Immunobiology 219 (2014) 179–188

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

Induction of innate immunity by *Aspergillus fumigatus* cell wall polysaccharides is enhanced by the composite presentation of chitin and beta-glucan

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ARTICLE INFO

Article history:
Received 18 July 2013
Received in revised form 3 October 2013
Accepted 3 October 2013
Available online 12 October 2013

Keywords:
β-Glucan
Chitin
Fungal cell wall polysaccharides
Inflammation
Innate immunity
Lung

ABSTRACT

Chitin and β-glucan are conserved throughout evolution in the fungal cell wall and are the most common polysaccharides in fungal species. Together, these two polysaccharides form a structural scaffold that is essential for the survival of the fungus. In the present study, we demonstrated that *Aspergillus fumigatus* alkali-insoluble cell wall fragments (AIF), composed of chitin linked covalently to β-glucan, induced enhanced immune responses when compared with individual cell wall polysaccharides. Intranasal administration of AIF induced eosinophil and neutrophil recruitment, chitinase activity, TNF-α and TSLP production in mice lungs. Selective destruction of chitin or β-glucan from AIF significantly reduced eosinophil and neutrophil recruitment as well as chitinase activity and cytokine expression by macrophages, indicating the synergistic effect of the cell wall polysaccharides when presented together as a composite PAMP. We also showed that these cell wall polysaccharides induced chitin-specific IgM in mouse serum. Our *in vivo* and *in vitro* data indicate that chitin and β-glucan play important roles in activating innate immunity when presented as composite cell wall PAMPs.

Introduction

Aspergillus fumigatus (*A. fumigatus*) is a common opportunistic fungal pathogen responsible for allergy and infection (Latge, 1999). *A. fumigatus*-mediated allergic and invasive disorders include allergic bronchopulmonary aspergillosis (ABPA), allergic rhinitis, allergic sinusitis, hypersensitivity pneumonitis, aspergilloma and invasive aspergillosis. Patients with cystic fibrosis are often reported as having complications of ABPA due to *A. fumigatus* colonisation in the lungs (Knutsen and Slavin, 2011; Romani, 2011). The resting conidia of *A. fumigatus* is made up of an outer rodlet layer which masks many of the pathogen-associated molecular patterns (PAMPs) that can trigger innate and adaptive immune responses (Aimanianda et al., 2009). However, the resting conidia can mediate an inflammatory effect through dectin-1 (Faro-Trindade et al., 2012), the germination of conidia results in exposure of specific PAMPs including β-glucan and chitin, which are readily recognised by pattern recognition receptors (PRRs) such as dectin-1 and FIBCD1, and leads to activation of the immune system (Steele et al., 2005; Thomsen et al., 2011).

The fungal cell wall is a physically rigid layer that shields fungal cells from the environment. Cell wall polymers isolated from fungi are classically divided in to two groups depending on their solubility in alkali. The alkali-insoluble cell wall fragments (AIF) of *A. fumigatus* mainly contain a glucan-chitin complex; however, other components such as galactomannan are also integral components (Fontaine et al., 2000). The growth and remodelling of *A. fumigatus* conidia to hyphae is accompanied by the synthesis of chitin and β-glucan. Simultaneously, synthesis of cell wall-associated proteases takes place, and secretory proteases are released into the fungal growth niche (Hearn and Sietsma, 1994). These proteases are responsible for inducing a Th2 response with eosinophilia, indicating their key role in allergy and asthma (Hansen et al., 1994; Yamashita et al., 2002). However, the role of the composite major cell wall polysaccharides in the development of the innate and adaptive immune responses is less well described.

We used purified AIF from *A. fumigatus* to study polysaccharide-mediated immune activation and compared AIF on a weight-to-weight basis with β-glucan and chitin. Our results show that intact AIF, representing a composite PAMP, strongly induces the innate

Abbreviations: AMCase, acidic mammalian chitinase; AIF, alkali-insoluble cell wall fragments; ABPA, allergic bronchopulmonary aspergillosis; *A. fumigatus*, *Aspergillus fumigatus*; BAL, bronchoalveolar lavage; FReD, fibrinogen-related domain; GlcNAc, N-acetylglucosamine; PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors.

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0171-2985/$ – see front matter © 2013 Published by Elsevier GmbH.
http://dx.doi.org/10.1016/j.imbio.2013.10.003

immune system and that destruction of either chitin or β-glucan results in a reduced immune response both *in vivo* and *in vitro*.

Materials and methods

Buffers and reagents

Tris-buffered saline (TBS) was composed of 140 mM NaCl, 10 mM Tris–HCl and 0.02% (w/v) NaN_2 (pH 7.4). TBS/Tw was composed of TBS containing 0.05% (v/v) Tween 20 (polyoxyethylene sorbitan monolaurate, MERCK-Schuchardt, Hohenbrunn, Germany). Phosphate buffered saline (PBS) was composed of 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 (pH 7.4). Coating buffer was composed of 60 mM Na_2CO_3, 35 mM $NaHCO_3$ and 0.02% (w/v) NaN_3 (pH 9.6). RPMI-1640 medium, penicillin and streptomycin, glutamine, trypsin–EDTA, Hanks' balanced salt solution (HBSS), foetal bovine serum (FBS), SuperScript® III Reverse Transcriptase and TRIzol were obtained from Life Technologies Europe BV, (Invitrogen) Denmark. Chitinase (from *Serratia marcescens*, C7809), β-glucanase (from *Trichoderma longibacterium*, G4423), crab shell chitin (C3641), curdlan (C7821), sodium metaperiodate, sodium borohydride, sodium nitrite, O-dianisidine dihydrochloride (ODD) tablets and the Chitinase Assay Kit (CS1030) were obtained from Sigma–Aldrich, Schnelldorf, Germany. Chitin beads (S6651) were obtained from New England Biolabs. Horseradish peroxidase-labelled rabbit anti-mouse IgG was obtained from DAKO, Denmark. Dehydrated Sabouraud dextrose broth (SD-broth) powder was obtained from BD-Difco, Albertslund, Denmark. Cell strainers (40-μm) were obtained from BD biosciences, Denmark. ELISA kits for TNF-α and TSLP (e-Biosciences) were obtained from AH diagnostics, Aarhus, Denmark and R&D systems, Oxon, United Kingdom, respectively.

