Immunology Letters 123 (2009) 14–20

Contents lists available at ScienceDirect

Immunology Letters

journal homepage: www.elsevier.com/locate/

Neutrophil activation induced by ArtinM: Release of inflammatory mediators and enhancement of effector functions

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ARTICLE INFO

Article history:
Received 26 October 2008
Received in revised form 18 January 2009
Accepted 19 January 2009
Available online 30 January 2009

Keywords:
Artocarpus integrifolia
Lectin
Neutrophil activation
Phagocytosis
LTB_4
TLR

ABSTRACT

The D-mannose binding lectin ArtinM from *Artocarpus integrifolia*, previously known as KM+ and artocarpin, is considered a stimulant of Th1-type immunity, which is able to confer resistance to some intracellular pathogens. In addition, ArtinM induces neutrophil migration by haptotaxis through simultaneous interactions of its carbohydrate recognition domains (CRDs) with glycans expressed on the extracellular matrix and the neutrophil surface. In the present study, we have expanded the characterization of ArtinM as a neutrophil activator. Exposure of neutrophils to ArtinM for 15 min resulted in tyrosine phosphorylation of intracellular proteins, a process that was selectively inhibited by D-mannose or mannotriose. Shortly after stimulation, neutrophils secreted high levels of LTB_4 and underwent shedding of L-selectin from their surface. Exposure to ArtinM enhanced neutrophil functions, such as respiratory burst and zymozan and *Listeria monocytogenes* phagocytosis. In addition, ArtinM-stimulated neutrophils displayed increased CXCL-8 secretion and TLR2 gene transcription. These results demonstrate that ArtinM is able to induce potent neutrophil activation, a feature that should be strongly considered in the assessment of the lectin capacity to confer resistance against infections.

1. Introduction

Neutrophils are major components of the innate immune system, and their accumulation within injured tissue is a hallmark of the acute inflammatory response. Neutrophil activities rely on their interaction with attractants, mainly chemokines. Indeed, these motile and phagocytic cells respond to a wide variety of exogenous and endogenous attractants, soluble or particulate, which are ligands for cell surface receptors of the superfamily of seven-transmembrane receptors associated with intracellular GTP (guanidine tri-phosphate)-binding heteroproteins. These "serpentine" molecules include receptors for C5a, formylpeptides, the PAF, leukotriene B_4 (LTB_4), and chemokines (mainly CXCR1 and CXCR2 for CXCL-8). The engagement of these G-protein-coupled receptors (GPCRs) activates phospholipases and conventional protein kinase C isoforms, including tyrosine kinases, mainly Lyn of the Src-family [1].

Some lectins are included among the multiple attractants that activate neutrophils. Through specific and reversible carbohydrate-binding activities, concanavalin-A (Con-A), wheat germ agglutinin (WGA), selectins, and some members of the galectin family have been shown to induce neutrophil responses [2–5]. It is most likely that glycans linked to cell surface receptors are targets for lectin recognition, which then triggers leukocyte activation. In this context, a few studies have demonstrated that glycosylation of GPCRs and Toll like receptors (TLRs) account for cell interaction with known activators [6,7].

Our previous work on the biological properties of a D-mannose binding lectin from the seeds of *Artocarpus integrifolia*, presently denoted ArtinM [8], showed that it induces macrophages to produce IL-12 [9]. This property is due to ArtinM interaction with TLR2 glycans [10], which promotes IL-12 production and a Th1 biased immune response. As a consequence, ArtinM has been shown to

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doi:10.1016/j.imlet.2009.01.009

confer resistance to *Leishmania* [9,11], and *Paracoccidioides brasiliensis* [10] infections. More recently, ArtinM was observed to abort precociously murine *Listeria monocytogenes* infection, suggesting the lectin enhances the innate mechanisms of immunity (Conrado et al., in preparation). The ability to modulate the immune response against intracellular pathogens renders ArtinM a good candidate for therapeutic application.

In addition to the immunomodulatory activity, ArtinM induces neutrophil haptotactic migration mediated by the simultaneous interaction of its carbohydrate recognition domains (CRDs) with cell surface *N*-glycans (linked to the CXCR2 molecule) [12] and extracellular matrix *N*-glycans (linked to laminin) [13–15]. An amplification loop for the *in vivo* ArtinM inflammatory activity is provided by mast cell degranulation, which is most likely due to the lectin interaction with glycans on FcεRI [16].

Due to its potential pharmaceutical application, ArtinM should be deeply studied regarding its action on human neutrophils, especially because these cells combine physiological and pathogenic roles [1]. In the current investigation we show that, following ArtinM stimulation, neutrophils present phenotypic and functional changes, which include intracellular tyrosine phosphorylation, shedding of L-selectin, release of inflammatory mediators, phagocytic and killing cell activities, and increased gene transcription of TLR2.

2. Material and methods

2.1. ArtinM preparations

ArtinM was extracted from *A. integrifolia* seeds and purified by sugar affinity chromatography as previously described by Santos-de-Oliveira et al. [17]. ArtinM preparations contained less than 0.05 ng/ml of bacterial endotoxin, as determined by the *Limulus* amoebocyte lysate assay (Sigma Chemical Co., St. Louis, MO).

2.2. Neutrophil isolation

Heparinized human blood from healthy volunteers was layered on a density gradient of a neutrophil isolation medium (Monopoly Resolving Medium, ICN Pharmaceuticals, Costa Mesa, CA, USA) as previously described [12]. After hypotonic lysis, cell preparations showed 98% purity and over 95% of the neutrophils were viable, as determined by trypan blue exclusion. This study was approved by the Ethics Committee of the University of Sao Paulo (Doc. Number: 9618/2004) and a written informed consent was obtained from all the volunteers prior to blood collection.

