



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

Lalit Kumar Dubey, Jesper Bonnet Moeller, Anders Schlosser, Grith Lykke Sorensen, Uffe Holmskov*

Institute of Molecular Medicine, Department of Cardiovascular and Renal Research, University of Southern Denmark, DK-5000 Odense, Denmark

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.

© 2013 Published by Elsevier GmbH.

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 (Knutson 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.

* Corresponding author. Tel.: +45 6550 3775; fax: +45 6550 3922.

E-mail address: uholmskov@health.sdu.dk (U. Holmskov).

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, Schnellendorf, 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 $\mu\text{g}/\text{ml}$) 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 anaesthetised with isoflurane before polysaccharide challenge. The mice received 3 intranasal doses of polysaccharides (100 $\mu\text{g}/50 \mu\text{l}$) 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 $\mu\text{g}/50 \mu\text{l}$) 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

trachea and gently aspirating back. This procedure was repeated twice, and the samples were pooled. The samples from each animal were centrifuged, and the supernatants were stored at -80°C until use. The BAL cell pellet was resuspended in 1 ml of PBS and used for total cell count determination. Total cells were counted using Beckman Coulter Z2 particle count and size analyser. Whole lung lysates were prepared using lysis buffer as previously described (Hartl et al., 2008) and used for chitinase, EPO and MPO measurements.

Eosinophil peroxidase (EPO) assay

The EPO level in BAL and lung homogenates, which mainly represents eosinophil infiltration, was measured as described previously (Oliveira et al., 2002). Briefly, o-phenylenediamine (OPD; 10 mg) was dissolved in 5.5 ml of distilled H_2O . Then, 1.5 ml of OPD solution was added to 8.5 ml of Tris buffer (pH 8.0) followed by the addition of 7.5 μl of H_2O_2 to produce EPO substrate solution. Using a 96-well plate, 100 μl of substrate solution was added to 100 μl of an undiluted BAL sample or 100 μl of lung homogenate (1 mg/ml). After 30 min of incubation at 37°C , the reaction was quenched with 50 μl of 4 M H_2SO_4 , and the absorbance was read at 492 nm. The results are expressed as the change in absorbance at 492 nm.

Myeloperoxidase (MPO) assay

The MPO level in BAL and lung homogenates, which mainly represents neutrophil infiltration, was measured as described previously (Balloy et al., 2005) with slight modifications. Briefly, 100 μl of BAL or 100 μl of lung homogenate (1 mg/ml) was added to 100 μl of substrate solution (O-dianisidine dihydrochloride, 1.25 mg/ml in HBSS) supplemented with 0.005% H_2O_2 . The reaction was incubated at room temperature and stopped by the addition of 50 μl of Na-azide (1% w/v). MPO activity was determined as the change in absorbance at 460 nm.

Chitinase assay

Chitinase activity in BAL fluid and lung homogenates was measured using a commercial kit according to the instructions provided by the manufacturer. Briefly, 10 μl of the test sample (BAL or lung homogenate) was mixed with 90 μl of substrate working solution (4-methylumbelliferyl β -D-N,N-diacetylchitobioside hydrate) in a 96-well black fluorescence plate (Nunc) and incubated for 30 min at 37°C in the dark. The reaction was stopped by adding 200 μl of sodium carbonate buffer, and fluorescence was measured using a fluorescence plate reader (Perkin Elmer). Chitinase activity was calculated using a standard curve obtained using a 4-methylumbelliferone standard solution and expressed as units/ml.

Lung histology

Lungs were excised and immediately fixed in 4% formaldehyde prior to embedding in paraffin wax for sectioning. Mice lung sections (5 μm thickness) were stained with haematoxylin and eosin (H&E) or PAS and analysed for peribronchial inflammation. Semi quantitation of images ($n = 4$ mice per group) was performed using Fiji software and inflammation score were determined.

Cytokine quantification

The levels of TNF- α and TSLP in BAL or whole lung homogenates were measured using commercial ELISA kits (R&D systems) according to the instructions provided by the manufacturer. The minimum detection limit for TNF- α and TSLP was 16 pg/ml.

In vitro macrophage stimulation

Murine macrophage-like RAW 264.7 cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 (Invitrogen) containing 10% heat-inactivated FBS, 2 mM L-glutamine and penicillin-streptomycin (referred to as complete medium) at 37°C in a 100% humidified incubator with 5% CO_2 . To maintain logarithmic growth, the cells were passaged every 2–3 days until they were used. RAW264.7 cells were seeded at a density of 1×10^6 cells/ml in complete medium and used for stimulation experiments. The cells were transferred to a serum-free medium overnight prior to stimulation with 100 $\mu\text{g}/\text{ml}$ of AIF, chitinase-treated AIF, glucanase-treated AIF and vehicle control (PBS) for various time points. The supernatants were harvested, centrifuged and stored at -80°C until use in cytokine assays.

Identification of an IgM antibody against chitin/chitosan

The identification of a specific IgM antibody targeted against polysaccharide antigen was determined as previously described with minor modifications (Rapaka et al., 2010). Low molecular weight chitin/chitosan (Sigma–Aldrich) derived from crab shells was dissolved in 2% acetic acid/PBS (v/v) and further diluted in PBS to a final concentration of 25 $\mu\text{g}/\text{ml}$. Chitin/chitosan was coated onto PolySorp 96-well plates at a concentration of 25 $\mu\text{g}/\text{ml}$ at 4°C . The plates were blocked with 10% FBS and 5% milk powder in PBS (blocking buffer), and blocking buffer was used as a dilution buffer for the serum and secondary antibodies. The samples were applied at 1:40 dilutions. The horseradish peroxidase-conjugated secondary antibody against mouse IgM was obtained from Southern Biotech. The plates were developed with TMB substrate, and the reaction was quenched with 2 N H_2SO_4 . The background for all ELISAs was set at an OD of <0.05 at 450 nm. The OD values at 450 nm were reported with the background subtracted.

Statistics

The data are expressed as the mean $\pm$ SEM unless otherwise stated. The data were assessed for significance using a non-parametric Mann–Whitney t -test or ANOVA using Prism 5 (GraphPad). A P -value <0.05 was considered as statistically significant, and the significance levels are represented as follows: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, ns = not significant.

