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Immunostimulatory activity of major membrane protein-II from *Mycobacterium leprae*

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Abstract

We examined the antigenicity of an immunomodulatory protein, major membrane protein (MMP)-II, from *Mycobacterium leprae*, since host defense against *M. leprae* largely depends on adaptive immunity. Both unprimed and memory T cells from healthy individuals were stimulated by autologous MMP-II-pulsed monocyte-derived dendritic cells (DCs) to produce IFN-γ. The DC-mediated IFN-γ production was dependent on the expression of MHC, CD86, and MMP-II antigens. Memory T cells from paucibacillary (PB) leprosy more extensively responded to MMP-II-pulsed DCs than T cells from healthy individuals, while comparable IFN-γ was produced by unprimed T cells. Memory T cells from multibacillary leprosy, which are normally believed to be anergic, were activated similarly to those from healthy individuals by MMP-II-pulsed DCs. These results suggest that memory T cells from PB leprosy are primed with MMP-II prior to the manifestation of the disease, and MMP-II is highly antigenic in terms of activation of adaptive immunity.

Keywords: *M. leprae*; Leprosy; Major membrane protein-II; T cell; Dendritic cell; IFN-γ; Memory T cell; Paucibacillary leprosy; Host defense

1. Introduction

Mycobacterium leprae is the causative agent of human leprosy, in which a chronic progressive peripheral nerve injury leading to systemic deformity is induced [1,2]. Most individuals infected with *M. leprae* do not manifest leprosy, but a few manifest the disease depending on their immunological status. Leprosy exhibits a wide range of clinical features and therefore, a broad disease spectrum is observed [3]. The representative spectra are the paucibacillary (PB) leprosy and multibacillary (MB)[1] leprosy. In the former disease spectrum, the localized skin and nerve lesions are observed and both $CD4^+$ and $CD8^+$ T cells chiefly act to localize the bacterial spread and, thus, disease lesion [4–6]. In contrast, in the latter disease spectrum, such cell-mediated immune responses are not efficiently evoked, but, rather, T cells show *M. leprae* Ag-specific anergic response [3]. The

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[1] *Abbreviation used:* MMP-II, major membrane protein-II; *M.*, *Mycobacterium*; DC, dendritic cell; MB, multibacillary; IFN, interferon; MHC, major histocompatibility complex; PB, paucibacillary; BCG, *M. bovis* bacillus Calmette-Guérin; Ab, antibody; Ag, antigen; APC, Ag-presenting cell; MDT, multi-drug therapy; TLR, Toll-like receptor; PBMCs, peripheral blood mononuclear cells; m, monoclonal; L, ligand; MLM, *M. leprae*-derived cell membrane; LPS, lipopolysaccharide; LAM, lipoarabinomannan.

vaccine currently examined for human use against the mycobacterial diseases is *M. bovis* bacillus Calmette-Guérin (BCG). However, its protective effect on *M. leprae* infection is not so convincing. Various efforts have currently been done for the development of new immunostimulatory agents, however, we still do not have an effective anti-leprosy vaccine. Also, in both forms of leprosy, the protective effects of antibody (Ab) in blood could not be observed. Therefore, the identification of the useful bacterial component antigens (Ags) which have immunomodulatory and immunostimulatory activities are desired.

Previously we demonstrated that dendritic cells (DCs), which are the most potent Ag-presenting cell (APC) capable of stimulating both memory and unprimed $CD4^+$ and $CD8^+$ T cell subsets [7–9], played a central role in stimulating T cells of both healthy individuals and PB leprosy patients [10,11]. However, macrophages less efficiently stimulated T cells [12]. Using DCs as APCs, we reported that cell membrane fraction of *M. leprae* was the most T cell stimulatory fraction [11], and therefore we identified major membrane protein-II (MMP-II) from this fraction as an immunomodulatory molecule.

