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Pulmonary Exposure to 1 → 3-β-Glucan Alters Adaptive Immune Responses in Rats

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1 → 3-β-Glucans have been associated with increased pulmonary inflammation in fungal-related indoor air problems. Epidemiological studies have shown a correlation between increases in T-cell proliferation and decreases in CD4+/CD8+ ratio after exposure to fungi. The objective of the present investigation was to determine the mechanisms by which 1 → 3-β-glucans affect immune responses using an animal model. Rats received a single dose of zymosan A (2.5 mg/kg body weight) via intratracheal instillation (IT) and were euthanized on days 1, 4, 6, 8, and 10 post IT. Bronchoalveolar lavage was performed at each time point post-IT. Inflammation and lung injury were assessed by measuring neutrophil infiltration into bronchoalveolar lavage fluid (BALF) and by measuring albumin and lactate dehydrogenase levels in BALF, respectively. Alveolar macrophage activation was determined by chemiluminescence. Immune response was characterized via immunophenotyping of bronchoalveolar lavage cells and lymphocytes isolated from the lung-associated lymph nodes. Upon challenge with zymosan, rats exhibited increased inflammation and injury at early time points (days 1 and 4) post IT exposure. Although elevations in neutrophil infiltration and chemiluminescence had returned to control levels on day 4, lymphocytes recovered from lung-associated lymph nodes continued to proliferate and reached a maximum on day 6. The CD4+/CD8+ T cell ratio from lymph nodes was lower in zymosan-treated rats than in control rats. Zymosan treatment increased tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10, and IL-12p70 secretion in BALF on day 1. In summary, rats exposed to zymosan had an increase in acute inflammation, and the altered lymphocyte profiles were consistent with the findings of epidemiology studies.

Mold and fungi have long been linked to respiratory problems in many occupations and environments. (Douwes et al., 2000; Fogelmark et al., 1992; Rylander, 1985, 1994, 1999a, 1999b; Rylander & Fogelmark, 1994; Rylander et al., 1992, 1994, 1999; Thorn et al., 1998; Urbain et al., 1999; Wan et al., 1999; Zejda & Dosman, 1993). The symptoms may include headache, wheeze, chest tightness, throat irritation, shortness of breath, cough and fatigue. 1 → 3-β-Glucans are major cell wall components of fungal spores and have been considered as a biomarker for fungi exposure in the workplace (Rylander, 1997).

Recently, epidemiological studies have shown a correlation between an alteration of immune marker expression and exposure to fungi (Beijer et al., 2003; Dales et al., 1998; Shin et al., 2004). Dales et al. (1998) found that children who lived in fungi-contaminated homes had an increase in T-cell proliferation and a reduced CD4+/CD8+ T-cell ratio when compared to those that lived in less contaminated homes. In contrast, a similar study examining lymphocyte subsets of peripheral blood mononuclear cells (PBMCs) in adults exposed to a higher 1 → 3-β-glucan concentration did not find a similar increase of cytotoxic CD8+ number compared to those exposed to a lower 1 → 3-β-glucan concentration (Beijer et al., 2003). A more recent study demonstrated an enhanced immune response to ubiquitous fungi in chronic rhinosinusitis patients (Shin et al., 2004). These investigators found that interleukin (IL)-5, IL-13, and interferon (INF)-γ were elevated in PBMCs from chronic rhinosinusitis patients when cocultured with a common fungi, *Alterneria* (Shin et al., 2004). These studies suggest that exposure to 1 → 3-β-glucans alters immunophenotyping and cytokine production by cells, but many questions remain about the mechanisms by which 1 → 3-β-glucans affect pulmonary immune responses.

In our previous studies, we have demonstrated that exposure to zymosan A, a particulate form of 1 → 3-β-glucan from

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The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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Saccharomyces cerevisiae, could induce pulmonary inflammation in rats (Young & Castranova, 2005; Young et al., 2001a, 2002, 2003a, 2003b). Briefly, zymosan elevated lung damage indicators and increased macrophage activation. The goal of the present study was to characterize the pulmonary immune response to zymosan in rats by examining lymphocyte population (lung-associated lymph nodes) and cytokine production by bronchoalveolar lavage cells and lymph node cells. We hypothesized that exposure to zymosan would alter the adaptive immune response in the lung of rats.