Results

Characterisation of *Aspergillus fumigatus* polysaccharides

We purified *A. fumigatus* cell wall polysaccharides based on their solubility in alkali. Furthermore, we prepared AIF without chitin and without β -glucan using enzymatic treatment. When the size distribution was analysed, we found that 70.6% of AIFs, 72.1% of glucans (AIF treated with chitinase) and 68.2% of chitins (AIF treated with glucanase) were less than 10 μm in size (Fig. 1A–C). The presence or absence of chitin in different preparations of AIF after enzymatic treatments was confirmed by binding to WGA. WGA successfully bound to AIF, glucanase-treated AIF and chitin beads but not to curdlan or chitinase-treated AIF as determined by pull-down experiments (Fig. 1D). Thus, chitinase treatment of AIF abolished its binding to WGA. To detect the presence of β -glucan in AIF, we used a monoclonal antibody raised against β -glucan in a pull-down approach. In a western blot using the pellet obtained from the pull-down experiment, the binding of β -glucan monoclonal antibody to AIF and chitinase-treated AIF was clear; however, this antibody did not bind to glucanase-treated AIF (Fig. 1E). The AIF preparations were adjusted to a concentration of 2 mg/ml, and each of the

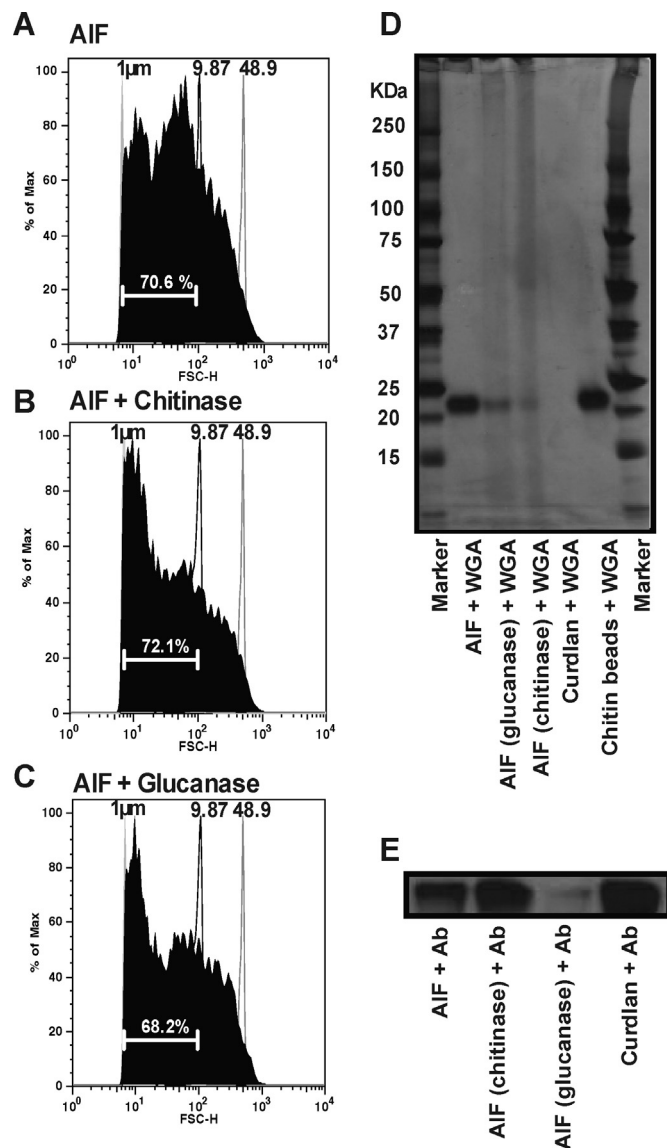


Fig. 1. Characterisation of *Aspergillus fumigatus* alkali-insoluble fragments (AIF). FACS analysis showing the size distribution of polysaccharides filtered through a 40-µm cell strainer. 1 µm, 9.87 µm and 48.9 µm beads were used as particle controls. Size distribution of (A) AIF, (B) AIF treated with chitinase, (C) AIF treated with glucanase. (D) Silver-stained SDS-PAGE of a WGA pull-down assay showing the presence of chitin in various polysaccharide preparations. WGA (5 µg/ml) was added to 1000 µg of AIF, AIF treated with glucanase or chitinase, curdlan or Chitin Beads in a total volume of 1000 µl, and a pull-down assay was performed as described in Materials and Methods. (E) SDS-PAGE and western blotting of a β-glucan pull-down assay using a monoclonal mouse anti-β-glucan antibody and developed with a rabbit anti-mouse antibody, showing presence of β-glucan in curdlan, AIF and chitinase-treated AIF but not in glucanase-treated AIF. The antibodies (5 µg/ml) were added to 1000 µg of polysaccharide in a total volume of 1000 µl.

polysaccharides (100 µg/50 µl) were used for intranasal challenge as described in Fig. 2A and B.

Aspergillus fumigatus polysaccharides recruit eosinophils and neutrophils

We performed intranasal administration of AIF, chitinase-treated AIF, glucanase-treated AIF and vehicle control (PBS) in C57BL/6 mice. Intranasal challenge of AIF and AIF treated with chitinase or glucanase resulted in significant lung inflammation as assessed by BAL fluid cell counts and lung histology (Figs. 2C and 3). The inflammation score for PBS, AIF, chitinase-treated AIF and

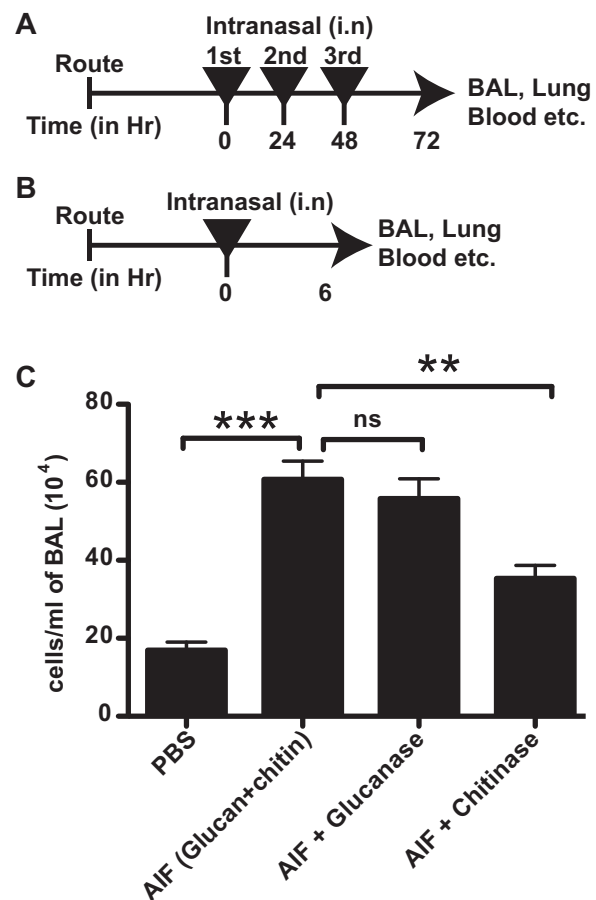


Fig. 2. Schematic presentation of the protocols for intranasal challenge with polysaccharide antigens. (A) In a 72-h experiment, C57BL/6 mice received 3 doses (100 µg/50 µl) of AIF, chitinase-treated AIF, glucanase-treated AIF or vehicle controls. At 24 h after the last intranasal dose, the mice were sacrificed, and BAL was performed. (B) In a 6-h experiment, C57BL/6 mice received one dose (100 µg/50 µl) of AIF, chitinase-treated AIF, glucanase-treated AIF or vehicle controls. The mice were sacrificed, and BAL was performed after 6 h. (C) In the 72-h experiment, total cell counts were obtained from BAL fluid. The results are presented as the mean ± SEM of a minimum of 6–8 mice per group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Mann–Whitney U -test.

