LSM510 Meta software and processed in LSM