MATERIALS AND METHODS

Animals

Specific-pathogen-free male Sprague-Dawley [H1a: (SD) CVF] rats approximately 7–8 wk old at arrival (∼225–250 g) were purchased from Hilltop Lab Animals (Scottdale, PA). The animals were housed in a room with restricted access under temperature- and humidity-controlled condition and HEPA-filtered air. Rats were allowed to acclimate in an AAALAC-approved animal facility for 1 wk before use. The rats were maintained on ProLab 3500 diet and tap water ad libitum. Alpha-Dri virgin cellulose chips and hardwood Beta-chips were used as bedding.

Experimental Design

At day 0, male Sprague-Dawley rats, ∼8–9 wk old, were intratracheally instilled with either saline (control) or zymosan at a dose of 2.5 mg/kg body weight. At days 1, 4, 6, 8, and 10 after instillation, bronchoalveolar lavage was performed on the right lung. The cells recovered were differentiated, and chemiluminescence (CL), a measure of macrophage function and production of reactive oxygen species, was determined. Albumin and lactate dehydrogenase (LDH) activity, indicators of lung injury, were measured in the acellular bronchoalveolar lavage fluid (BALF). At the same time points, the left lungs were inflated with 10% buffered formalin and preserved. The lobes of the left lungs were embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). The lung-associated lymph nodes were removed and lymphocytes were recovered. Immunophenotyping was performed on the lymphocyte suspension and bronchoalveolar lavage cells via flow cytometry. The cytokines in the BALF and lymphokines from cultured lymphocytes were also determined by using enzyme-linked immunosorbent assay (ELISA) kits. The following cytokine levels from the first fraction of bronchoalveolar lavage (BAL) were determined: tumor necrosis factor (TNF)-α, IL-2, IL-4, IL-6, IL-10, IL-12p70 on days 1, 4, 6, 8, and 10 post IT. The levels of IL-2, IL-4, IL-10, IL-12p70, and IFN-γ were determined for lymphocytes, cultured supernatants.

Zymosan Treatment

Zymosan A was purchased from Sigma Chemical Company (St. Louis, MO). On day 0, rats were lightly anesthetized by an intraperitoneal injection of 0.6 ml of a 1% (w/v) solution of sodium methohexital (Brevital; Eli Lilly, Indianapolis, IN) and intratracheally instilled with zymosan at a dose of 2.5 mg/kg body weight or saline as a vehicle control. The selection of this optimal zymosan dose was based on a previous study (Young et al., 2001a) demonstrating a dose response where 2.5 mg/kg body weight was the concentration that induced a significant difference in measurable inflammation and injury (LDH and albumin).

Bronchoalveolar Lavage and Cell Differentials

At 1, 4, 6, 8, and 10 days after IT, the rats were anesthetized with an overdose of sodium pentobarbital and then exsanguinated by severing the abdominal aorta. The left bronchus was clamped off, and bronchoalveolar lavage was performed on the right lungs of rats. The lungs were first lavaged with 1 ml/100 g body weight (BW) of Ca^{2+}- and Mg^{2+}-free phosphate-buffered solution (PBS) at pH 7.4. The first lavage fluid remained in the lung for 30 s with constant massaging of the lung and was repeated twice. This first fraction of BALF was centrifuged at 500× g for 10 min and supernatant used for analysis lactate dehydrogenase (LDH), albumin and cytokines. The lung was further lavaged with 6-ml aliquots of PBS until a total of 30 ml BALF was collected. These samples were also centrifuged for 10 min at 500× g and the cell pellets from all washes for each rat were combined and used for cell differentials and for phenotyping. Total cell number was determined with a Coulter Multisizer II (Coulter Electronics, Hialeah, FL), and 1×10^5 cells were spun for 5 min at 72× g and pelleted onto a slide using a cytospin (Shandon Cytospin III, Shandon, Inc., Pittsburgh, PA). Cells (200 cells/rat) were differentiated into alveolar macrophages, lymphocytes, eosinophils, and neutrophils on cytocentrifuge-prepared slides after staining with Leukostat stain (Fisher Scientific, Pittsburgh, PA).