glucanase-treated AIF were 0.868, 1.527, 1.002 and 1.426 respectively. Mice group which received AIF and glucanase treated AIF had a significant inflammation score (PBS vs. AIF, $P = 0.013$), (PBS vs. AIF + glucanase, $P = 0.009$) compared to PBS treated group. Chitinase treated AIF inflammation score was not significant when compared to PBS treated group. Further more chitinase treatment of AIF reduced the inflammation score compared to AIF group however this reduction was not significant ($P = 0.0651$). Treatment with AIF alone induced a >2-fold increase in BAL fluid cell numbers compared with PBS treatment. Chitinase-treated AIF reduced the total cell number in BAL compared with untreated AIF, whereas glucanase treatment did not influence the total cell number compared to untreated AIF. Similarly, the EPO level and MPO level, which reflect the number of eosinophils and neutrophils, respectively, were increased in mice receiving AIF. The EPO levels were reduced in BAL and lung homogenates after treatment with chitinase- and glucanase-treated AIF when compared with intact AIF; however, the reduction was not significant (Fig. 4A and B). The MPO levels were also reduced in BAL and lung homogenates after treatment with chitinase- and glucanase-treated AIF compared with intact AIF, and this reduction reached significance in lung homogenates (Fig. 5A and B). Taken together, these results indicate that, on a weight-to-weight basis, intact AIF is a stronger

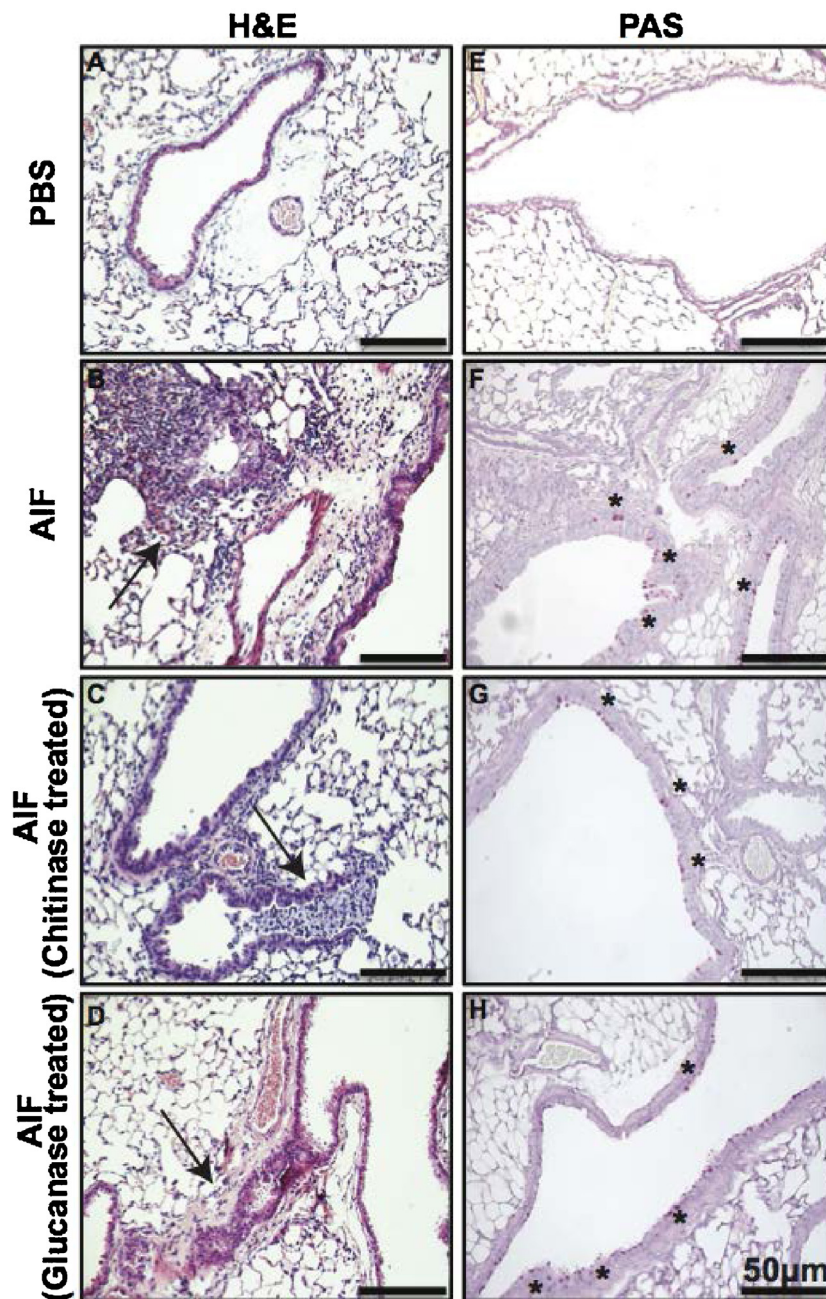


Fig. 3. AIF induces inflammation in lung tissue. Haematoxylin and eosin (H&E) or PAS staining of mice lung sections from the 72-h experiment. Representative airways ($n=4$ mice per group) from mouse lungs treated with PBS, AIF, chitinase-treated AIF and glucanase-treated AIF are shown. Arrows and stars indicate regions with inflammation and PAS positive cells respectively. Magnification: $20\times$ objective. Scale bar = $50\mu\text{m}$.

inducer of inflammation in the lung compared with the decomposed polysaccharides.

Aspergillus fumigatus polysaccharides induce chitinase expression in mice lungs

We tested the ability of AIF to induce chitinase expression in mice lungs. Mice that received intranasal AIF showed significantly higher chitinase levels both in BAL and lung homogenates when compared with the PBS group (Fig. 6A and B). Depletion of chitin from AIF via treatment with chitinase significantly reduced the chitinase level compared with intact AIF; however, the same result was not observed when AIF was treated with glucanase.

We next studied the influence of cell wall-associated proteases on eosinophil recruitment and chitinase activity in BAL using heat-killed *A. fumigatus* mycelium fragments and comparing the results with those obtained using AIF. There was no difference observed in the EPO level (Fig. 7A) or the chitinase level (Fig. 7B) in mice, indicating that these responses may be protease-independent.

