

# Adjuvant effect of zymosan after pulmonary treatment in a mouse ovalbumin allergy model

Shih-Houng Young, Michael G. Wolfarth, Jenny R. Roberts, Michael L. Kashon, and James M. Antonini

*Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia, USA*

## ABSTRACT

An association has been observed between indoor mold contamination and lung allergy and asthma. This relationship is not fully understood. 1→3-β-Glucan is the major cell wall component of fungi and a good marker of fungi exposure. The objective was to evaluate the adjuvant effect of zymosan, a crude yeast cell wall preparation of 1→3-β-glucan, during ovalbumin (OVA) sensitization in an allergy model. BALB/c mice were sensitized by pharyngeal aspiration with saline, 50 μg of OVA, or OVA with 1, 10, 50, or 75 μg of zymosan on days 0, 7, and 14. One week after sensitization, each sensitized animal group was challenged with an aspiration dose of 50 μg of OVA once a week for 2 weeks. At 1 day after the last aspiration, bronchoalveolar lavage fluid and blood was collected, and markers of lung allergy and inflammation were assessed. An adjuvant effect of zymosan on OVA allergy during sensitization was observed as indicated by significant elevations in lung eosinophils, serum OVA-specific IgE, and lung IL-5 in the groups sensitized with zymosan and OVA. Pulmonary treatment with zymosan also amplified lung inflammation. Elevations were observed in lung neutrophils, TNF-α, and parameters of lung injury in the groups primed with both zymosan and OVA. In nearly all parameters, a non-linear dose–response relationship was observed in the groups primed with OVA and zymosan. The optimum adjuvant dose of zymosan was 10 μg. This study demonstrated an adjuvant effect of zymosan when exposures occurred during the sensitization phase in an OVA-induced allergy model in BALB/c mice.

**KEYWORDS** 1→3-β-glucan, allergy, fungi, inflammation, zymosan

## INTRODUCTION

Filamentous microfungi (mold) can become airborne and threaten human health after inhalation [1]. Both fungi and molds are ubiquitous and exist in the natural environment in indoor and outdoor settings. Water content (e.g., dampness) has been shown to promote fungi growth [2]. Massive flooding or major water intrusion can trigger fungi and molds to grow exponentially, as was seen in more than 40% of the homes in New Orleans after Hurricane Katrina [3]. Exposure to elevated levels of mold and other microbial agents

has been implicated in diseases associated with damp indoor environments and has become a major public concern in the indoor environment.

Repeated exposure to significant quantities of fungal material can result in respiratory irritation and allergic sensitization in some individuals [4]. Sensitized individuals may subsequently respond to much lower concentrations of airborne fungal material. Fungi and mold exposure has been associated with coughing, wheezing, chest tightness, shortness of breath, allergic reactions, hypersensitivity pneumonitis, and has been shown to exacerbate respiratory symptoms in sensitized asthmatics [3]. Importantly, mold levels in dust were associated with new-onset asthma among employees in a water-damaged building [5, 6]. The mechanisms by which mold and fungi induce asthma and allergic reactions have not been fully elucidated.

1→3-β-Glucans are the major cell wall constituents of fungi and have been considered as a biomarker for fungi exposure in the workplace [7].

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Address correspondence to James M. Antonini, PhD, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, 1095 Willowdale Road (M/S 2015), Morgantown, WV 26505, USA. E-mail: jga6@cdc.gov

Zymosan A is a commercially available yeast cell wall preparation containing  $\beta$ -glucan. The composition of Zymosan A has been reported to be ~70–75% total polysaccharide, ~13–17% protein, 0.8% chitin, 3–3.5% ash, and 6–7% fat [8]. Among the total polysaccharides, 50–57% are glucans and 16–22% are mannan. Zymosan has been shown to induce lung inflammation [9], alter adaptive immune response [10], and affect lung clearance of a bacterial pathogen [11, 12] after pulmonary treatment when evaluated using an animal model. In a comparison of the pulmonary inflammatory potential of different components of yeast and fungal cell walls, 1 $\rightarrow$ 3- $\beta$ -glucan was the most potent inflammatory agent tested, and zymosan, the crude B-glucan preparation, had a greater inflammatory activity than a partially purified particulate 1 $\rightarrow$ 3- $\beta$ -glucan [13]. In addition, specific  $\beta$ -glucans [14, 15] and soluble mold extracts [16] have been observed to have adjuvant effects in an allergic response to ovalbumin (OVA) in mice.

The goal of the current study was to further evaluate the adjuvant effect of zymosan, a crude yeast cell wall preparation containing  $\beta$ -glucan, during sub-optimal lung OVA sensitization in a mouse allergy model. BALB/c mice were sensitized by pharyngeal aspiration with saline (vehicle control), OVA, or OVA with various doses of zymosan on days 0, 7, and 14. One week after sensitization, each sensitized animal group was challenged by pulmonary aspiration with OVA once a week for 2 weeks. At 1 day after the last aspiration, the presence of lung allergy, inflammation, and injury were assessed in the animals from the different treatment groups.