Lung-Associated Lymphocyte Harvest and Culture

Lung-associated lymph nodes were harvested in a 15-ml conical tube that contained 1 ml RPMI 1640 medium (Sigma) with 2 m*M* glutamine, 100 μg/ml. streptomycin, 100 U/ml penicillin, 5×10^{-5} *M* 2-β-mercaptoethanol, 5 m*M* HEPES and 10% heat-inactivated FBS (Sigma). A Teflon fluorocarbon resin and stainless-steel shaft pestle (Fisher Scientific, Pittsburgh, PA, catalog number 05-559-26) were used to gently release lymphocytes into medium. Aliquots of 1.5-ml cell suspensions adjusted to 6×10^6 lymphocytes were added to each well of a 24-well tissue culture plate (Becton Dickinson, Franklin Lakes, NJ) and incubated (37°C and 5% CO_2) for 24 h with or without concanavalin A (Con A, Sigma, St. Louis, MO) at a concentration of 3.3 μg/ml. The lymphocyte-conditioned media was collected and centrifuged (834 × g for 5 min) and aliquots of the supernatants were stored at −80°C until assayed.

Immunophenotyping and Flow Cytometry

Immunophenotyping was performed on lymphocyte suspensions via flow cytometry. Briefly, 50 μl of lymphocyte suspensions (∼0.5×10^6 cells) was added to a 12×75 mm polystyrene

tube on ice. A blocking buffer (100 μl) containing 300 μg/ml mouse immunoglobulin G (IgG) was added to this suspension for 10 min. Anti-CD3 and anti-CD45R were used for T- and B-cells enumeration, respectively. 7-Aminoactinomycin D (7-AAD) and monoclonal antibody such as anti-CD3, anti-CD4, anti-CD8a, anti-CD45R, and NKR-P1A were purchased from Becton Dickinson (San Diego, CA). Appropriate amounts of antibody for different cell membrane surface markers were prepared in FACS buffer (PBS with 0.2% bovine serum albumin [BSA] and 0.09% NaN_3) and added to a tube (50 μl/tube) for 30 min on ice. Each antibody was titrated for optimum amount of antibody. Each tube was then washed 1× with 1 ml FACS buffer to remove supernatant. The cells were then fixed with 1% paraformaldehyde (concentration at final) and analyzed on FACSCalibur (flow cytometer, BD Biosciences, San Diego, CA). Two panels of three- or four-color analysis were set up for immunophenotyping: CD4-FITC/NKR-P1A-PE/CD8-PerCP/CD3-APC and CD45R-FITC/CD3-PE/7-AAD. Flow data was expressed as percent of positive stained cells. The live lymphocyte population was selected based on forward-scatter and side scatter light signal intensity, and was identified by 7-AAD staining. In total, 10,000 events were collected per sample.

Biochemical Parameters of Injury

The albumin content and LDH activity in the acellular first fraction of BALF were measured. 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 bromcresol green using an albumin BCG diagnostic kit (Sigma, 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 autoanalyzer (Roche Diagnostic Systems, Montclair, NJ).

Alveolar Macrophage Chemiluminescence

Luminol-dependent chemiluminescence (CL) was performed on bronchoalveolar lavage cells as a measure of the light generated by reactive species produced by activated alveolar macrophages. CL was performed with an automated Berthold Autolumat LB 953 luminometer (Wallac, Inc., Gaitherburg, MD) at 390–620 nm for 15 min. Nonopsonized, insoluble zymosan (2 mg/ml, Sigma Chemicals Co., St. Louis, MO) was used as a stimulant of alveolar macrophages (5×10^5 macrophages per test as calculated from the Coulter count) and was added to the assay immediately prior to measurement of CL. Resting CL was determined by incubating 5×10^5 macrophages at 37°C for 10 min in 0.008 mg/dl (weight/volume) luminol in a total of 0.5 ml of 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid buffer followed by the measurement of CL for 15 min. Zymosan-stimulated CL was calculated as the counts per minute (cpm) in the zymosan-stimulated assay minus the cpm in the resting assay.

BALF and Lymphocyte Cytokines

Cytokine protein concentrations were determined with enzyme-linked immunosorbent assay (ELISA) kits from Biosource International (Camarillo, CA). The results of this colorimetric assay were obtained with a Spectramax 250 plate spectrophotometer using Softmax Pro 2.6 software by (Molecular Device Corp., Sunnyvale, CA). The following cytokine levels from the first fraction of BALF were determined: TNF-α, IL-2, IL-4, IL-6, IL-10, and IL-12p70 on days 1, 4, 6, 8, and 10 post IT. The levels of IL-2, IL-4, IL-10, IL-12p70, and IFN-γ were determined for lymphocytes, cultured supernatants on days 1, 4, 6, 8, and 10 post IT.