AIF induces TNF- α and TSLP production in mouse lungs

We next studied the ability of AIF as well as chitinase- and glucanase-treated AIF to induce TNF- α and TSLP production at early (6 h) and late (72 h) time points in mouse lungs. We observed a significant increase in both TNF- α and TSLP production as early as 6 h after intranasal administration of AIF (Fig. 8A and B). AIF

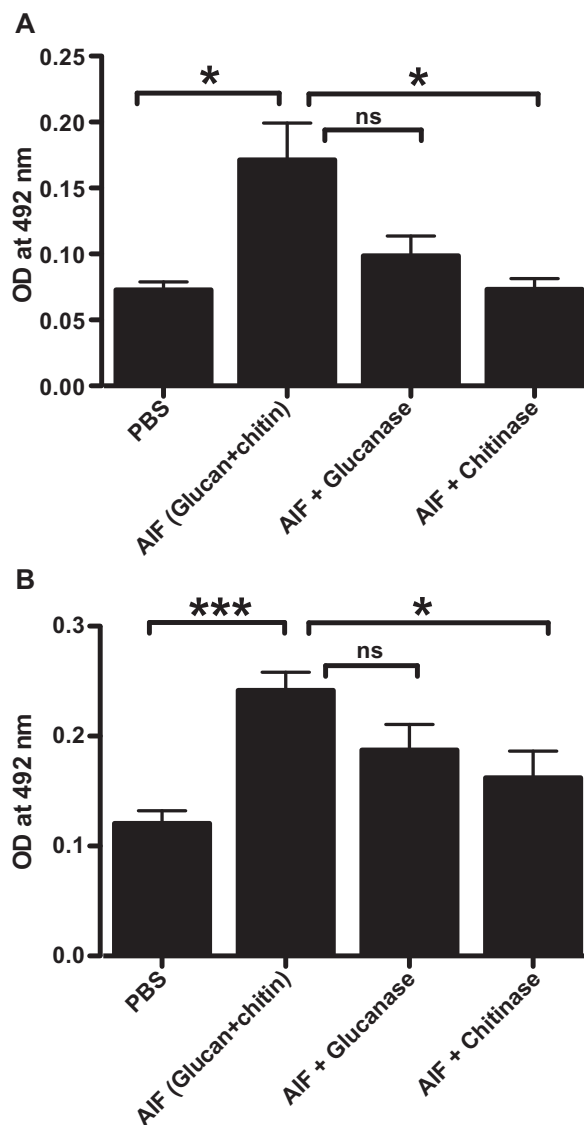


Fig. 4. Eosinophil recruitment by AIF requires the presence of both chitin and β -glucan. In the 72-h experiment, C57BL/6 mice received 3 doses (100 μ g/50 μ l) of AIF, chitinase-treated AIF, glucanase-treated AIF or vehicle control. At 24 h after the last intranasal dose, the mice were sacrificed and (A) BAL and (B) lung homogenate were analysed for EPO. The results are presented as the mean $\pm$ SEM of a minimum of 4–6 mice per group. * P < 0.05, ** P < 0.01, *** P < 0.001, Mann–Whitney t -test.

treated with chitinase or glucanase failed to induce significant TNF- α production compared with intact AIF at both time points. However, chitinase-treated AIF significantly induced TNF- α and TSLP production from 6 h to 72 h (chitinase-treated AIF at 6 h vs. 72 h; # P = 0.0087; Fig. 8A and B).

Degradation of chitin or β -glucan in AIF attenuates TNF- α production in macrophages

To support our *in vivo* findings, we studied the ability of AIF as well as chitinase- and glucanase-treated AIF towards TNF- α expression in mouse macrophage cell line. Stimulation of RAW264.7 cells with AIF resulted in the production of large amounts of TNF- α within 2 h, which further increased up to 12 h (Fig. 9). We observed a clear reduction in TNF- α production when the cells were stimulated with chitinase- or glucanase-treated AIF. During the first 6 h of stimulation, TNF- α production was significantly higher in AIF-stimulated RAW264.7 cells compared with cells stimulated with

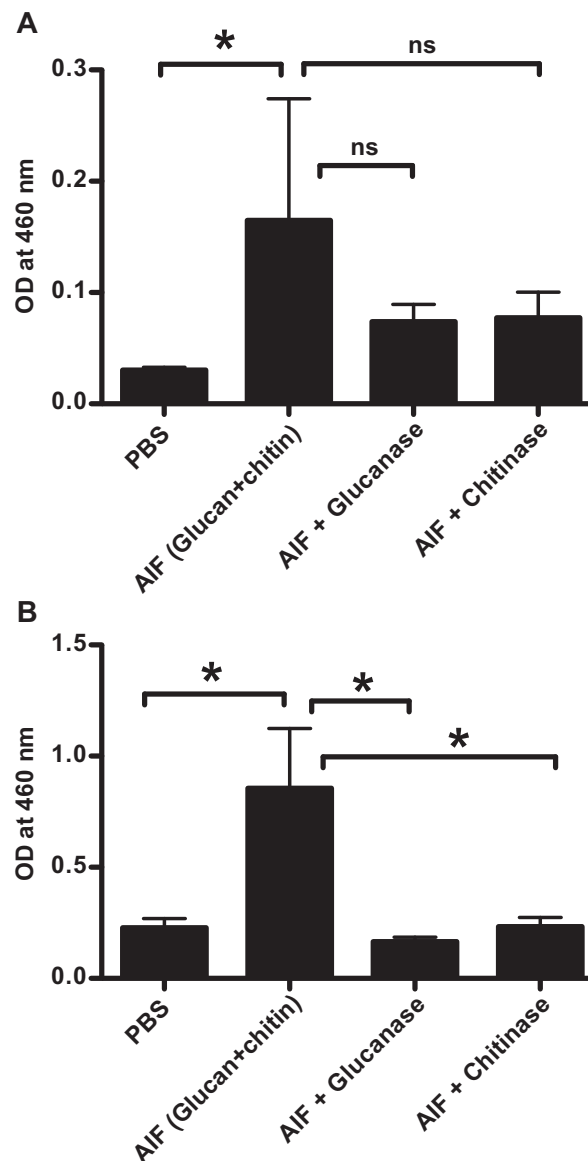


Fig. 5. Neutrophil recruitment by AIF requires the presence of both chitin and glucan. In the 72-h experiment, C57BL/6 mice received 3 doses (100 μ g/50 μ l) of AIF, chitinase-treated AIF, glucanase-treated AIF or vehicle control. At 24 h after the last intranasal dose, the mice were sacrificed and (A) BAL and (B) lung homogenates were analysed for MPO. The results are presented as the mean $\pm$ SEM of a minimum of 4–6 mice per group. * P < 0.05, ** P < 0.01, *** P < 0.001, Mann–Whitney t -test.

chitinase- or glucanase-treated AIF. However, the difference disappeared over time and was abolished after 12 h.

Challenge with AIF enhanced the production of a chitin-specific IgM

We evaluated chitin-specific IgM production in mice that received AIF intranasally for 3 consecutive days. We found that the AIF-treated mice had significantly higher chitin/chitosan-specific IgM compared with the PBS-treated group (Fig. 10). These results suggest a possible role for cell wall polysaccharides in the induction of lymphocyte activation.