## METHODS

### Animals and Pulmonary Treatment

Specific pathogen-free male BALB/c mice (Charles River Lab, Wilmington, MA) were used in the experiments. BALB/c mice have a Th2-biased immune response, and have been described as developing evidence of allergy subsequent to OVA immunization [17]. All mice used in the study were treated according to an Animal Care and Use Committee approved in-house animal protocol. The animals were housed in a room with restricted access and HEPA-filtered air, and were allowed to acclimate for 1 week before use in an animal facility approved by the Association for Assessment and Accreditation of Laboratory Animal Care. The mice were maintained on Harlan NIH-31, 6% irradiated diet, and tap water ad libitum. Beta-chips were used as bedding.

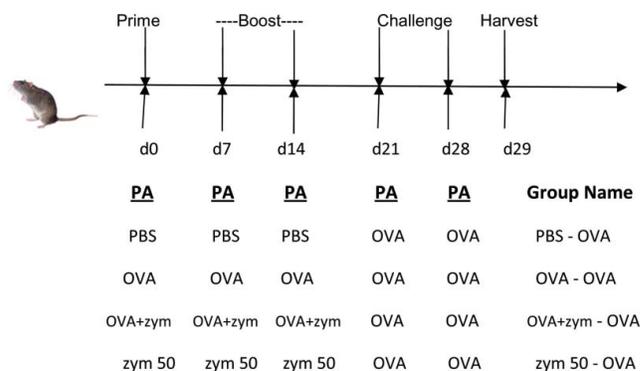


FIGURE 1. Experimental design: dosing schedule. BALB/c mice were sensitized by pharyngeal aspiration (PA) with PBS, OVA (50  $\mu$ g), zymosan (50  $\mu$ g), or a combination of OVA (50  $\mu$ g) + zymosan (1, 10, 50, and 75  $\mu$ g) once a week for 3 weeks ( $n = 12$ ). One week later, these groups of mice were challenged by PA with 50  $\mu$ g OVA once a week for 2 weeks. All mice were euthanized 1 day after the final treatment on day 29. The treatment groups based on the above dosing schedule were designated as: (1) PBS-OVA, (2) OVA-OVA, (3) OVA+zym 1-OVA, (4) OVA+zym 10-OVA, (5) OVA+zym 50-OVA, (6) OVA+zym 75-OVA, and (7) zym 50-OVA.

For the study, Zymosan A (Sigma-Aldrich Co., St. Louis, MO), a crude yeast cell wall preparation containing 1 $\rightarrow$ 3- $\beta$ -glucan, was used. Mice (20–25 g) were sensitized 3 times (once a week for 3 weeks) by pharyngeal aspiration of 40  $\mu$ L of sterile phosphate-buffered saline (PBS; vehicle control), a 50  $\mu$ g solution of OVA (Sigma-Aldrich Co., St. Louis, MO), a 50  $\mu$ g suspension of zymosan, or a combination of OVA (50  $\mu$ g) + zymosan (1, 10, 50, and 75  $\mu$ g). One week after the third treatment, the groups of mice were challenged by pharyngeal aspiration with 50  $\mu$ g OVA once per week for 2 weeks (see Figure 1 for a detailed diagram of the study's dosing regimen). The treatment groups based on the above dosing schedule were designated as: (1) PBS-OVA, (2) OVA-OVA, (3) zym 50-OVA, (4) OVA+zym 1-OVA, (5) OVA+zym 10-OVA, (6) OVA+zym 50-OVA, and (7) OVA+zym 75-OVA. It was the goal to produce a suboptimal sensitization response in the OVA-OVA group as compared to the PBS-OVA group to allow for the evaluation of a possible adjuvant effect of OVA+zym treatment.

The pharyngeal aspiration method was conducted according to Rao *et al.* [18]. Briefly, each mouse was anesthetized with isoflurane (Abbott Laboratories, North Chicago, IL). When fully anesthetized, the mouse was placed on a slanted board with the tongue gently pulled aside by small forceps. Then a 40  $\mu$ L suspension of the different samples was pipetted at the base of the tongue, and tongue restraint was continued until at least 2 deep breaths were completed.

This pharyngeal aspiration technique has been shown to provide a more even distribution of particles in the lung of mice than intratracheal instillation [18]. Mice ( $n = 12/\text{treatment group}$ ) were euthanized on day 29 at 1 day after the final treatment, and bronchoalveolar lavage (BAL) and blood collection were performed.

### Whole Blood Collection and Bronchoalveolar Lavage

At 1 day after the final treatment on day 29, mice ( $n = 8/\text{group}$ ) were deeply anesthetized with an intraperitoneal injection of sodium pentobarbital ( $> 100 \text{ mg/kg}$  body weight; Sleepaway, Fort Dodge Animal Health, Wyeth, Madison, NJ). Whole blood was collected by cardio-puncture, and the animals were exsanguinated by severing the abdominal aorta. BAL was performed using 0.6 mL of  $\text{Ca}^{2+}$ - and  $\text{Mg}^{2+}$ -free cold PBS at pH 7.4. The first fraction of BAL fluid was retained in the lungs for 30 seconds with constant massaging of the lungs until collection. This first fraction of BAL fluid was centrifuged at  $500 \times g$  for 10 minutes, and the supernatant was used for analyzing lactate dehydrogenase (LDH) activity, albumin, and cytokines levels. The lungs were further lavaged with 1-mL aliquots of PBS until a total of 5 mL BAL fluid was collected. These samples were also centrifuged for 10 minutes at  $500 \times g$ , and the cell pellets from all washes for each mouse were combined and used for cell differentials. Total cell number was determined with a Multisizer 3 Coulter Counter (Beckman Coulter, Miami, FL).