Measurement of Nitric Oxide

The presence of nitric oxide in acellular BAL was determined as the accumulation of nitrite using a modified microplate assay using the Greiss reagent (Green et al., 1982). Briefly, total nitrite was measured after 1 h of incubation with a nitrate reductase to convert any nitrate in the sample to nitrite. The microplate was then centrifuged, and the supernatant was transferred to another microplate to stop the enzymatic reaction and incubated with the Greiss reagent (Molecular Probes, Eugene, OR) for 10 min. The absorbance of the samples was analyzed on a SPECTRAmax 250 spectrophotomter (Molecular Devices Co., Sunnydale, CA) at 550 nm. The measurement of total nitrite represents the presence of both nitrate and nitrite (NOx) in the sample.

Statistical Analysis

All data are presented as means ± standard error of measurement (SEM). Statistical analysis was performed using SigmaStat v3.11 software (Systat Software, Inc.). The significance was set at $p \leq .05$. A paramatic t-test was used to compare zymosan-treated group with saline or zymosan + Con A with saline + Con A at each time point. For multiple comparisons, a two-way analysis of variance (ANOVA) with Student–Newman–Keuls procedure method was used.

RESULTS

Pulmonary Inflammation, Cytotoxicity and Lung Injury

LDH Activity and Albumin

Figure 1A ($n = 8$–12) shows that LDH activity was elevated approximately two- to threefold in the zymosan-treated group at days 1, 4, and 6 post zymosan IT. LDH activity level then decreased over time in the zymosan-treated group, which suggests a recovery from pulmonary injury. On days 8 and 10, there was no significant difference from control rats. Zymosan-treated rats had approximately three- to fourfold higher albumin level than that of control at days 1, 4, 6, and 10 post IT (Figure 1B).

BALF Cell Differential

Zymosan induced local inflammation and recruitment of inflammatory cells in lung. The results showed that on day 1, a

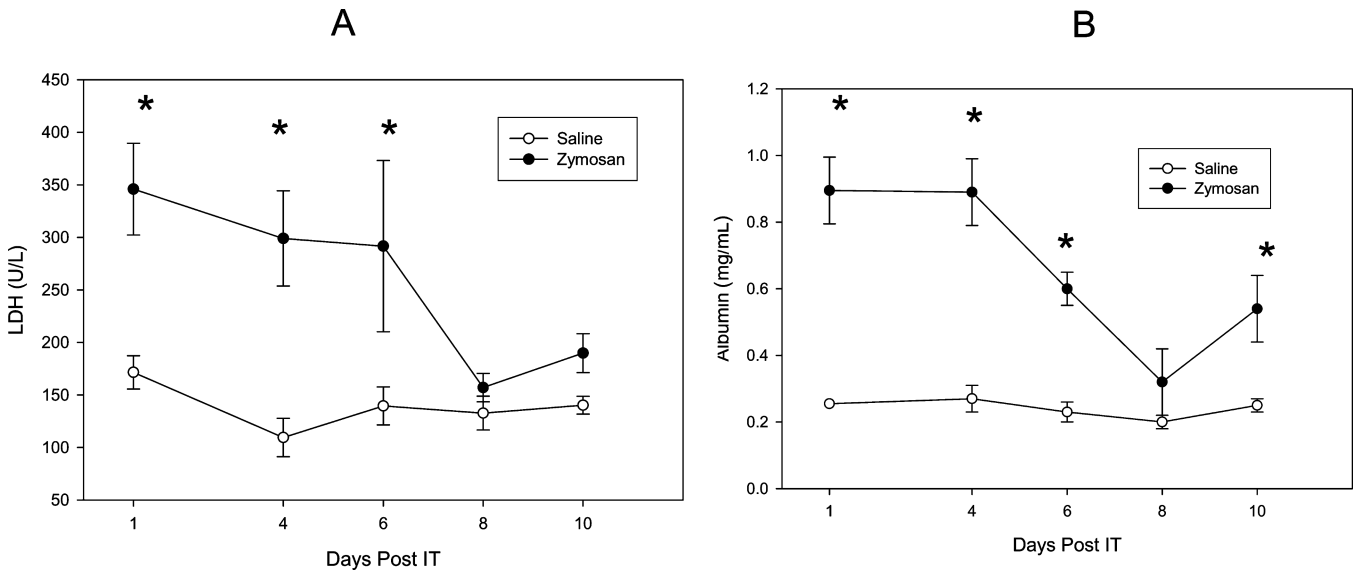

FIG. 1. Lactate dehydrogenase (LDH) activity (A) and albumin concentrations (B) on different days post IT. Values are means ± SEM of 8–12 rats per exposure group. Asterisk indicates a significant increase versus the saline control level ($p < .05$).