2.3. Immunocytochemistry assay for phosphotyrosine

Human neutrophils (10^4 cells) were placed on coverslips coated with 2% Biobond (EM Science, Fort Washington, PA, USA) and incubated with either ArtinM (2.5 μg/ml) or phosphate-buffered saline (PBS; 3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl, pH 7.4) alone for 0, 15 and 30 min at 37 °C. For some assays, ArtinM was pretreated for 60 min at room temperature with 0.2 M D-mannose, 0.2 M D-galactose (Sigma Chemical Company, St Louis, MO, USA), or 0.01 M mannotriose (Manα1-3[Manα1-6]Man) (Dextra Laboratories, Reading, UK). After being rinsed with PBS, the cells were fixed for 20 min with 2% formaldehyde and incubated for 45 min with anti-phosphotyrosine antibody (clone PY20—BD Transduction Lab, Canada) diluted in PBS containing 0.01% saponin (Sigma Chemical Company). Cells were rinsed and then incubated for 30 min with donkey anti-mouse IgG (Fab′2) conjugated with FITC (Jackson ImmunoResearch, West Grove, PA, USA), diluted in PBS containing 0.01% saponin (1:50). The coverslips were submitted to extensive rinse and mounted with Fluormount-G (EM Science) for further examination by fluorescence microscopy (Zeiss Axiophot—capture program: Case Data Manager). Images were analyzed using the morphometric program Image Pro Plus.

2.4. Leukotriene B_4 production

Human neutrophils (5×10^5 cells) were incubated with ArtinM (2.5 μg/ml), PAF (10^{-8} M) (Sigma Chemical Company), or CXCL-8 (50 ng/ml) (*E. coli*; R&D Systems, Minneapolis, MN, USA) diluted in RPMI medium (GIBCO, Gaithersburg, MD) supplemented with 5% fetal calf serum (GIBCO, Gaithersburg, MD) for 10 min at 37 °C. The LTB_4 concentration in the culture medium was quantified using a commercially available ELISA kit (LTB_4 Immunoassay—R&D System) according to the manufacturer's instructions. LTB_4 concentrations (pg/0.1 ml) were determined from an appropriate standard curve.

2.5. CD62L expression

Human neutrophils were incubated at 37 °C with ArtinM (2.5 μg/ml), fMLP (10^{-8} M) (Sigma Chemical Company), or CXCL-8 (50 ng/ml) for 5, 15, 30, or 60 min. After being rinsed with PBS containing 1% BSA and 0.01% azide (PBS–BSA–azide), the cells were incubated with FITC-conjugated anti-CD62L antibody (mouse IgG2a – clone SK11 – BD Biosciences, San Jose, CA, USA) (10 μg/ml) or control isotype IgG2a conjugated-FITC (10 μg/ml—BD Biosciences) in the presence of rabbit serum diluted in the PBS–BSA–azide. After a 40-min incubation, the cells were washed with PBS containing 0.01% azide (PBS–azide), fixed with 1% formaldehyde in PBS, and analyzed by FACScan flow cytometer (Becton Dickinson, San, CA, USA).

2.6. Phagocytosis assays

2.6.1. Phagocytosis of zymozan

A neutrophil suspension (1×10^4 cells/ml) was distributed to the wells of a microplate (0.1 ml/well) and incubated with ArtinM (2.5 μg/ml), PMA (1 μg/ml), or CXCL-8 (50 ng/ml). A suspension of neutral red-stained zymozan (Sigma Chemical Company) (2.3×10^8 particles/ml) was added to the wells. After a 30-min incubation at 37 °C, the cells were fixed with Baker's formaldehyde–calcium (4% formaldehyde, 2% sodium chloride, 1% calcium acetate) for 30 min. They were then washed two times by centrifugation ($453 \times g$ for 5 min) to eliminate non-phagocyted neutral red-stained zymosan. The stained particles phagocyted by the cells were solubilized by addition of 0.1 ml acidified alcohol (10% acetic acid, 40% ethanol in distilled water) to each well. After 30 min, the absorbance at 550 nm was read (Benchmark Microplate Reader—Bio-Rad Laboratories, San Francisco, CA, USA).

2.6.2. Phagocytosis of L. monocytogenes

L. monocytogenes (10403S strain) was inoculated into 100 ml of brain–heart infusion broth (BHI, Oxoid, Hampshire, England). Cultures were incubated in a rotary shaker at 200 rpm, 37 °C, for approximately 5 h (optical density, $OD_{600} = 0.8$). For preparation of bacterial suspension, the cultures of *L. monocytogenes* were precipitated by centrifugation ($3000 \times g$; 15 min), the pellet was washed twice and resuspended with phosphate-buffered saline. *L. monocytogenes* were labeled with 10 μM CFSE (carboxyfluorescein diacetate succinimidyl ester—Molecular Probes, Eugene, Oregon, USA) and incubated for 15 min at 37 °C. They were washed with PBS by centrifugation until the supernatant was clear of CFSE and fixed with PBS containing 10% formaldehyde.

Human neutrophils (5×10^5 cells) were incubated with ArtinM (2.5 μg/ml), PMA (1 μg/ml), CXCL-8 (50 ng/ml), 0.2 M D-mannose, 0.2 M D-galactose, for 30 min at 37 °C. For some assays, ArtinM