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Mannan binding lectin attenuates double-stranded RNA-mediated TLR3 activation and innate immunity

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ABSTRACT

Mannan binding lectin (MBL) functions as a pattern recognition molecule (PRM) which is able to initiate complement activation. Here, we characterize a previously unrecognized attribute of MBL as a double-stranded RNA (dsRNA) binding protein capable of modifying Toll like receptor 3 (TLR3) activation. MBL interacts with poly(I:C) and suppresses poly(I:C)-induced activation of TLR3 pathways and subsequent cytokine production. In addition, MBL binds to TLR3 directly. Surprisingly, disrupting the interaction between MBL and complement receptor 1 (CR1) or restraining the traffic of MBL to phagosome reversed the MBL limited TLR3 activation. We demonstrate the importance of MBL guided ligands intracellular localization, emphasizing the significance of understanding the dynamics of TLR agonists complexed with MBL or other PRMs inside the cell in immune defense.

Structured summary of protein interactions::
TLR3 physically interacts with **MBL** by anti bait coimmunoprecipitation (View interaction)
TLR3 and **MBL** colocalize by fluorescence microscopy (View interaction)

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1. Introduction

The innate immunity system represents our first line of host defense against invading microorganisms. Innate pattern recognition receptors/molecules (PRRs/PRMs), including toll-like receptor (TLR), are involved in the recognition of conserved motifs of microbial origins or pathogen-associated molecular patterns (PAMPs), and play crucial roles in innate immunity response to invading microorganisms [1,2]. Engagement of PRMs by their cognate ligands triggers activation of protein kinases as well as transcription factors involved in inflammation and immunity [3]. Among the biochemically diverse pathogen molecules, double-stranded RNA (dsRNA) or its synthetic analog poly(I:C) is a ligand for TLR3 [4]. When activated by poly(I:C), TLR3 induces type I interferon (IFN), inflammatory cytokine production via the recruitment of adaptor molecules [5].

Mannan binding lectin (MBL, also called mannose binding lectin or mannose binding protein) is a member of the collectin family that is produced by the liver and can initiate the complement cascade. The polypeptide chain of MBL consists of a 20-residue 'cysteine-rich' region (containing 3 cysteines), followed by a collagen-like region (CLR) containing 19 Gly–X–Y triplets, a 'neck' region and then a C-terminal calcium dependent carbohydrate-recognition domain (CRD) [6]. MBL recognizes carbohydrate patterns, found on the surface of a large number of pathogenic microorganisms, including bacteria, viruses, protozoa and fungi, resulting in activation of the lectin pathway of the complement system. MBL is able to differentiate between the carbohydrates found on self glycoproteins and the carbohydrate patterns found

Abbreviations: CLR, collagen-like region; CR, complement receptor; CRD, carbohydrate recognition domain; dsRNA, double-stranded RNA; IFN, interferon; MBL, mannan binding lectin; MDCs, monocyte-derived dendritic cells; GlcNAc, *N*-acetylglucosamine; PAMP, pathogen-associated molecular pattern; PRR/PRM, pattern recognition receptor/molecule; poly(I:C), polyinosinic–polycytidylic acid; TLR, toll-like receptor; TNF-α, tumor necrosis factor α

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on infectious non-self surfaces and, hence, functions as a prototypic pattern-recognition molecule [7,8]. A number of studies have demonstrated that MBL deficiency increases susceptibility to certain infection, and the mechanisms associated with this susceptibility to infection include decreased opsonic function and reduced activation of the lectin-complement pathway [9]. In addition, accumulating studies suggested that MBL is able to modulate inflammatory responses during bacterial and viral infection [10–12]. Our previous studies showed that MBL could suppress LPS-induced tumor-necrosis factor-alpha (TNF-α) and IL-12 production in THP-1 cells and monocyte-derived dendritic cells (mDCs) by inhibiting LPS-induced NF-kappaB (NF-κB) DNA binding and translocation [13,14]. In addition, MBL attenuated allogeneic T cell proliferation induced by LPS-primed mDCs [14]. Eddie Ip et al. also observed a novel collaboration between MBL and TLR2/6 initiated upon engulfment of *Sacchromyces aureus* [11]. MBL traffics into early phagosomes and colocalizes with recruited TLR2, promoting the inflammatory response [11]. However, the immunomodulatory role of MBL in activation of TLR3 signaling has not been investigated.

In this study, we showed that MBL modified poly(I:C) triggered TLR3 activation. We demonstrated that MBL bound to poly(I:C) and intracellularly colocalized with TLR3. Furthermore, we investigated the essential contribution of MBL receptor on cell surface and the phagosome compartment in coordinating these responses. These data indicated a novel mechanism by which soluble PRM such as MBL integrated with TLRs in the innate immune network to orchestrate host defense responses.