4.5-fold increase of total cells recovered by BALF was observed in the zymosan-treated group (Table 1). At later time points (days 4, 6, 8, and 10), total cell counts were approximately 2.5- to 3-fold higher in the zymosan group.

PMN infiltration has been shown to have a dose-response relationship with zymosan (Young et al., 2001a). In this study, zymosan induced a massive neutrophil infiltration on day 1 (Table 1). In the saline-treated control group, 99% of bronchoalveolar lavage cells were alveolar macrophages (AM). In comparison, on day 1, the zymosan-treated group only had 28.7% AM, but had significantly more neutrophils (67.1%). In addition, the zymosan-treated group also had a small, but measurable, percentage of lymphocytes (1.1%) and eosinophils (3%) on day 1. On day 4, neutrophil infiltration rapidly decreased in the zymosan-treated group. However, there were still differences in neutrophil counts between saline-treated and zymosan-treated groups for days 6, 8, and 10.

Histology

Histological staining was done on the left lungs of saline and zymosan-treated groups at days 1 and 4 (Figure 2). Lungs appeared normal for the saline group on days 1 and 4 (Figures 2A and C); however, the lungs of the zymosan-treated animals displayed inflammation with increased airway thicking, as well as a significant infiltration of neutrophils (Figures 2, B and D).

Immunophenotyping : BALF + Lymph-Node Lymphocytes

Table 2 shows the immunophenotyping of bronchoalveolar lavage cells. Each phenotype cell number was calculated by using value = (percent positive) × (total cell count). Zymosan treatment induced a significant ($p < .05$) increase in both CD3+CD4+ and CD3+CD8+ T cells, B cells (CD45R+), and NK cells (NKR-P1A+CD3−) in recovered BALF cells at all time points compared to the saline-treated group.

TABLE 1
Bronchoalveolar lavage cell differentials

	Total cells (10^6)		Macrophages (10^6)		Neutrophils (10^6)	
Time	Saline	Zymosan	Saline	Zymosan	Saline	Zymosan
Day 1	9.64 ± 0.66	46.42 ± 9.11[a]	9.60 ± 0.65	11.45 ± 1.77	0.02 ± 0.01	33.42 ± 7.75[a]
Day 4	9.38 ± 0.64	23.91 ± 2.79[a]	9.36 ± 0.64	21.19 ± 2.71[a]	0.01 ± 0.01	1.54 ± 0.29[a]
Day 6	10.26 ± 0.75	36.90 ± 2.43[a]	10.20 ± 0.75	35.44 ± 2.30[a]	0.05 ± 0.05	0.68 ± 0.30[a]
Day 8	11.25 ± 0.61	36.59 ± 1.99[a]	11.25 ± 0.61	36.29 ± 1.95[a]	0 ± 0	0.15 ± 0.07[a]
Day 10	9.99 ± 0.55	30.84 ± 2.18[a]	9.99 ± 0.55	30.67 ± 2.15[a]	0 ± 0	0.16 ± 0.06[a]

Note. Values are means ± SEM ($n = 8$–12).
[a]Significantly greater than mean value of saline control rats.

TABLE 2
Immunophenotyping on BAL cells (10^3)