Discussion

The cell wall shields fungal cells from hostile environments, and evolution has conserved chitin and β -glucan as cross-linked

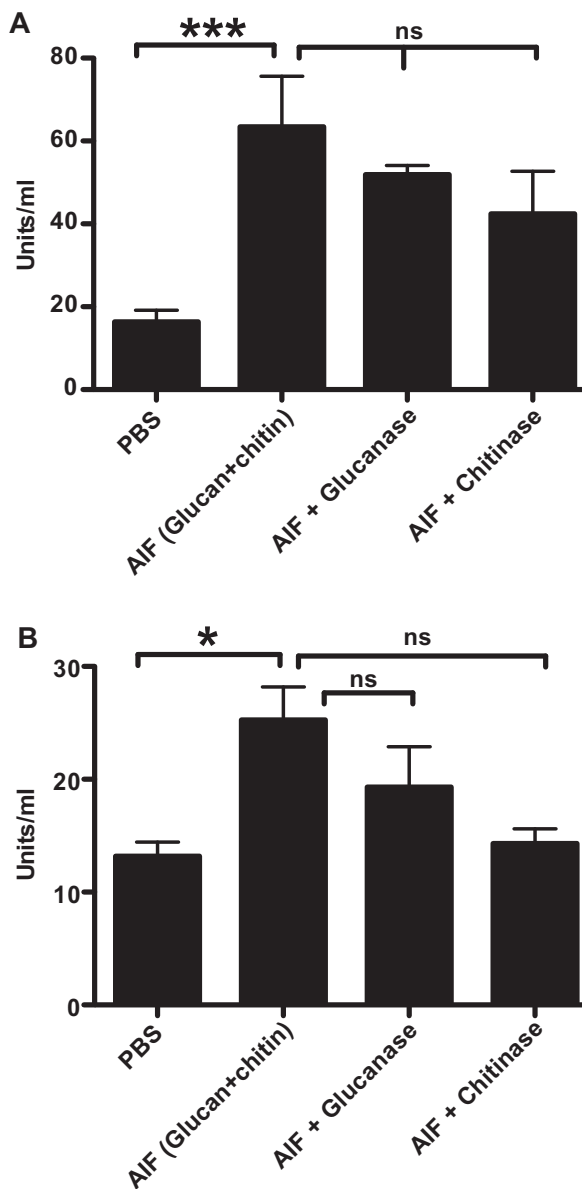


Fig. 6. Chitinase induction by AIF in mouse lungs. In the 72-h experiment, C57BL/6 mice received 3 doses (100 μ g/50 μ l) of AIF, chitinase-treated AIF, glucanase-treated AIF or vehicle control. At 24 h after the last intranasal dose, total chitinase activity was measured in (A) BAL and (B) lung homogenate using a fluorogenic substrate (4-methylumbelliferyl β -D-N,N-diacetylchitobioside hydrate). The data are expressed in units/ml. The results are presented as the mean $\pm$ SEM of a minimum of 6–8 mice per group. * P < 0.05, ** P < 0.01, *** P < 0.001, Mann–Whitney t -test.

composite carbohydrates that confer both rigidity and flexibility to the fungal cell wall during the different life states of the fungus (Latge, 1999). These structures are not present in mammalian hosts, and immune receptors have evolved to recognise chitin and β -glucan (Knutsen and Slavin, 2011; Romani, 2011). Fungi also secrete proteases that activate the immune system through specific receptors (Knutsen and Slavin, 2011; Romani, 2011). In the present study, we have studied the effect of *A. fumigatus* AIF on a weight-to-weight basis and compared AIF-mediated immune activation with that induced by chitinase- and glucanase-treated AIF fragments representing isolated β -glucan and chitin, respectively.

The AIF fragments were free of endotoxin, and enzymatic treatment with glucanase and chitinase destroyed β -glucan and chitin, respectively, as determined by pull-down assays using anti- β -glucan antibody and WGA. However, the enzyme treatment did

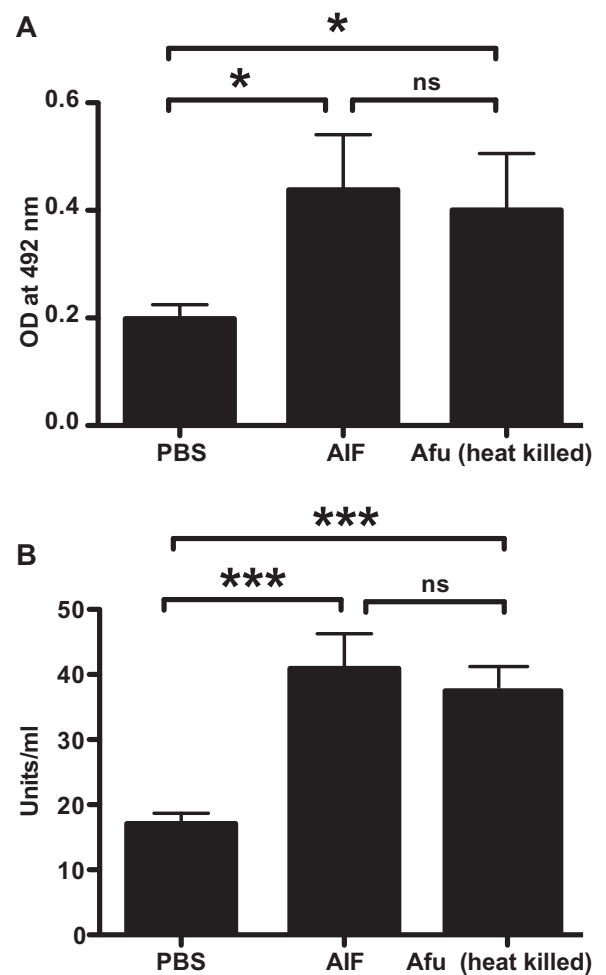


Fig. 7. Eosinophil peroxidase and chitinase expression in mice lungs induced by heat-killed *A. fumigatus* mycelium fragments. C57BL/6 mice received 3 intranasal doses of heat-killed *A. fumigatus* mycelium fragments (100 μ g/50 μ l) or AIF as described in Fig. 2A. At 24 h after the last intranasal dose, the mice were killed, and BAL was performed as described in Materials and Methods. EPO and total chitinase activity in BAL were measured as described earlier. The results are presented as the mean $\pm$ SEM of a minimum of 6–8 mice per group. * P < 0.05, ** P < 0.01, *** P < 0.001, Mann–Whitney t -test.

not result in an overall change in size distribution of the particles as judged by flow cytometry, which is an important point because different particle sizes can influence immune activation, as observed for both chitin particles (Da Silva et al., 2009) and β -glucan (Goodridge et al., 2011).

Intranasal instillation of AIF resulted in an increase in BAL cell number from $16.93 \times 10^4 \pm 5.45$ cells/ml to $60.73 \times 10^4 \pm 11.36$ cells/ml. Destruction of chitin by chitinase resulted in a significant decrease in cell number to $35.39 \times 10^4 \pm 7.45$ cells/ml. Destruction of β -glucan by glucanase did not alter the cell number compared with intact AIF. Mice treated with intact AIF, glucanase-treated AIF and chitinase-treated AIF exhibited massive inflammatory cell infiltration in the tissues as well as the induction of PAS-positive alveolar epithelial cells based on examinations of histological sections.