2. Materials and methods

2.1. Cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized whole blood by use of Ficoll density gradient centrifugation and monocytes were purified from the PBMCs by immunomagnetic negative selection using the human Monocyte Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). DCs were prepared from human PMBCs of health donors. The cells were suspended in IMDM (Gibco BRL, Gaithersburg, MD, USA) and allowed to adhere to 12-well tissue culture plates (Corning-Costar, MA, USA). After incubation for 1 h at 37 °C, non-adherent lymphocytes were removed. Adherent monocytes were harvested and washed twice, then cultivated for 7 days in IMDM containing rHuGM-CSF (25 ng/ml; Peprotech, Inc., Rocky Hill, N.J.) and rHu-IL-4 (20 ng/ml; Peprotech, Inc.) and supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% FBS. Human THP-1 cells were cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U/ml of penicillin, 100 μg/ml of streptomycin and 10% FBS.

2.2. Preparation of MBL

MBL was isolated from human plasma by affinity chromatography on a mannan-agarose column (Sigma–Aldrich, St. Louis, MO, USA) and subsequent anion-exchange chromatography using Mono-Q HR 5/5 column (Pharmacia Biotech Europe, Orsay, France) as previously described [13]. Possible residual endotoxin in the purified protein was removed by a Detoxi-Gel Endotoxin Removing Column (Pierce, Rockford, IL, USA) and endotoxin levels in the protein preparations were undetected by a limulus amebocyte lysate kit (Biowhittaker, Walkersville, MD, USA). Recombinant CLR and CRD of MBL were respectively expressed in *Escherichia coli* using the pET expression system (Novagen, Madison, WI, USA) and purified by nickel-chelating resins (GE Healthcare, Piscataway, NJ, USA) according to the protocols.

2.3. Direct binding of MBL to poly(I:C)

For poly(I:C) pull down assay, poly(C) (Sigma–Aldrich) coated agarose beads were resuspended with poly(I) (Sigma–Aldrich) in 50 mM Tris (pH 7.0)-150 mM NaCl to form poly(I:C) beads as previously described [15]. The beads were incubated with 2 μg of MBL protein with gentle agitation at 4 °C for overnight. Eluted immunoprecipitates were resolved on SDS–PAGE and examined for MBL using anti-MBL monoclonal antibodies (Abcam, Cambridge, MA, USA). Reactions were visualized using enhanced chemiluminescence (ECL) reagents (Thermo Fisher Scientific, Fremont, CA, USA) for immunoblotting. For ELISA assays, microtiter wells (Nunc, Kamstrup, Denmark) were coated overnight at 4 °C with 20 μg/ml of poly(I:C). Plates were incubated at RT for 1 h with different concentrations of recombinant CRD or CLR of MBL or purified native MBL protein followed with corresponding antibodies incubation. The levels of bound MBL were determined using colorimetric assays after incubation with HRP-conjugated secondary antibodies. In ligand competition assay, MBL protein was incubated with pre-coated poly(I:C) in the presence of increasing concentrations of mannan (Sigma–Aldrich).

2.4. Cell stimulation

Human monocytes or mDCs were incubated with poly(I:C) (20 μg/ml) in the presence or absence of MBL (10 μg/ml). MBL protein was pre-incubated with poly(I:C) for 30 min at room temperature for complex formation prior to the incubation. In some cases, cells were pretreated with anti-CR1 antibodies (10 μg/ml, BD Biosciences, San Jose, CA, USA), chloroquine (200 μM, Sigma–Aldrich), NH_4Cl (5 mM) or bafilomycin A1 (50 nM, EMD Millipore Corporation, Billerica, MA, USA) for 30 min before stimulation with MBL–poly(I:C) complex.

2.5. Quantitative RT-PCR and ELISA

RNAs were extracted from cells using Trizol reagent (Invitrogen, Carlsbad, CA, USA) 4 h after MBL–poly(I:C) stimulation. Reverse transcription (RT) was performed using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Glen Bumie, MD, USA), followed by PCR assays using the primers as follows, human TNF-α, sense primer: 5′-TGG CCT GCA CAG TGA AGT GCT G-3′, antisense primer: 5′-TGG CCA GAA CCA AAG GCT CCC T-3′; human IL-6, sense primer: 5′-AAA TTC GGT ACA TCC TCG ACG G-3′, antisense primer: 5′-GGA AGG TTC AGG TTG TTT TCT GC-3′; GAPDH, sense primer: 5′-CTC CTC CTG TTC GAC AGT CAG C-3′, antisense primer: 5′-CCC AAT ACG ACC AAA TCC GTT-3′. Real-time PCR was performed in the Rotor-Gene 6000 real-time PCR detection system (Qiagen, Hilden, Germany). Reactions were completed in a 20 μl volume containing a mixture of cDNA, specific primers of each gene, and the SYBR Green Master Mix (Takara, Otsu, Shiga, Japan). Gene expression was quantified relative to the expression of GAPDH, and normalized to that measured in control by standard $2^{(-\Delta\Delta CT)}$ calculation. Culture supernatants were collected at 24 h after stimulation and cleared of debris by centrifugation. TNF-α and IL-6 expression levels in the culture supernatants were assayed with ELISA kits (eBiosciences, San Diego, CA, USA) according to the manufacturer's instructions and IFN-β levels in the supernatants were measured with ELISA kits from Uscn life Science (Wuhan, China).

2.6. Immunoprecipitation and immunoblotting

Protein lysates prepared using RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, pH7.4) were separated by SDS–PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in Tris-Buffered saline containing 0.05%

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