### OVA-specific IgE Measurement

The murine anti-OVA IgE Ab was detected in serum samples collected from the treated animals using an IgE capture ELISA procedure modified from Hogan et al. [19]. The following reagents were used: monoclonal anti-mouse IgE (BD PharMingen, San Diego, CA), PBS/1% skim milk; OVA (25 mg/mL), rabbit anti-OVA-HRP conjugate (GenWay Biotech, San Diego, CA); and tetramethylbenzidine (Sigma-Aldrich Co., St. Louis, MO) substrate solution. After incubation for 10 minutes at room temperature, the reaction was stopped by adding 2N sulfuric acid and color development evaluated as OD450 using an automated plate reader.

### Flow Cytometry: Whole Blood Differentiation and Quantification

One hundred  $\mu\text{L}$  of the red blood cell suspension was added into a flow cytometry tube with 100  $\mu\text{L}$  of 10% rat serum in FACS buffer for 10

minutes. Then, 50  $\mu\text{L}$  of pre-mixed antibodies in FACS buffer was added to this tube and stained for 30 minutes at room temperature on a shaker. The antibody mix contained the final concentration of the following monoclonal antibodies: MHC II-FITC (2.5  $\mu\text{g/mL}$ , 2G9), Gr-1-APC (2  $\mu\text{g/mL}$ , RBC-8C5), CCR3-PE (0.625  $\mu\text{g/mL}$ , 83.101.111), CD3-PerCP (10  $\mu\text{g/mL}$ , 145-2C11), B220-Per-CP (2  $\mu\text{g/mL}$ , RA3-6B2), and NK1.1-PE (2  $\mu\text{g/mL}$ , PK136). All the antibodies were purchased from PharMingen (Becton Dickinson, San Diego, CA) except CCR-3, which was purchased from R&D Systems (Minneapolis, MN). To prevent nonspecific binding to Fc receptors, 2.4G2 blocking reagent (6  $\mu\text{g/mL}$ ) was added to the monoclonal antibody mix. Red blood cells were lysed by adding 100  $\mu\text{L}$  of Caltag Cal-lyse lysing solution (GAS-010, Invitrogen, Carlsbad, CA) for 10 minutes in the dark and then adding 1 mL of de-ionized water. The Caltag counting beads (PCB-100, Invitrogen, Carlsbad, CA) were added for cell enumeration prior to analysis in FACSCalibur (Becton Dickinson Biosystems, San Jose, CA). Samples were acquired through a live gate without compensation. After collecting 3500 counting beads, the data of all cells were exported to analysis software, FlowJo (Treestar, Costa Mesa, CA). The data were then analyzed according to the following gating strategy. First, leukocytes were separated by side scattering and forward scattering into 3 gates: lymphocytes, monocytes, and eosinophils + neutrophils. Lymphocytes were identified by FSC/SSC and expressing CD3 or B220. B cells were distinguished from T cells by MHC-II expression in lymphocytes gate. Eosinophils were defined as cells expressing the cotaxin receptor CCR3. Neutrophils were identified as cells expressing myeloid differentiation antigen Gr-1 and lack of CCR3 expression. Monocytes were identified by FSC/SSC and expressing Gr-1.

### Flow Cytometry: Bronchoalveolar Lavage Cells Differentiation and Quantification

BAL cell differentiation was done according to Stevens et al. [20] with some modifications. BAL cells were re-suspended in 250  $\mu\text{L}$  PBS, and 100  $\mu\text{L}$  of the suspension was added into a flow cytometry tube with 100  $\mu\text{L}$  of 10% rat serum in FACS buffer for 10 minutes. Then 50  $\mu\text{L}$  of pre-mixed antibodies in FACS buffer was added and cells were stained for 30 minutes at room temperature on a shaker. The mixture contained the final concentration of 5  $\mu\text{g/mL}$  of the following antibodies: Fc block, Ly6G-FITC, Siglec-F-PE, CD45-PerCP, and CD11c-APC. All the antibodies were purchased from PharMingen. The Caltag

counting beads (PCB-100, Invitrogen, Carlsbad, CA) were added for cell enumeration prior to analysis in FACSCalibur. Samples were acquired through a live gate without compensation. After collecting 4000 counting beads, the data of all cells were exported to the analysis software, FlowJo. The leukocytes were identified by cells expressing CD45+. Neutrophils were identified as cells expressing CD45+Ly6G+. Eosinophils were identified as cells expressing CD45+Siglec-F+ and macrophages were defined as cells expressing CD45+CD11c+.

### Biochemical Parameters of Injury

Albumin content and LDH activity were measured in the acellular, first fraction of BAL fluid. These measures reflect the permeability of the bronchoalveolar-capillary barrier and general cytotoxicity, respectively. Albumin content was determined colorimetrically at 628 nm based on albumin binding to bromocresol green using an albumin BCG diagnostic kit (Sigma-Aldrich Co., St. Louis, MO). LDH activity was determined by measuring the reduction of lactate to pyruvate coupled with the formation of NADH at 340 nm. Measurement was performed with a COBAS MIRA auto-analyzer (Roche Diagnostic System, Montclair, NJ).