Neutrophils play an important role in killing fungal hyphae (Bruns et al., 2010), and they produce chitinase (van Eijk et al., 2005), which has antifungal properties (Chen et al., 2009). Eosinophils also kill fungal hyphae by releasing their cellular components, including major basic protein (MBP) and eosinophil-derived neurotoxins (Yoon et al., 2008). We measured myeloperoxidase (MPO) and eosinophil peroxidase (EPO) in BAL

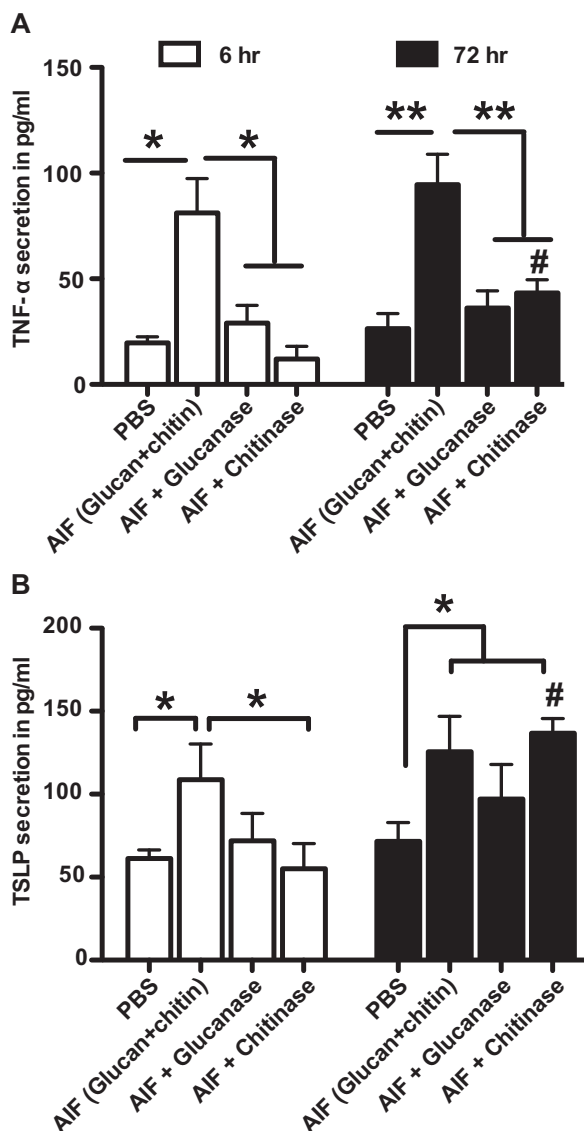


Fig. 8. AIF induces TNF- α and TSLP production *in vivo*. In the 6-h and 72-h experiments, C57BL/6 mice received PBS, AIF, chitinase-treated AIF and glucanase-treated AIF as outlined in Fig. 2A and B. Whole lung homogenates were prepared and analysed for (A) TNF- α and (B) TSLP production by ELISA. When mice received chitinase-treated AIF, both TNF- α and TSLP production increased significantly (#) from 6 h to 72 h. The results are presented as the mean $\pm$ SEM of 5–6 mice per/group. * P < 0.05, ** P < 0.01, *** P < 0.001, # P < 0.05 for chitinase-treated AIF at 6 h vs. chitinase-treated AIF at 72 h, Mann–Whitney t -test.

and lung tissue homogenates after intranasal administration of intact or degraded AIF. AIF induced both EPO and MPO in BAL fluid and lung tissue compared to PBS. Although enzymatic pretreatment of AIF reduced EPO levels, it did not significantly influence EPO levels in either BAL or lung tissue. Earlier reports stated that eosinophils release eosinophil-derived neurotoxin and MBP after β -glucan exposure, whereas chitin does not elicit the same effect, indicating that eosinophils respond differently towards these PAMPs (Yoon et al., 2008). The MPO levels were reduced significantly (to the PBS background level) by enzymatic treatment of AIF, suggesting that the neutrophil granulocytes are dependent on composite carbohydrates for the release of MPO.

Both chitin and β -glucan are known to induce chitinase expression (Lalaker et al., 2009; Reese et al., 2007; van Eijk et al., 2007, 2010) in epithelial and phagocytic cells, and chitinase activity plays an important role in the antifungal immune defence (Chen et al.,

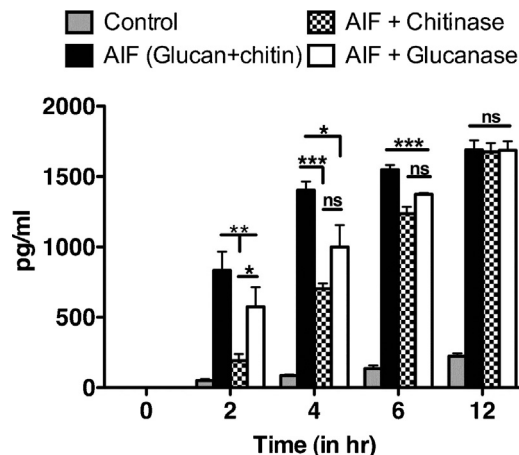


Fig. 9. AIF induces TNF- α production by macrophages. The mouse macrophage cell line RAW264.7 (1×10^6 ml $^{-1}$) was grown in complete medium. After washing in PBS, the cells were stimulated with 100 μ g/ml of AIF, chitinase-treated AIF, glucanase-treated AIF or vehicle control in serum-free media. The TNF- α concentrations were measured by ELISA at different time points. The data from 3 individual experiments (each performed in triplicate) are presented. * P < 0.05, ** P < 0.01, *** P < 0.001, Mann–Whitney t -test.

2009). We found that AIF treatment induced chitinase production in mouse lungs. Pretreatment of AIF with chitinase and glucanase reduced the chitinase activity; however, this reduction was only significant after pretreatment with chitinase, indicating the relative importance of the presence of a substrate PAMP vs. a non-substrate PAMP for the induction of chitinase activity.

The proinflammatory cytokine tumour necrosis factor- α (TNF- α) is produced in response to *A. fumigatus* challenge *in vivo* (Hohl et al., 2005; Mehrad et al., 1999), and chitin has been shown to induce TSLP production in keratinocytes (Koller et al., 2011). We observed that TNF- α was strongly induced by AIF in lung homogenates both 6 h after one instillation and 24 h after 3 consecutive instillations. Additionally, isolated β -glucan and chitin are known to induce Th1 and Th2 cytokines, respectively (Da Silva et al., 2010; Kawashima et al., 2012). It was therefore surprising to observe that chitinase and glucanase treatment of AIF abolished the induction of TNF- α cytokine. Particle size may be one explanation for this discrepancy. We are currently investigating whether intact β -glucan triggers differential immune activation in the lungs compared to chitin-treated AIF on a weight-to-weight basis.

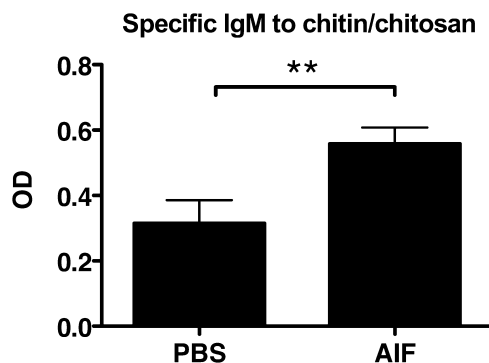


Fig. 10. Intranasal challenge with AIF increased the level of a specific IgM against chitin/chitosan. In the 72-h experiment, C57BL/6 mice received 3 intranasal doses of AIF or PBS as described in Fig. 2A. At 24 h after the last intranasal dose, the mice were sacrificed, and serum was prepared. Serum was diluted 1:40 in blocking buffer, and specific IgM antibodies against chitosan/chitin were measured by ELISA. The data are shown as OD₄₅₀ values with the secondary antibody background signal subtracted. In all experiments, the secondary antibody alone yielded an OD₄₅₀ value of < 0.050. The results are presented as the mean $\pm$ SD of 6 mice per group from 2 independent experiments. * P < 0.05, ** P < 0.01, *** P < 0.001, Mann–Whitney t -test.