	CD3+CD4+ T cells		CD3+CD8a+ T cells		CD3+ T cells		CD45R+ B cells		NKR-P1A+CD3- NK cells	
	Saline	Zymosan	Saline	Zymosan	Saline	Zymosan	Saline	Zymosan	Saline	Zymosan
Day 1	9.55 ± 1.95	178.97 ± 41.35^a	$4.63 \pm .093$	92.49 ± 23.75^a	13.27 ± 3.06	350.23 ± 72.22^a	20.69 ± 6.99	323.36 ± 83.32^a	2.06 ± 0.95	150.82 ± 39.28^a
Day 4	7.51 ± 0.89	90.96 ± 7.53^a	4.02 ± 0.81	253.23 ± 34.04^a	10.69 ± 1.11	456.57 ± 59.81^a	9.68 ± 1.57	294.15 ± 22.09^a	1.73 ± 0.48	163.85 ± 20.9^a
Day 6	7.88 ± 1.48	388.79 ± 65.06^a	4.65 ± 1.04	346.47 ± 81.55^a	10.75 ± 1.36	1303.13 ± 354.04^a	14.44 ± 2.77	312.63 ± 77.16^a	1.63 ± 0.37	125.36 ± 27.44^a
Day 8	6.98 ± 1.28	159.7 ± 48.91^a	3.51 ± 1.17	59.43 ± 24.69^a	13.64 ± 1.75	531.52 ± 86.69^a	17.78 ± 2.44	127.4 ± 18.66^a	1.44 ± 0.63	59.68 ± 7.23^a
Day 10	9.00 ± 1.44	361.36 ± 67.35^a	3.23 ± 0.69	102.20 ± 19.83^a	9.02 ± 1.26	436.61 ± 69.84^a	14.97 ± 2.17	68.18 ± 10.99^a	0.84 ± 0.31	46.14 ± 7.82^a

Note. Values are means ± SEM ($n = 8$–12). Each phenotype cell number was calculated by using value = (% positive) × (total cell count).
[a]Significantly greater than mean value of saline control rats ($p < .05$).

TABLE 3
Immunophenotyping on lung-associated lymphocytes (10^6)

	CD3+CD4+ T cells		CD3+CD8a+ T cells		CD3+ T cells		CD45R+ B cells		NKR-P1A+CD3- NK cells	
	Saline	Zymosan	Saline	Zymosan	Saline	Zymosan	Saline	Zymosan	Saline	Zymosan
Day 1	9.89 ± 1.75	10.94 ± 1.63	5.10 ± 1.10	6.76 ± 1.27	15.23 ± 2.72	19.89 ± 3.17	4.91 ± 0.90	4.43 ± 0.90	0.08 ± 0.02	0.07 ± 0.01
Day 4	5.56 ± 0.74	12.88 ± 2.97^a	2.71 ± 0.36	8.16 ± 1.68^a	9.09 ± 1.38	23.87 ± 5.55^a	2.78 ± 0.26	10.45 ± 2.29^a	0.04 ± 0.01	0.29 ± 0.07^a
Day 6	8.31 ± 1.23	26.41 ± 3.97^a	3.88 ± 0.34	23.25 ± 4.32^a	12.67 ± 1.65	52.83 ± 6.50^a	5.41 ± 0.82	16.38 ± 4.06^a	0.05 ± 0.01	1.11 ± 0.72^a
Day 8	8.30 ± 1.02	25.05 ± 2.98^a	3.59 ± 0.48	14.03 ± 1.91^a	12.27 ± 1.23	43.29 ± 5.32^a	4.89 ± 1.19	15.15 ± 3.17^a	0.07 ± 0.01	0.55 ± 0.18^a
Day 10	7.50 ± 1.02	26.97 ± 3.24^a	3.60 ± 0.56	15.20 ± 1.86^a	11.74 ± 1.59	43.77 ± 6.26^a	5.26 ± 1.09	14.91 ± 1.62^a	0.10 ± 0.03	0.53 ± 0.12^a

Note. Values are means ± SEM ($n = 8$–12). Each phenotype cell number was calculated by using value = (% positive) × (total cell count).
[a]Significantly greater than mean value of saline control rats ($p < .05$).

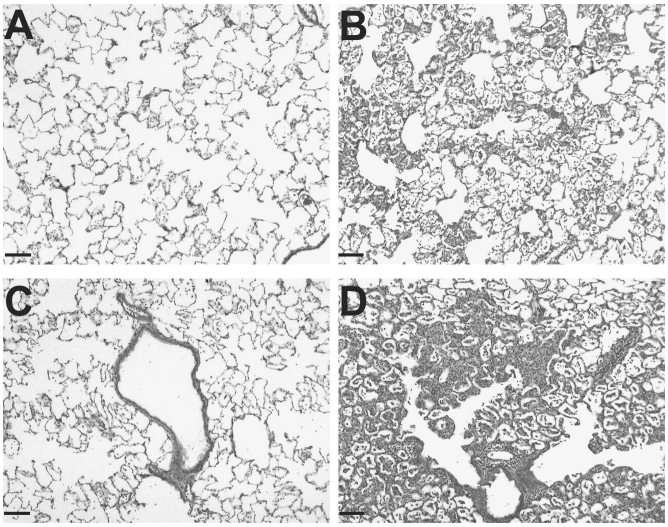

FIG. 2. Micrographs of one rat lung stained with hematoxylin and eosin after exposure to (A) saline, day 1; (B) zymosan, day 1; (C) saline, day 4; and (D) zymosan, day 4. The original magnification is ×10. The bar is 100 μm.