### BAL Fluid Cytokines

Analysis of cytokines in the acellular BAL fluid was conducted using a Mouse Th1/Th2 Cytokine Kit (BD Biosciences, San Diego, CA) and was analyzed on a FACSCalibur flow cytometer. Standard curves were determined for each cytokine, ranging from 20 pg/mL to 5000 pg/mL. The lower limit of detection of the assay was 2.5–6.3 pg/mL, depending on the cytokine. The following cytokines were measured: interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5), interferon- $\gamma$  (IFN- $\gamma$ ), and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ). Only the cytokines (e.g., IL-5, TNF- $\alpha$ ) that had a significantly different response from the OVA–OVA group are presented.

### Lung Histopathology

A separate set of non-lavaged BALB/c mice ( $n = 4$ /group) from each treatment group were reserved for lung histopathology analysis. The lungs were inflated and fixed with 10% neutral buffered formalin for a minimum of 24 hour. The left lung lobes were embedded as individual lobes in paraffin, and then a 5  $\mu$ m standard section was cut. Slides were stained with hematoxylin and eosin and interpreted by a contracted board certified veterinary pathologist

in a blinded fashion for morphological changes related to lung allergy, injury, and inflammation. If abnormal changes were found, severity was scored as follows: 0 = normal (change not present), 1 = minimal, 2 = mild, 3 = moderate, 4 = marked, and 5 = severe.

### Statistical Analysis

All data are presented as means  $\pm$  standard error (SE) of measurement. Differences were considered statistically significant at  $P < 0.05$ . Statistical analyses for Figures 2–6 were performed using SigmaStat v3.11 software (Systat Software, Inc., Chicago, IL). An analysis of variance (ANOVA) with the Student-Newman-Keuls post-hoc test was used for comparing the different treatment groups. The statistical analyses for the lung histopathology scores (data not shown) were conducted using SAS/STAT software, Version 9.1, of the SAS System for Windows (SAS Institute, Inc., Cary, NC). Histopathology severity scores were analyzed using an omnibus Kruskal-Wallis nonparametric test followed by pairwise comparisons using the Wilcoxon rank-sum test.

## RESULTS AND DISCUSSION

The objective of the current study was to evaluate the adjuvant effect of zymosan, a crude yeast cell wall preparation containing  $\beta$ -glucan, during sub-optimal lung OVA sensitization in a mouse allergy model. Multiple studies have shown that different preparations or extracts of 1 $\rightarrow$ 3- $\beta$ -glucan have potential adjuvant effects in mouse models of allergy. Ormstad *et al.* [15] demonstrated that a crude  $\beta$ -glucan suspension extracted from barley enhanced a Th2-dependent antibody response to OVA, as indicated by increased levels of IgE and IgG1 compared to OVA. In a related investigation, Instanes *et al.* [16] observed that soluble extracts of different molds had adjuvant effects on the allergic response to OVA in mice. Wan *et al.* [14] utilized an airway OVA mouse allergy model by which animals were sensitized by an aerosolized crude  $\beta$ -glucan extract and OVA. They observed that the crude  $\beta$ -glucan preparation induced Th2-mediated responses by abrogating inhalation-induced IgE down-regulation and promoting airway eosinophil infiltration to inhaled antigen. In contrast, using a similar design by which animals were co-exposed to OVA by inhalation, Rylander and Holt [21] showed that a crude  $\beta$ -glucan suppressed OVA-induced antibody formation and eosinophilia, as well as abolished the stimulation

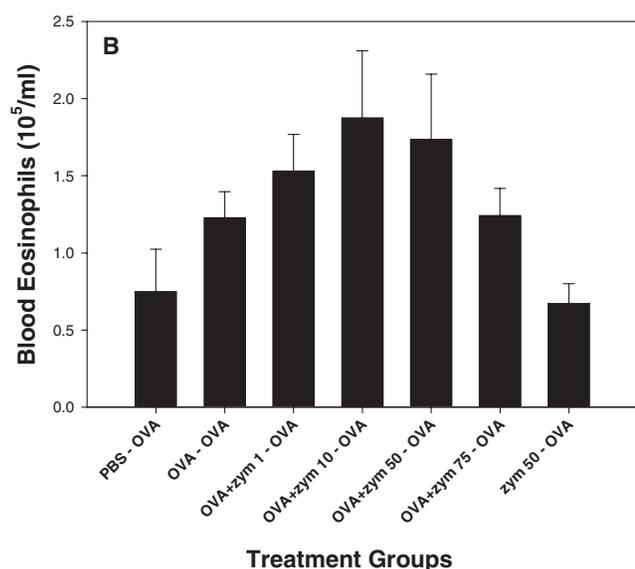
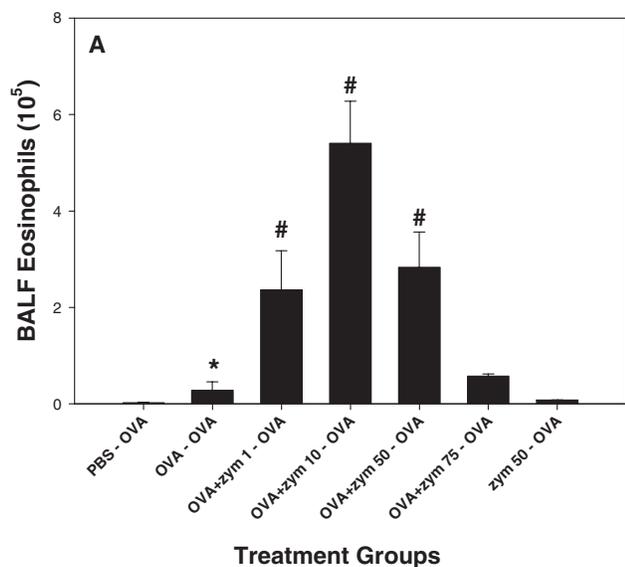