In vitro stimulation of macrophages (RAW264.7) with AIF triggered strong induction of TNF- α . At early time points, glucanase and especially chitinase treatment of AIF dampened TNF- α induction; however, over time, the difference between the 3 treatments was diminished, and after 12 h, no difference was observed between the treatments.

The kinetics of the *in vitro* stimulation of RAW264.7 cells therefore appear to be different, and inhibitory components may exist that dampen the *in vivo* response against AIF. Other cells, including alveolar epithelial cells, may also be involved in the response against the administered components.

TSLP is known to regulate barrier immunity in the lung and to promote Th2-associated inflammation (Zhou et al., 2005; Ziegler and Artis, 2010). We observed an increase in TSLP production 6 h after AIF instillation. As observed for the induction of TNF- α , both chitinase- and glucanase-treated AIF failed to induce significant TSLP production at this time point. At the late time point, chitinase-treated AIF was as efficient as intact AIF for inducing TSLP production, indicating that β -glucan alone can induce TSLP production as reported previously (Kreindler et al., 2010).

The combined presentation of β -glucan and chitin in AIF may therefore be involved in the TSLP-mediated recruitment of dendritic cells, which are known to transport conidia and hyphae of *A. fumigatus* to draining lymph nodes (Bozza et al., 2002), thus instructing and stimulating the T-helper cell response.

It has recently been demonstrated that natural IgMs are involved in the earliest aspects of fungal carbohydrate antigen recognition in the host and influence the trafficking of antigen-loaded CD11c⁺ cells to regional lymph nodes (Rapaka et al., 2010). We observed a distinct increase in IgM antibodies reacting to chitin/chitosan as early as 72 h after the first AIF instillation. We speculate that intranasally administered AIF enhanced native IgM production through TSLP-dependent recruitment of CD11c⁺ cells.

Our data demonstrate that AIF, representing a composite carbohydrate structure, is an efficient stimulator of the innate immune system. Degrading β -glucan or chitin within AIF by enzymatic treatment resulted in differential attenuation of the observed responses both *in vivo* and *in vitro*. Future studies will reveal whether well-known receptors for β -glucan and chitin (such as Dectin-1 and FIBCD1, respectively) can recognise composite PAMPs more efficiently than non-composite PAMPs or if composite PAMPs induce synergy between individual receptors.

Acknowledgements

This work was supported by The Danish Medical Research Council, The Novo Nordisk Foundation, The Lundbeck Foundation, The Gangsted Foundation, The Augustinus Foundation and Fonden til Lægevidenskabens Fremme.