Mice

Female C57BL/6 mice (Charles River, UK) were housed under specific-pathogen-free conditions in individual ventilated cages and used at 6–12 weeks of age. The animals had *ad libitum* access to pelleted food and water. The mice were randomised into various groups before commencement of the experiments. All experiments involving mice were performed under a license obtained from The Danish National Animal Experiments Inspectorate, who also approved the study (ref. no. 2012-15-2934-00354).

Alkaline insoluble fragments (AIF)

A. fumigatus cell wall polysaccharides and secretory protease antigens were prepared as described previously (Arruda et al., 1992; Fontaine et al., 2000; Strong et al., 2002) with slight modifications. Briefly, *A. fumigatus* (293) (CBS, The Netherlands) was grown in SD broth for 7 days in stationary form. Subsequently, the mycelium was harvested by filtration and used for preparation of polysaccharide antigens. For preparation of polysaccharide antigens, the mycelium was washed in PBS and subjected to hot alkali treatment (1 M NaOH/65 °C) for 30 min followed by 5 washes with sterile ultra-pure water and one wash with Tris–HCl (pH 7.4). This process was repeated twice. AIF were then ground using a mortar and pestle, washed and filtered through a 40-μM cell strainer (BD). Filtered AIFs were analysed by flow cytometry and tested to determine endotoxin contamination using the Limulus amoebocyte lysate assay (Lonza). The absence of cell wall-associated proteases in AIF preparations was ensured by boiling AIF in TBS/Tw with 5 mM Ca^{2+}. No protein bands were observed upon analysis of the AIF by SDS-PAGE, confirming that the AIF preparations were free from cell wall-associated proteases. *A. fumigatus* mycelium fragments (heat-killed *A. fumigatus*) containing cell wall-associated proteases were prepared by vigorous vortexing of mycelia with glass beads in PBS. Broken fragments were washed in PBS, filtered through a 40-μm cell strainer and heat killed by incubating at 80 °C for 30 min. The heat-killed fragments were washed again with PBS, quantified and stored at 4 °C until use.

Enzymatic destruction of chitin or β-glucan from alkali-insoluble fragments

Chitin and β-glucan from AIF were destroyed *via* enzymatic degradation as described previously (Bozza et al., 2009; Fontaine et al., 2000) with minor modifications. We used chitinase (from *Serratia marcescens*) to destroy chitin in AIF, whereas to obtain chitin, AIFs were treated with recombinant β-glucanase (NZY Tech, Portugal). This process was repeated 2–3 times, and the resulting fragments were washed with water and PBS, filtered through a 40-μm cell strainer and stored in sterile endotoxin-free PBS at 4 °C until they were used for intranasal administration. The presence and absence of chitin and β-glucan were analysed by pull-down experiments as indicated in the following section. The bound proteins were analysed by SDS-PAGE and silver staining. The size distribution of particles was analysed using flow cytometry.

Pull-down assay

To detect the presence of chitin and β-glucan in various polysaccharide preparations, we performed pull-down assays. AIF, β-glucan (AIF treated with chitinase) or chitin (AIF treated with β-glucanase) were washed 3 times in TBS/Tw/Ca^{2+} before use. Wheat germ agglutinin (WGA) was used as a chitin binding protein, and a β-glucan monoclonal antibody was used for β-glucan detection. WGA or antibody (at 5 μg/ml) was added to 1000 μg of each polysaccharide in a total volume of 1000 μl. WGA mixed with chitin beads or curdlan (β-glucan) were used as positive and negative polysaccharide controls, respectively. Polysaccharides and protein mixtures were incubated for 16 h at 4 °C, and pull-down was performed by centrifuging the tubes at 8000 rpm for 5 min at 4 °C. The insoluble pellets were washed 3 times in TBS/Tw/Ca^{2+}, and the bound protein was eluted by boiling the pellet in TBS/Tw/Ca^{2+} containing SDS-PAGE buffer. Eluted proteins were separated on 4–12% polyacrylamide gradient gels in a discontinuous buffer system and visualised by silver staining or blotted onto a polyvinylidene difluoride membrane (GE Healthcare). The membrane was incubated with horseradish peroxidase-coupled rabbit anti-mouse IgG diluted 1:10,000 in TBS/Tw. The membrane was washed and developed using the standard enhanced chemiluminescence method according to the manufacturer's recommendations (Amersham Biosciences).

Intranasal challenges with polysaccharides

Mice were anesthetised with isofluorane before polysaccharide challenge. The mice received 3 intranasal doses of polysaccharides (100 μg/50 μl) for 3 consecutive days. The animals were sacrificed 24 h after the last intranasal challenge, and bronchoalveolar lavage (BAL) fluid as well as whole lung tissues were obtained and used for further analysis. In other experiments, mice received a single dose (100 μg/50 μl) of polysaccharides and were sacrificed after 6 h.

Bronchoalveolar lavage (BAL) fluid and whole lung homogenate preparation

The animals were sacrificed by CO_2 asphyxiation. Subsequently, a median sternotomy was performed, and the trachea was exposed by blunt dissection. A 22-gauge catheter was inserted into the trachea, and BAL was performed by instilling 1 ml of PBS into the