FIGURE 2. Allergic response: (A) lung eosinophils, and (B) blood eosinophils recovered from the bronchoalveolar lavage fluid (BALF) and blood at 1 day after the final treatment. Values are means  $\pm$  SE of 8 mice per exposure group; \* indicates significant increase ( $P < 0.05$ ) versus the PBS-OVA group; # indicates a significant increase ( $P < 0.05$ ) versus the OVA-OVA group.

of OVA-induced antibody production by endotoxin (lipopolysaccharide).

Multiple parameters of lung allergy, inflammation, and injury were examined in the blood and BAL fluid of mice from the different treatment groups 1 day after the final OVA challenge on day 29 of the dosing regimen in the current study (Figure 1). Eosinophil influx into the lungs, indicative of a local allergic response, indicated that a suboptimal level of

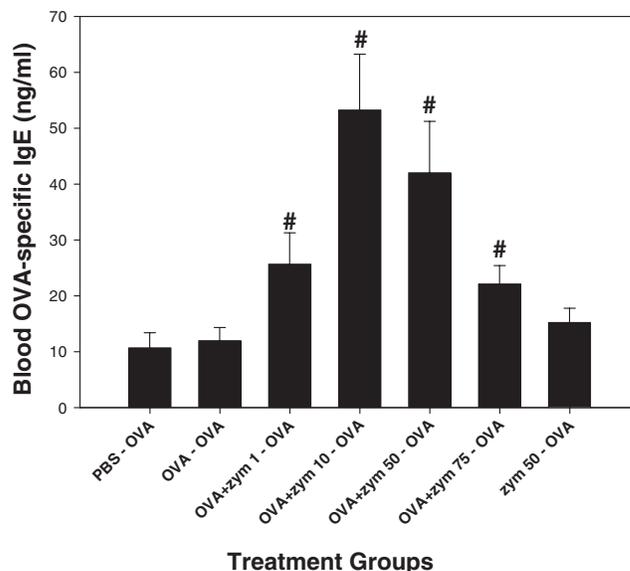


FIGURE 3. OVA-specific IgE levels in the blood serum. Values are means  $\pm$  SE of 8 mice per exposure group; # indicates a significant increase ( $P < 0.05$ ) versus the OVA-OVA group.

sensitization was achieved as the OVA-OVA group was significantly elevated above the PBS-OVA group (Figure 2A). An adjuvant effect of zymosan on OVA allergy also was observed as evidenced by significant elevations in lung eosinophils in the OVA+zym-OVA groups compared to the OVA-OVA group

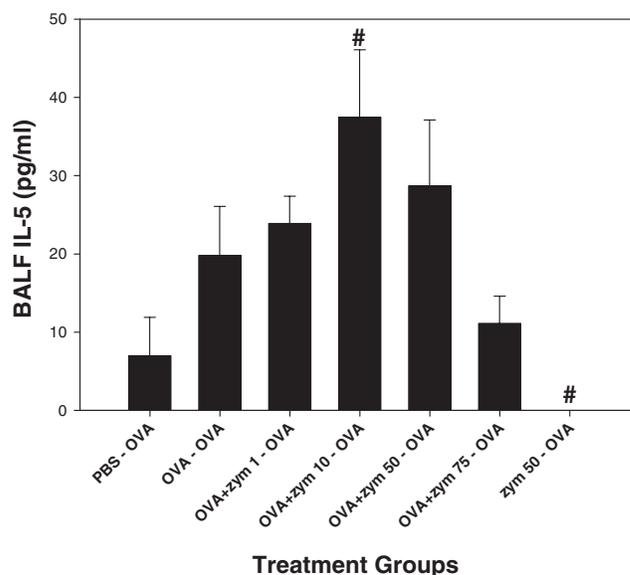


FIGURE 4. Pro-allergic cytokine: IL-5 in the acellular bronchoalveolar lavage fluid (BALF) at 1 day after the final treatment. Values are means  $\pm$  SE of 8 mice per exposure group; # indicates a significant difference ( $P < 0.05$ ) versus the OVA-OVA group.