MMP-II was originally identified from *M. leprae* as a major native protein in 1990 and was recognized to be identical to *M. paratuberculosis* bacterioferritin. Purification of MMP-II by reverse-phase chromatography, revealed a large molecular mass of 380 kDa, which has a ferroxidase-center residue. MMP-II contained 1000–4000 atoms of iron per molecule of protein. In the previous study, we showed that purified MMP-II stimulated DCs to produce IL-12 p70, and TNFα through the ligation to toll-like receptor (TLR)-2 [13]. In this study, we evaluated the immunostimulatory activity of purified MMP-II using DCs as APCs, since type 1 T cells response is most closely associated with host defense against *M. leprae* [1,2,14]. Furthermore, we assessed if MMP-II is associated with the activation of T cells in PB leprosy patients.

2. Materials and methods

2.1. Preparation of cells and bacteria

Peripheral blood was obtained under informed consent from healthy volunteers who were PPD-positive due to *M. bovis* BCG vaccination at childhood, and from five cases each of PB and MB leprosy patients. The status of patients used in this study are as follows: PB leprosy: 2 female and 3 male, age range (31–56), and MB leprosy: 1 female and 4 male, age range (21–53). All patients were under multi-drug therapy (MDT) for less than 7 months. We are aware that PPD-negative individuals would help to provide full information for these experiments because *M. leprae* and *M. bovis* BCG share some common Ags. However, in Japan, such individuals are not available for study, because *M. bovis* BCG vaccination was compulsory for children (0–4 year-old) until some years ago. PPD-negative individuals in Japanese population are the ones who do not respond to BCG vaccination; and therefore, it is likely that they may suffer from unknown human disease or immuno-insufficiency. Therefore these individuals cannot be used for our experiments. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll–Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described [15]. For preparation of the monocytes, $CD3^+$ T cells were removed from either freshly isolated heparinized blood, or cryopreserved PBMCs using immunomagnetic beads coated with anti-CD3 monoclonal (m) Ab (Dynabeads 450, Dynal, Oslo, Norway). The $CD3^-$ fraction of the PBMCs were plated on collagen-coated plates and cultured for 60 min at 37 °C. The non-plastic-adherent cells were then removed by extensive washing and the remaining adherent cells were used as monocytes and precursors of DCs [14]. Monocyte-derived DCs were differentiated from the plastic-adherent cells as described [15,16]. Briefly, the plastic-adherent cells were cultured in 3 ml of RPMI 1640 medium containing 10% FCS for 5 days in the presence of 50 ng of rGM-CSF (Pepro Tech EC, London, England) and 10 ng of rIL-4 (Pepro Tech) per milliliter. rGM-CSF and rIL-4 were supplied every 2 days and 400 μl of medium was replaced as described previously [16]. In some cases, DCs unpulsed or pulsed with Ags were further treated with a soluble form of CD40 ligand (L) (Pepro Tech) to obtain fully matured DCs capable of efficiently activating T cells. The purity of DCs obtained was 90.5% as judged by the expression of CD1a.

2.2. Purification of whole cell membrane fraction of *M. leprae* and MMP-II

The whole cell membrane fraction (MLM) was obtained according to previous report [13]. Briefly, the mycobacterial suspension was mixed with Zirconium beads in the presence of protease inhibitors at a ratio of approximately 1:1 (v/v) and homogenized using Beads Homogenizer Model BC-20 (Central Scientific Commerce, Tokyo), at 1500 rpm for 90 s 3–4 times. The beads were separated and the suspension was centrifuged at 10,000*g* for 30 min. The supernatant was then further ultra-centrifuged at 100,000*g* for 1 h. The resulting pellet was suspended in PBS, washed 2 times and taken as the membrane fraction. The *MMP-II* gene was PCR amplified from *M. leprae* chromosomal DNA and cloned into *Escherichia coli* expression vector as described previously [13]. Briefly, the *MMP-II* gene was inserted into the expression plasmid pET28 (Novagen, Madison, WI) and transformed into *E. coli* strain ER2566 (New England