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Short communication

Role of Malt1 protease activity in pathogenesis of inflammatory disorders mediated by FcγR signaling

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ABSTRACT

MALT lymphoma-translocation protein 1 (Malt1) protease activity is triggered by stimulation of various immune receptors. Activation of Malt1 protease induces cleavage of negative regulators for immune responses, resulting in lymphocytes activation. Although Malt1 protease mediates the signaling process downstream of the T cell, B cell, and dectin receptors, its contribution in Fcγ receptor (FcγR) signaling has not been elucidated. In this study, we investigated the role of Malt1 protease activity in FcγR signaling using Malt1 protease-deficient (PD) mouse. In addition, role of Malt1 protease for the development of FcγR-mediated autoimmune disease was also investigated *in vivo*. Malt1 protease cleaves their substrates, such as RelB and cylindromatosis (CYLD). However, the Malt1 proteolytic activity was silenced in the Malt1 PD mice. Production of inflammatory cytokines *via* FcγR stimulation was decreased on dendritic cells prepared from Malt1 PD mice. In FcγR-dependent murine immune thrombocytopenia (ITP) model, gene expressions of the inflammatory cytokines in the spleen of Malt1 PD mice were lower than those of WT mice. Then, Malt1 PD mice protected the development of thrombocytopenia. These results clearly figured out that Malt1 protease activity plays an important role in the activation of innate immune cells *via* FcγR, and the development of FcγR-mediated autoimmune diseases. Therefore, Malt1 is an attractive target for the treatment of inflammatory diseases mediated by FcγR.

1. Introduction

IgG autoantibodies play an important role in the development of several autoimmune diseases, such as systemic lupus erythematosus (SLE) [1] and immune thrombocytopenia (ITP) [2]. Effector functions of IgG autoantibodies are largely dependent on the formation of immune complexes (ICs) with the specific antigens. IgG-ICs can activate the complement system and Fcγ receptor (FcγR) on innate immune cells including dendritic cells (DCs) and macrophages. Especially, the activation of FcγR leads to a variety of immune reactions, including phagocytosis, cytokine production, and tissue injury, as well as IC clearance in the development of autoimmunity [3]. Indeed, mice lacking FcR γ-chain are deficient in activating FcR, and are thus protected from various autoimmune diseases such as arthritis, nephritis, and thrombocytopenia [4,5]. Moreover, human soluble FcγR IIB SM101, which inhibits the binding of IC to FcγR, results in clinical benefits in patients with SLE and ITP [6,7]. These facts show that FcγR signaling pathway is an attractive target for treating IgG autoantibody-mediated autoimmune diseases.

MALT lymphoma-translocation protein 1 (Malt1), or paracaspase, is a cysteine protease expressed in T cells, B cells, and innate immune cells. Malt1 mediates the signaling pathway downstream of the spleen tyrosine kinase (Syk) in B cells and innate immune cells [8]. Once these pathways are activated, Malt1 induces the activation of lymphocytes with two distinct mechanisms. Firstly, Malt1 works as a scaffold protein, leading to TRAF6-dependent NF-κB activation [9]. Secondly, Malt1 works as a cysteine protease, which catalytically processes the cleavage of negative regulators of immune reactions, such as RelB [10], cylindromatosis (CYLD) [11], resulting in the lymphocyte activation. These different signaling pathways play essential roles in the lymphocyte activation and the development of autoimmune diseases.

Malt1 protease-deficient (PD) mouse has been established to reveal the specific role of Malt1 enzymatic activity. The studies using Malt1 PD mouse indicated that Malt1 protease activity has a significant role in

Abbreviations: BAC, bacteria artificial chromosome; BMDC, bone marrow-derived dendritic cell; CYLD, cylindromatosis; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; FcγR, Fcγ receptor; FcR, Fc receptor; GM-CSF, Granulocyte macrophage colony-stimulating factor; IC, immune complex; IgG, Immunoglobulin; G, ITP, immune thrombocytopenic purport; mAb, monoclonal antibody; MALT, mucosa-associated lymphoid tissue; Malt1, MALT lymphoma-translocation protein 1; NF-κB, nuclear factor-κB; PD, protease-deficient; P/I, PMA/Ionomycin; Syk, spleen tyrosine kinase; SLE, systemic lupus erythematosus; TRAF6, tumor necrosis factor receptor-associated factor 6; TNF-α, tumor necrosis factor-α; WT, wild-type