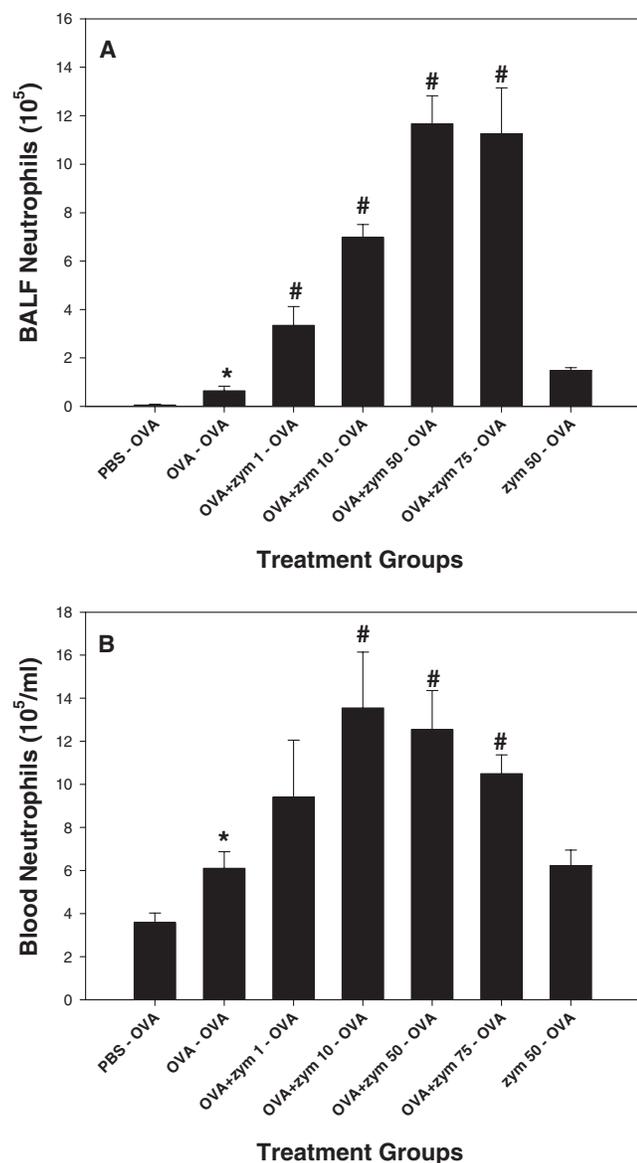


FIGURE 5. Inflammatory neutrophilic response: (A) lung neutrophils, and (B) blood neutrophils recovered from the bronchoalveolar lavage fluid (BALF) and blood at 1 day after the final treatment. Values are means  $\pm$  SE of 8 mice per exposure group; \*indicates significant increase ( $P < 0.05$ ) versus the PBS-OVA group; #indicates a significant increase ( $P < 0.05$ ) versus the OVA-OVA group.

(Figure 2A). In regards to the systemic response, the adjuvant effect was not as dramatic. The combination of OVA and zymosan priming in the lungs did not cause a statistically significant increase in peripheral blood eosinophil numbers compared to the OVA-OVA group (Figure 2B).

Further evidence for an adjuvant effect of zymosan on OVA allergy was observed in that serum OVA-specific IgE was significantly increased at all concentrations in the OVA+zym-OVA groups compared to

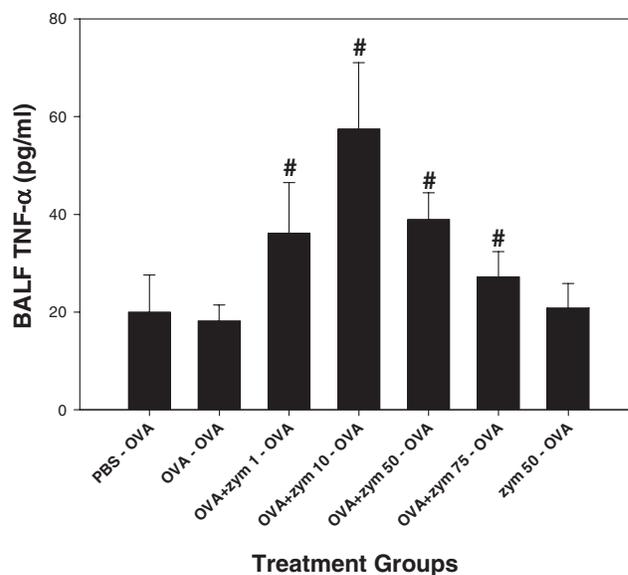


FIGURE 6. Pro-inflammatory cytokine: TNF- $\alpha$  in the acellular bronchoalveolar lavage fluid (BALF) at 1 day after the final treatment. Values are means  $\pm$  SE of 8 mice per exposure group; #indicates a significant increase ( $P < 0.05$ ) versus the OVA-OVA group.

OVA-OVA group (Figure 3). A similar trend was observed when evaluating the BAL fluid concentrations of IL-5 (Figure 4). Allergy in both humans and animal models has been shown to be caused by polarization of CD4<sup>+</sup> T-helper lymphocytes to the Th2 subclass [22]. IL-5 is a marker for the Th2 phenotype and has an important role in allergy. IL-5 is involved in the differentiation and maturation of eosinophils and in the migration of eosinophils to tissue sites in the presence of an allergic response and has been identified across animal models as being a key common denominator in allergic inflammatory pathways [23–25].

The current study differs from similar previous animal studies in that zymosan, an insoluble, particulate form of crude  $\beta$ -glucan, was shown to have a strong adjuvant effect. Ormstad *et al.* [15] indicated that the adjuvant effect of an insoluble  $\beta$ -glucan had a much less pronounced adjuvant effect than a soluble form of  $\beta$ -glucan. Our group has previously demonstrated that insoluble crude  $\beta$ -glucan, but not soluble crude  $\beta$ -glucan, induced the majority of pulmonary inflammation, injury, and irritation (e.g., increased breathing frequency) in an animal model [26]. Also, the results of the current study differ from the findings of the investigation by Rylander and Holt [21]. The reasons for the contradictory results may be due to the dose levels and pulmonary treatment methods used in the studies. In the current study, a pulmonary aspiration dose, 1–75  $\mu$ g of zymosan, was used compared to an inhalation dose of 8  $\mu$ g/m<sup>3</sup> for

4 hour/day for 5 days/week for 5 weeks in the study of Rylander and Holt [21]. The rationale for the doses chosen in the current study was based on the fact that no response was observed below 1  $\mu\text{g}$  pharyngeal aspiration dose of zymosan in preliminary experiments. It is important to note that realistic environmental exposures range from 1 ng/kg to 5 ng/kg body weight [27].

