



SIGNR1-mediated phagocytosis, but not SIGNR1-mediated endocytosis or cell adhesion, suppresses LPS-induced secretion of IL-6 from murine macrophages



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ABSTRACT

C-type lectin receptors (CLRs) serve as phagocytosis receptors for pathogens and also function as adhesion molecules and in the recognition and endocytosis of glycosylated self-antigens. In the present study, we demonstrated that phagocytosis mediated by a mouse mannose-binding CLR, SIGNR1 significantly suppressed the LPS-induced secretion of the specific pro-inflammatory cytokines from the resident peritoneal macrophages and the mouse macrophage-like cells that express SIGNR1 (RAW-SIGNR1). LPS-induced secretion of IL-6 from peritoneal macrophages suppressed in response to uptake of oligomannose-coated liposomes (OMLs), and the suppression was partly inhibited by treatment with an anti-SIGNR1 antibody. LPS-induced secretion of IL-6 from RAW-SIGNR1 cells was also clearly inhibited by treatment of the cells with OMLs >0.4 μm in diameter, but treatment with OMLs <0.4 μm in diameter did not affect the IL-6 secretion. In contrast, LPS-induced TNF- α secretion from the cells was not affected on treatment of the cells with OMLs. Suppression of the IL-6 secretion was not observed following treatment with oligomannose-containing soluble polymers or when cells were bound to an oligomannose-coated solid phase. Phagocytosis of oligomannose-coated liposomes did not interfere with the transcription of IL-6 mRNA, but did affect IL-6 mRNA stability, leading to suppression of IL-6 secretion. Interestingly, treatment of the cells with Ly290042, a PI3 kinase inhibitor, partly blocked the suppression of LPS-induced secretion of IL-6 by OML. Thus, we conclude that SIGNR1-mediated phagocytosis but not SIGNR1-mediated endocytosis and cell adhesion, suppresses the TLR4-mediated production of specific proinflammatory cytokines via PI3 kinase signaling.

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1. Introduction

It is becoming clear that C-type lectin receptors (CLRs), which are expressed on antigen presenting cells (APCs), are particularly important pattern recognition molecules that recognize carbohydrate structure and take up pathogens into the cellular compartments of APCs via recognition of surface glycans of pathogens, leading to processing and presentation of their antigens on MHC class I and class II molecules [1,2]. In addition, the interaction of CLRs with pathogens can directly lead to the induction of intracellular signaling cascades that initiate or modulate specific cytokine responses and thus tailor T cell polarization to the pathogens [3–5]. These characteristic abilities suggest that CLRs expressed on APCs are potentially useful for antigen delivery for vaccination, as well as immune modulation.

Among these CLRs, DC-SIGN, which interacts with a wide range of pathogens through the recognition of mannose- and fucose-containing glycans, has emerged as a key player in the induction of immune responses against numerous pathogens by modulating TLR-induced activation. It has been demonstrated that the interaction of DC-SIGN with mannose- or fucose-containing components of pathogens affects TLR4-mediated immune responses by impairing DC-maturation and enhancing IL-10 production [6,7]. The binding of ligands to DC-SIGN leads to activation of the serine/threonine kinase, Raf-1, which mediates the phosphorylation of the NF- κB subunit, p65, at Ser276, and in turn leads to increasing transcription of IL-10 through p65 acetylation, thus, also resulting in increased IL-10 production [4]. It has been also shown that Salp15, an immunomodulatory protein produced by the salivary glands of ticks, interacts with DC-SIGN and inhibits TLR2- and TLR4-mediated production of the pro-inflammatory cytokines IL-6 and TNF- α [8]. The modulation of TLR-responses by Salp15 is also dependent on the activation of Raf-1 by the binding of Salp15 to

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SIGNR1, which is mediated through enhanced degradation of their respective mRNAs [8].

We have demonstrated that liposomes coated with a neoglycolipid (NGL) constructed from mannotriose and dipalmitoyl-phosphatidylethanolamine (Man3-DPPE) (oligomannose-coated liposomes, OMLs) can induce a strong cellular immune response against encapsulated antigens in mice [9]. We also showed that OMLs were preferentially taken up peritoneal phagocytic cells (CD11b⁺ cells), and the uptake leads to upregulation of costimulatory molecules and MHC class II molecules on peritoneal phagocytic cells, with enhanced production of IL-12 [10], which is essential for induction of a Th1 immune response. Interestingly, spontaneous secretion of IL-6 from these peritoneal phagocytic cells was suppressed following *in vivo* incorporation of OMLs [10]. SIGNR1 appears to be a structural and functional mouse homologue of human DC-SIGN. It has been shown that significant levels of SIGNR1 are expressed on murine peritoneal phagocytic cells (macrophages) [11,12], and SIGNR1 expressed on the peritoneal macrophages is involved in uptake of OMLs [12]. We also showed that SIGNR1 directly mediates the uptake of several NGL-coated liposomes, including OMLs, using non-phagocytic CHO cells transfected with SIGNR1 and murine macrophage-like RAW264.7 cells that stably expressed SIGNR (RAW-SIGNR1 cells) [13,14].

Since spontaneous secretion of IL-6 from peritoneal phagocytic cells was suppressed by OML uptake and certain levels of SIGNR1 are expressed murine peritoneal phagocytic cells [10,12], SIGNR1-mediated recognition of ligands may affect the production of IL-6 from phagocytic cells, as is the case for human DC-SIGN. In addition to mediating uptake of carbohydrate-decorated particles, SIGNR1 also mediates internalization of carbohydrate-bearing soluble polymers, such as ovalbumin and dextran, and cell adhesion to carbohydrate-coated solid phases [11,14]. These results indicate that SIGNR1 on the cells act as phagocytic, endocytotic and adhesive receptors. However, there have been no reports describing the abilities of SIGNR1 affect cytokine production from phagocytic cells.