References

- Aimanianda, V., Bayry, J., Bozza, S., Kniemeyer, O., Perruccio, K., Elluru, S.R., Clavaud, C., Paris, S., Brakhage, A.A., Kaveri, S.V., Romani, L., Latge, J.-P., 2009. Surface hydrophobin prevents immune recognition of airborne fungal spores. *Nature* 460, 1117–1121.
- Arruda, L., Mann, B., Chapman, M., 1992. Selective expression of a major allergen and cytotoxin, Asp f 1, in *Aspergillus fumigatus*. Implications for the immunopathogenesis of *Aspergillus*-related diseases. *Journal of Immunology* 149, 3354–3359.
- Ballou, V., Huerre, M., Latge, J.-P., Chignard, M., 2005. Differences in patterns of infection and inflammation for corticosteroid treatment and chemotherapy in experimental invasive pulmonary *Aspergillus* infection. *Infection and Immunity* 73, 494–503.
- Bozza, S., Clavaud, C., Giovannini, G., Fontaine, T., Beauvais, A., Sarfati, J., D'Angelo, C., Perruccio, K., Bonifazi, P., Zagarella, S., Moretti, S., Bistoni, F., Latge, J.-P., Romani, L., 2009. Immune sensing of *Aspergillus fumigatus* proteins, glycolipids, and polysaccharides and the impact on Th immunity and vaccination. *Journal of Immunology* 183, 2407–2414.
- Bozza, S., Gaziano, R., Spreca, A., Bacci, A., Montagnoli, C., di Francesco, P., Romani, L., 2002. Dendritic cells transport conidia and hyphae of *Aspergillus fumigatus* from the airways to the draining lymph nodes and initiate disparate Th responses to the fungus. *Journal of Immunology* 168, 1362–1371.
- Bruns, S., Kniemeyer, O., Hasenberg, M., Aimanianda, V., Nietzsche, S., Thywissen, A., Jeron, A., Latge, J.-P., Brakhage, A.A., Gunzer, M., 2010. Production of extracellular traps against *Aspergillus fumigatus* *in vitro* and in infected lung tissue is dependent on invading neutrophils and influenced by hydrophobin RodA. *PLoS Pathogens* 6, e1000873.
- Chen, L., Shen, Z., Wu, J., 2009. Expression, purification and *in vitro* antifungal activity of acidic mammalian chitinase against *Candida albicans*, *Aspergillus fumigatus* and *Trichophyton rubrum* strains. *Clinical and Experimental Dermatology* 34, 55–60.
- Da Silva, C.A., Chalouni, C. c., Williams, A., Hartl, D., Lee, C.G., Elias, J.A., 2009. Chitin is a size-dependent regulator of macrophage TNF and IL-10 production. *Journal of Immunology* 182, 3573–3582.
- Da Silva, C.A., Pochard, P., Lee, C.G., Elias, J.A., 2010. Chitin particles are multifaceted immune adjuvants. *American Journal of Respiratory and Critical Care Medicine* 182, 1482–1491.
- Faroo-Trindade, I., Willment, J.A., Kerrigan, A.M., Redelingshuys, P., Hadebe, S., Reid, D.M., Srinivasan, N., Wainwright, H., Lang, D.M., Steele, C., Brown, G.D., 2012. Characterisation of innate fungal recognition in the lung. *PLoS ONE* 7, e35675.
- Fontaine, T., Simenel, C., Dubreucq, G., Adam, O., Delepierre, M., Lemoine, J., Vorgias, C.E., Diaquin, M., Latge, J.-P., 2000. Molecular organization of the alkali-insoluble fraction of *Aspergillus fumigatus* cell wall. *Journal of Biological Chemistry* 275, 27594–27607.
- Goodridge, H.S., Reyes, C.N., Becker, C.A., Katsumoto, T.R., Ma, J., Wolf, A.J., Bose, N., Chan, A.S.H., Magee, A.S., Danielson, M.E., Weiss, A., Vasilakos, J.P., Underhill, D.M., 2011. Activation of the innate immune receptor Dectin-1 upon formation of a phagocytic synapse. *Nature* 472, 471–475.
- Hansen, M.Y., Wold, J.K., Paulsen, B.S., Cohen, E.H., Karlsson-Borgå, Å., 1994. Allergens in *Aspergillus fumigatus*. *Allergy* 49, 235–241.
- Hartl, D., He, C.H., Koller, B., Da Silva, C.A., Homer, R., Lee, C.G., Elias, J.A., 2008. Acidic mammalian chitinase is secreted via an ADAM17/epidermal growth factor receptor-dependent pathway and stimulates chemokine production by pulmonary epithelial cells. *Journal of Biological Chemistry* 283, 33472–33482.
- Hearn, V.M., Sietsma, J.H., 1994. Chemical and immunological analysis of the *Aspergillus fumigatus* cell wall. *Microbiology* 140, 789–795.
- Hohl, T.M., Van Epps, H.L., Rivera, A., Morgan, L.A., Chen, P.L., Feldmesser, M., Pamer, E.G., 2005. *Aspergillus fumigatus* triggers inflammatory responses by stage-specific β -glucan display. *PLoS Pathogens* 1, e30.
- Kawashima, S., Hirose, K., Iwata, A., Takahashi, K., Ohkubo, A., Tamachi, T., Ikeda, K., Kagami, S.-i., Nakajima, H., 2012. β -glucan curdlan induces IL-10 producing CD4⁺ T cells and inhibits allergic airway inflammation. *Journal of Immunology* 189, 5713–5721.
- Knutsen, A.P., Slavin, R.G., 2011. Allergic bronchopulmonary aspergillosis in asthma and cystic fibrosis. *Clinical Developmental Immunology*, 2011.
- Koller, B., Müller-Wiefel, A.S., Rupec, R., Korting, H.C., Ruzicka, T., 2011. Chitin modulates innate immune responses of keratinocytes. *PLoS ONE* 6, e16594.
- Kreindler, J.L., Steele, C., Nguyen, N., Chan, Y.R., Pilewski, J.M., Alcorn, J.F., Vyas, Y.M., Aujla, S.J., Finelli, P., Blanchard, M., Zeigler, S.F., Logar, A., Hartigan, E., Kurs-Lasky, M., Rockette, H., Ray, A., Kolls, J.K., 2010. Vitamin D3 attenuates Th2 responses to *Aspergillus fumigatus* mounted by CD4⁺ T cells from cystic fibrosis patients with allergic bronchopulmonary aspergillosis. *Journal of Clinical Investigation* 120, 3242–3254.
- Lalaker, A., Nkrumah, L., Lee, W.-K., Ramanathan, M., Lane, A.P., 2009. Chitin stimulates expression of acidic mammalian chitinase and eotaxin-3 by human sinonasal epithelial cells *in vitro*. *American Journal of Rhinology and Allergy* 23, 8–14.
- Latge, J.-P., 1999. *Aspergillus fumigatus* and *Aspergillosis*. *Clinical Microbiology Reviews* 12, 310–350.
- Mehrad, B., Strieter, R.M., Standiford, T.J., 1999. Role of TNF- α in pulmonary host defense in murine invasive *Aspergillosis*. *Journal of Immunology* 162, 1633–1640.
- Oliveira, S.H.P., Taub, D.D., Nagel, J., Smith, R., Hogaboam, C.M., Berlin, A., Lukacs, N.W., 2002. Stem cell factor induces eosinophil activation and degranulation: mediator release and gene array analysis. *Blood* 100, 4291–4297.
- Rapaka, R.R., Ricks, D.M., Alcorn, J.F., Chen, K., Khader, S.A., Zheng, M., Plevy, S., Bengten, E., Kolls, J.K., 2010. Conserved natural IgM antibodies mediate innate and adaptive immunity against the opportunistic fungus *Pneumocystis murina*. *Journal of Experimental Medicine* 207, 2907–2919.
- Reese, T.A., Liang, H.E., Tager, A.M., Luster, A.D., Van Rooijen, N., Voehringer, D., Locksley, R.M., 2007. Chitin induces accumulation in tissue of innate immune cells associated with allergy. *Nature* 447, 92–96.
- Romani, L., 2011. Immunity to fungal infections. *Nature Reviews Immunology* 11, 275–288.
- Steele, C., Rapaka, R.R., Metz, A., Pop, S.M., Williams, D.L., Gordon, S., Kolls, J.K., Brown, G.D., 2005. The β -glucan receptor Dectin-1 recognizes specific morphologies of *Aspergillus fumigatus*. *PLoS Pathogens* 1, e42.
- Strong, P., Clark, H., Reid, K., 2002. Intranasal application of chitin microparticles down-regulates symptoms of allergic hypersensitivity to *Dermatophagoides pteronyssinus* and *Aspergillus fumigatus* in murine models of allergy. *Clinical & Experimental Allergy* 32, 1794–1800.
- Thomsen, T., Schlosser, A., Holmskov, U., Sorensen, G.L., 2011. Ficolins and FIBCD1: soluble and membrane bound pattern recognition molecules with acetyl group selectivity. *Molecular Immunology* 48, 369–381.
- van Eijk, M., Scheij, S.S., van Roemen, C.P.A.A., Speijer, D., Boot, R.G., Aerts, J.M.F.G., 2007. TLR- and NOD2-dependent regulation of human phagocyte-specific chitinase. *FEBS Letters* 581, 5389–5395.

- van Eijk, M., van Roomen, C.P.A.A., Renkema, G.H., Bussink, A.P., Andrews, L., Blommaart, E.F.C., Sugar, A., Verhoeven, A.J., Boot, R.G., Aerts, J.M.F.G., 2005. Characterization of human phagocyte-derived chitotriosidase, a component of innate immunity. *International Immunology* 17, 1505–1512.
- van Eijk, M., Voorn-Brouwer, T., Scheij, S.S., Verhoeven, A.J., Boot, R.G., Aerts, J.M.F.G., 2010. Curdlan-mediated regulation of human phagocyte-specific chitotriosidase. *FEBS Letters* 584, 3165–3169.
- Yamashita, Y., Okano, M., Yoshino, T., Hattori, H., Yamamoto, T., Watanabe, T., Takishita, T., Akagi, T., Nishizaki, K., 2002. Carbohydrates expressed on *Aspergillus fumigatus* induce in vivo allergic Th2-type response. *Clinical & Experimental Allergy* 32, 776–782.
- Yoon, J., Ponikau, J.U., Lawrence, C.B., Kita, H., 2008. Innate antifungal immunity of human eosinophils mediated by a beta-2 integrin, CD11b. *Journal of Immunology* 181, 2907–2915.
- Zhou, B., Comeau, M.R., Smedt, T.D., Liggitt, H.D., Dahl, M.E., Lewis, D.B., Gyarmati, D., Aye, T., Campbell, D.J., Ziegler, S.F., 2005. Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. *Nature Immunology* 6, 1047–1053.
- Ziegler, S.F., Artis, D., 2010. Sensing the outside world: TSLP regulates barrier immunity. *Nature Immunology* 11, 289–293.