It has been reported that zymosan has distinct biological activities compared to purified yeast particulate 1 $\rightarrow$ 3- $\beta$ -glucan [13]. A recent study by Huang et al. [28] indicated that the adjuvant activity of purified 1 $\rightarrow$ 3- $\beta$ -glucan led to a Th1/Th17 response, and not the Th2 response as reported in this paper and by others [14–16] for zymosan, further differentiating the activities of 1 $\rightarrow$ 3- $\beta$ -glucan from zymosan. It was observed that OVA-loaded purified  $\beta$ -glucan particles induced strong humoral and Th1- and Th17-dependent CD4+ T-cell responses in mice. This difference in immune response may be due to the fact that zymosan also is composed of a significant amount of mannan, which is a highly immunogenic polysaccharide. Mannan is an important fungal cell wall antigen responsible for the specificity of different serologic reactions and induction of humoral responses

[29, 30]. Because of the presence of mannan, chitin,  $\beta$ -glucan, and other components of zymosan, a study is needed that examines the influence these other fungal cell wall components individually have on the allergy/adjuvant dose response observed in the current study.

Evidence of an adjuvant effect of zymosan on inflammation also was observed by significant elevations in lung neutrophils (Figure 5A), blood neutrophils (Figure 5B), and BAL fluid concentrations of TNF- $\alpha$  (Figure 6) compared to the OVA–OVA group. In addition, OVA and zymosan priming caused a significant increase in lung injury as determined by LDH activity and albumin concentrations in the BAL fluid (data not shown). Previously, our group has shown that pulmonary exposure to particulate zymosan increased lung neutrophil influx, LDH activity, and alveolar macrophage activation [26]. Others have shown that different lung pro-inflammatory agents, such residual oil fly ash [31, 32], diesel exhaust particles [33], ambient particulate matter [34, 35], and house dust mites [36] have adjuvant effects in animal respiratory allergy models.

The lung histopathologic changes in the current study were consistent with an allergic response

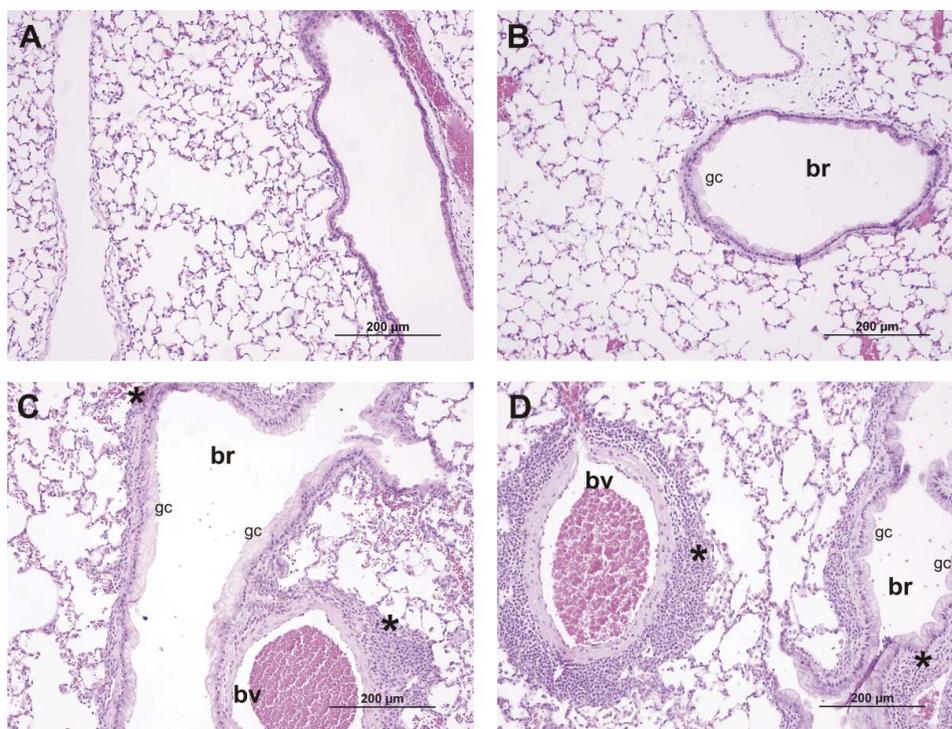


FIGURE 7. Lung histopathology: (A) Mice were sensitized with zymosan (50 mg) then challenged with OVA. (B) Mice were sensitized with OVA then challenged with OVA. (C) Mice were sensitized with OVA + zymosan (10  $\mu\text{g}$ ) then challenged with OVA. (D) Mice were sensitized with OVA + zymosan (50  $\mu\text{g}$ ) then challenged with OVA. Micron bar = 200  $\mu\text{m}$ ; br = bronchiole; bv = blood vessel; gc = goblet cell; \* = leukocyte infiltration.

after OVA challenge and included perivascular, peribronchiolar, and alveolar eosinophilic infiltrates, as well as goblet cell hyperplasia. Minimal to mild intimal changes were noted in pulmonary vessels in many mice, associated with surrounding inflammation. These changes included intimal thickening, endothelial cell hypertrophy, and leukocyte infiltration, probably due to emigration of leukocytes. In the mice primed with 50 mg of zymosan only then challenged with OVA, the bronchiolar epithelium was normal and there was no eosinophil or lymphocyte infiltration around either the vessel on the left or the one on the right (Figure 7A). In the group primed with OVA then challenged with OVA, the bronchiole epithelium is shown to be lined with an increase in goblet cells (Figure 7B). The surrounding alveoli appeared empty and free of leukocytes.