The murine macrophage-like cell line, RAW264.7 cells and its gene-transfected cells have been used for investigations of TLR- or CLR-mediated cytokine production [15–18]. Therefore, in this study, we examined the effects of different abilities of SIGNR1, e.g., SIGNR1-mediated phagocytosis, endocytosis and cell adhesion, on LPS-induced production of inflammatory cytokines using SIGNR1-expressing resident peritoneal macrophages and RAW264.7 cells that stably expressed SIGNR (RAW-SIGNR1 cells).

2. Materials and methods

2.1. Cells and materials

The mouse macrophage-like RAW264.7 cell line that stably expresses SIGNR1 (RAW-SIGNR1 cells) was used in these experiments, as described previously by ourselves [19]. The cells were maintained in DMEM containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, and 0.5 mg/ml G418. A hamster anti-SIGNR1 monoclonal antibody (clone 22D1, hamster IgG) and an irrelevant hamster IgG isotype were purchased from eBioscience (Boston, MA, USA). Oligosaccharide-conjugated BSA probes and oligosaccharide-conjugated polyacrylamide (PAA) probes were obtained from Dextra Laboratory Inc. (Reading, UK) and Syntesome (Munich, Germany), respectively. The following oligosaccharides were purchased from Dextra Laboratory Inc. for the preparation of neoglycolipids (NGLs): mannotriose (Man3; Man α 1–6(Man α 1–3)Man), lacto-N-fucopentaose 2 (LNFP2, Le^a; Gal β 1–3(Fuc α 1–4)GlcNAc β 1–3Gal β 1–4Glc), lacto-N-fucopentaose 3 (LNFP3, Le^x;

Gal β 1–4(Fuc α 1–3)GlcNAc β 1–3Gal β 1–4Glc), biantennary N-linked core pentasaccharide (BNCP; GlcNAc β 1–2Man α 1–6(GlcNAc β 1–2Man α 1–3)Man). NGLs were prepared in our laboratory, as described previously [9]. The NGL-coated liposomes with a diameter of 1 μ m and comprised of cholesterol, DPPC, and NGL at molar ratios of 10:10:1 were prepared as described previously [9]. The liposomes with diameters of 0.4, 0.2 and 0.1 μ m were prepared from the 1 μ m-diameter liposomes by serial extrusion through 0.4-, 0.2- and 0.1- μ m pore polycarbonate membrane (Nucleopore, Pleasanton, CA). The particle sizes of the liposomes were determined by dynamic light scattering using a particle size analyzer (LB-550, Horiba, Kyoto, Japan). The median particle sizes of the liposomes with diameters of 0.4, 0.2 and 0.1 μ m were 1.18, 0.44, 0.21, and 0.12 μ m, respectively.

2.2. Preparation of peritoneal macrophages and assessment of *in vitro* uptake of carbohydrate-coated liposomes

Six- to 8-week-old female C57BL/6 mice were purchased from Shizuoka Laboratory Animal Corporation (Hamamatsu, Japan). All animal experiments were conducted in compliance with the ethical requirements of the Animal Committee at Tokai University. Peritoneal cells were harvested by lavage using 5 ml of ice-cold PBS and washed twice with PBS. The cells obtained from the peritoneal cavity were then seeded onto a culture dish with a surface grafted with a temperature-responsive polymer (UpCell[®], CellSeed Inc., Tokyo, Japan) and incubated at 37 °C for 1 h. After non-adherent cells were removed by extensive washing with pre-warmed culture medium, adherent cells were recovered by culturing the cells at 20 °C for 30 min and were used as resident peritoneal macrophages [19].

2.3. Assay for SIGNR1-mediated ligand uptake and cell adhesion

The uptake of liposomes into resident peritoneal macrophages and RAW-SIGNR1 cells was performed as described previously [14]. Briefly, the cells (5×10^5 /500 μ l of medium) were placed in a 1.5-ml siliconized tube (Sumitomo Bakelite Co. Ltd., Tokyo, Japan), FITC-BSA-containing NGL-coated liposomes (10 μ g of cholesterol) were added, and the cells were then incubated for 1 h at 37 °C with gentle rotation. After incubation, the cells were washed twice with HBSS containing 1 mM EDTA to remove liposomes bound to the cell surface and fixed with 1% paraformaldehyde. The fluorescence from FITC was then determined to calculate the specific uptake of the liposomes by the cells.

Cell adhesion was assessed, as described previously [14]. For the preparation of NGL-coated solid phases, 100 μ l of Man3-DPPE dissolved in isopropanol (100 pmol/well) were added into each well of a 96-well plate, and the wells were dried at 37 °C, washed with PBS, and blocked with 1% BSA in PBS for 1 h at room temperature. For the preparation of oligosaccharide-conjugated BSA-coated solid phases, 100 μ l of Man3-DPPE dissolved in water (50 μ g/ml) were added to each well of a 96-well plate, and incubated at 4 °C for overnight. The wells were then washed with PBS and blocked with 1% BSA in PBS for 1 h at room temperature. For determination of cell adhesion, RAW-SIGNR1 cells (1×10^6 /well) suspended in medium containing 2% FBS were added to the NGL- or oligosaccharide-conjugated BSA probe-coated wells of a 96-well plate, and the plate was centrifuged at 180g (1000 rpm) for 1 min. After incubation of the plate for 30 min at 37 °C, each well was filled with pre-warmed medium, and the plate was then sealed with a plate sealer and centrifuged upside down at 180g for 1 min to remove any unbound cells. After removal of the unbound cells and medium, cells bound to the solid phases were measured by absorbance at 450 nm using a cell counting kit-8 (Domino, Kumamoto, Japan).

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