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the activation of T cells, B cells, and DCs upon T cell, B cell, and dectin receptors, respectively [12–14]. Inactivation of Malt1 protease protected the development of experimental autoimmune encephalomyelitis and experimental colitis in these studies. However, the contribution of Malt1 protease to FcγR signaling in innate immune cells remains unknown. In the present study, we aimed to elucidate the relation of Malt1 protease to the activation of innate immune cells *via* FcγR signaling using Malt1 PD mice. Firstly, activation of the bone marrow-derived DC (BMDC) prepared from Malt1 PD mice was compared *in vitro* to that of wild-type (WT) mice. In addition, effect of Malt1 protease on the FcγR-mediated innate immune cell activation was also investigated *in vivo* in passive ITP model. These experiments indicate that the crucial role of Malt1 protease in the activation of innate immune cells upon FcγR stimulation, which is involved in the pathogenicity of ITP.

2. Materials and methods

2.1. Generation of Malt1 PD mice

Malt1 PD mice with BALB/c genetic background were established at Takeda Pharmaceutical Company, Ltd. (Japan). Detailed procedures for generations of Malt1 PD mice are presented in Supplemental Appendix.

2.2. Stimulation and western blotting

Spleens from Malt1 PD and WT mice were dissociated by passage through a nylon mesh, followed by passing tissues through a cell strainer. Single cell suspensions were hemolyzed with ammonium chloride solution, followed by purification of $CD4^+$ T cells in the splenocytes using $CD4^+$ T cell isolation kit II (Miltenyi Biotec, USA). Purified $CD4^+$ T cells were seeded onto the 6-well plate and incubated for 30 min with or without proteasome inhibitor MG-132 (5 μM). Then, the cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (Wako Pure Chemical, Japan) at 20 ng/mL and ionomycin (Wako Pure Chemical, Japan) at 250 ng/mL for indicated time points. As a positive control, Jurkat cells were stimulated with PMA and ionomycin (P/I) for 60 min with MG-132. After incubation, the cell pellets were lysed with RIPA buffer and subsequently denatured. Cellular lysates were fractionated by SDS-PAGE and subjected to immunoblot assays with antibodies described below. Primary antibodies used in this study were as follows: anti-RelB (C1E4), anti-CYLD (D1A10) (all purchased from Cell Signaling, USA), anti-Malt1 (H300), anti-IκBα (C-21) (all purchased from Santa Cruz, USA), and anti-β-Actin (Wako Pure Chemical, Japan). HRP-conjugated anti-mouse or anti-rabbit IgG (all purchased from GE Healthcare, USA) were used as secondary antibodies.

2.3. BMDC generation and stimulation

BMDCs were differentiated by granulocyte macrophage colony-stimulating factor (GM-CSF). Briefly, the bone marrow cells from Malt1 PD and WT mice were obtained from the femurs and tibias. Bone marrow cells were cultured in RPMI-1640 containing 10% FBS and 20 ng/mL of murine GM-CSF (Peprotech, USA). Medium was replaced on day 3, followed by the collection of BMDCs on day 6 by pipetting with PBS containing 2 mM EDTA. The frequency of $CD11c^+$ cells was determined by flow cytometry using FITC-conjugated anti-CD11c monoclonal antibody (mAb) (BD Biosciences, USA). BMDCs were seeded onto 6-well plate coated with 30 μg/mL of anti-CD16/CD32 mAb (2.4G2) (Bio X Cell, USA), followed by culturing for 24 h. Concentrations of TNF-α, IL-6, IL-12p40, and IL-23 in the medium were determined by ELISA (all obtained from R&D Systems, USA).