However, in mice primed with OVA + zymosan (10  $\mu\text{g}$ ) then challenged with OVA, the bronchioles were lined almost entirely by goblet cells (e.g., goblet cell hyperplasia) and surrounded by a mild infiltration of leukocytes (Figure 7C). Leukocyte infiltration was present around a blood vessel, in thickened alveolar septa, and within alveolar lumina. In the group primed with OVA + zymosan (50  $\mu\text{g}$ ) then challenged with OVA, bronchioles were observed to have diffused goblet cell hyperplasia, and there were thick band of lymphocytes and eosinophils that encircled pulmonary vessels (Figure 7D). In addition, alveolar septa were thickened and contained increased numbers of macrophages and eosinophils. Similar changes in lung architecture and lung cell and inflammatory responses previously have been observed after exposure to residual oil fly ash particles [37] and urban air particulate matter [38] in an allergic pulmonary inflammation model.

In ranking the severity of the histopathologic changes, statistically significant increases were observed for perivascular lymphocyte infiltration in the OVA+zym 10–OVA, OVA+zym 50–OVA, and OVA+zym 75–OVA groups compared to the OVA–OVA group (data not shown). In addition, the severity of alveolar neutrophil–eosinophil and histiocytic infiltrates was significantly increased in the OVA+zym 10–OVA group compared to the OVA–OVA group. Peribronchiolar lymphocyte infiltration was significantly increased, in terms of severity, in the OVA+zym 75–OVA group compared to the OVA–OVA group.

In most cases, the changes in lung histopathology followed the same non-linear, bell-shaped zymosan dose–response relationship as the BAL parameters of allergy, inflammation, and injury for the OVA+zym–OVA groups. For every parameter except lung neutrophils, the response peaked in the group

primed with OVA + 10  $\mu\text{g}$  zymosan with the response returning towards OVA–OVA levels in the group primed with OVA + 75  $\mu\text{g}$  zymosan. A phenomenon that is common in allergy studies has been the observation that greater adjuvant responses occur at lower doses compared to higher doses. Instanes *et al.* [16] indicated that the adjuvant effect of mold extracts was most pronounced at the lowest dose (12.5  $\mu\text{g}$ ) examined, which was comparable to the peak adjuvant dose of 10  $\mu\text{g}$  of zymosan in the current study.

Human studies also have observed a similar dose–response adjuvant effect. A significant, non-linear relationship between endotoxin exposure and a number of common allergens was seen in adult pig farmers as the risk of sensitization was strongly decreased with increasing exposure to endotoxin [39]. Schram-Bijkerk *et al.* [40] observed similar findings in house dust mites and mite sensitization in farming and non-farming children. Interestingly, exposure to high levels of crude sources of  $\beta$ -glucans was observed to be associated with decreased risk for recurrent wheezing among infants [41, 42], indicating that  $\beta$ -glucan exposure may protect against asthma development. This protective effect is believed to be due to microbial exposure early in life and development of tolerance.

## CONCLUSIONS

In the current study, we assessed the potential for particulate fungi and yeast, as tested using zymosan, for the observed adjuvant activity during the sensitization phase in a mouse model of asthma. This response was characterized by increased eosinophils in the lungs as well as elevations in IgE and IL-5 levels after sensitization with a combination of zymosan and OVA compared to sensitizing with OVA alone. Pulmonary treatment with zymosan also amplified lung inflammation and injury in the OVA allergy model. Elevations were observed in lung neutrophils, TNF- $\alpha$ , and biochemical parameters of lung injury in the groups primed with both zymosan and OVA. In nearly all parameters assessing lung allergy and inflammation, a non-linear dose–response relationship was observed in the groups primed with a combination of OVA and different doses of zymosan, a phenomenon that is commonly observed in the assessment of allergic responses. A study is needed that examines the influence of other fungal cell wall components, such as mannan and chitin as well as purified  $\beta$ -glucan, on the allergy/adjuvant response to OVA observed in the current study. The findings from this study may be useful in evaluating the pulmonary

allergic and asthmatic responses observed in non-sensitized and sensitized individuals who live or work in areas that are contaminated with dusts that contain yeast and molds with high levels of crude  $\beta$ -glucans.

## ABBREVIATIONS

ANOVA	Analysis of variance
BAL	Bronchoalveolar lavage
BALF	Bronchoalveolar lavage fluid
br	Bronchiole
bv	Blood vessel
gc	Goblet cell
IL	Interleukin
LDH	Lactate dehydrogenase
OVA	Ovalbumin
PA	Pharyngeal aspiration
PBS	Phosphate-buffered saline
SE	Standard error of measurement
zym	Zymosan

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