2.4. Mouse ITP model

To determine *in vivo* cytokine expressions through FcγR activation, Malt1 PD and WT mice were intraperitoneally injected with 3 μg/mouse of anti-CD41 (gpIIb/IIIa) mAb (MWReg30) (BD Biosciences, USA). Spleens were harvested 3 h after the stimulation, followed by storing in RNA later at 4 °C. Total RNA from the spleen was isolated using RNeasy mini kit (Qiagen, Germany) and DNaseI (Qiagen, Germany) to avoid genomic DNA contamination. High capacity cDNA reverse transcription kit (Life Technologies, USA) was used for cDNA synthesis. Quantitative PCR was performed using TaqMan fast advanced master mix (Life Technologies, USA) with specific primers on TaqMan gene expression assays (Life Technologies, USA). The values were normalized to β-actin (*Actb*) gene expression.

To develop the thrombocytopenia, anti-CD41 mAb (1 μg/mouse) or isotype rat IgG1 antibody (Bio X Cell, USA) was intraperitoneally administered to Malt1 PD and WT mice. At the next day, whole blood was obtained by cardiac puncture, followed by platelet counting using XT-1800i automated hematology analyzer (Sysmex, Japan).

2.5. Statistics

Values are presented as mean ± S.E. Statistical analysis was performed using SAS System for Windows (SAS Institute). The difference between two groups was analyzed as follows. Statistical analysis was carried out using Student's *t*-test when equality of variances was indicated by *F*-test. When there were no equal differences, Aspin-Welch's *t*-test was conducted. A probability value of $p < 0.05$ was considered statistically significant.

3. Results and discussion

To investigate physiological functions of the protease activity of Malt1, we established Malt1 PD mice, which harbored the C461A mutation in the catalytic domain whereas retained the scaffold domain. The expression level of Malt1 protein of Malt1 PD mice was unchanged compared to that of WT mice (Supplemental Fig. 1A). The protease activity of Malt1 was evaluated by measuring cleavage of its substrate proteins, such as RelB and CYLD, in T cells stimulated by P/I in the presence of MG-132, which prevented further proteasomal degradation of the cleavage products. As shown in Supplemental Figs. 1A and B, stimulation led to cleaving of the Malt1 substrates, RelB and CYLD, on T cells isolated from WT mice. On the other hand, cleavage products of RelB and CYLD were not observed in those obtained from Malt1 PD mice. The processing levels of RelB and CYLD in the Malt1 PD and WT cells were similar to those reported by Jaworski et al. [12]. Hence, it can be concluded that this particular strain had destroyed Malt1 protease activity. On the other hand, IκBα was found to be degraded in the cells of both strains (Supplemental Fig. 1C). Since IκBα degradation was suppressed in Malt1 knockout mice, Malt1 PD mouse had a normal scaffold function [15].

Then, we investigated the role of Malt1 protease in the activation of DCs upon FcγR stimulation. To confirm that the bone marrow cells derived from Malt1 PD mice were appropriately differentiated into DCs, the population of $CD11c^+$ cells was determined by flow cytometry. The population of $CD11c^+$ cells differentiated from Malt1 PD mice was found to be similar to that from WT mice, revealing that Malt1 inactivation does not affect the differentiation to DC (Supplemental Fig. 2).

Then, we compared the cytokine release from BMDCs stimulated with anti-FcγR mAb. As shown in Fig. 1A–C, the concentrations of TNF-α, IL-6 and IL-12p40 from Malt1 PD cells were significantly lower than those from WT cells. The tendency of decrease in the production of IL-23 from Malt1 PD cells was also observed (Fig. 1D). These results figure out that Malt1 proteolytic activity plays an essential role in the FcγR-mediated activation of DCs.

Next, we examined *in vivo* role of Malt1 protease in the development of anti-CD41 mAb-induced ITP model in mice. CD41 is expressed on the platelets, and anti-CD41 mAb leads to the activation of FcγR signaling, followed by the platelet clearance by innate immune cells [16]. Indeed,