Table 3 shows the immunophenotyping results from lung-associated lymph nodes. There was a slight increase in lymphocyte counts in the zymosan-treated group on day 1 (24.9×10^6 vs 21.4×10^6 cells). Significant increases of 2.8-, 3.7-, 3.3-, and 3.4-fold in lymphocyte counts were observed on days 4, 6, 8, and 10 in the zymosan-treated group when compared to the control (data not shown). Zymosan-induced lymphocyte proliferation reached a maximum on day 6. On day 1, the number of lymphocytes that expressed CD3 in zymosan-treated rats was not different from saline control. However, zymosan induced a significantly higher proliferation of T cells on days 4, 6, 8, and 10 (CD3+ number in Table 3). The number of NK cells (NKR-P1A+CD3−) was significantly increased in the zymosan-treated group on days 4, 6, 8, and 10. Zymosan treatment not only induced proliferation of lymphocytes, but also altered the proliferation of lymphocytes. The result of lymphocyte phenotyping showed that both the numbers of double positive CD3+CD4+ and CD3+CD8a+ cells were increased in zymosan-treated rats (Table 3). However, the increase in the CD8a+ subpopulation appeared to be faster than the increase in the CD4+ subpopulation, which resulted in the ratio of CD3+CD4+/CD3+CD8a+ being lower in the zymosan-treated group (Figure 3).

Production of Reactive Species

Figure 4 shows that AMs from zymosan-treated rats had a 15-fold greater oxidative potential than control on day 1, as indicated by an increase in CL. On day 4, the zymosan-treated group had four-fold higher CL activity than the control. CL was the same for both groups on days 6, 8, and 10. Figure 5 demonstrated that on days 1 and 4, there was no difference in NOx level between control and zymosan-treated rats in the first BALF.

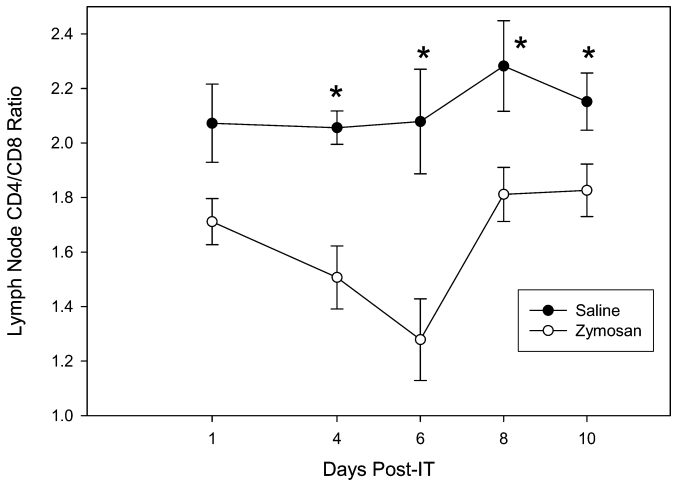

FIG. 3. CD4/CD8 ratio in lung-associated lymph nodes from saline- or zymosan-treated rats. Values are means ± SEM of 8–12 rats per exposure group. Asterisk indicates a significant increase versus the saline control level ($p < .05$). The CD3+CD4+/CD3+CD8+ ratio was calculated by using CD3+CD4+ phenotype cell number divided by CD3+CD8+ phenotype number from each individual rat. The mean ± SEM values of CD3+CD4+/CD3+CD8+ then plot as shown.

However, NOx increased two-fold in the zymosan-treated rats on days 6, 8, and 10.

BALF Cytokine

Expression of various cytokines in BALF was increased in the zymosan-treated rats on day 1 post IT (Figure 6). There was an 18-fold, 46.5-fold, 4.5-fold, and 35-fold, increase post 1 day in TNF-α, IL-6, IL-10, and IL-12p70, respectively, when compared to the saline group. The cytokines levels returned to control

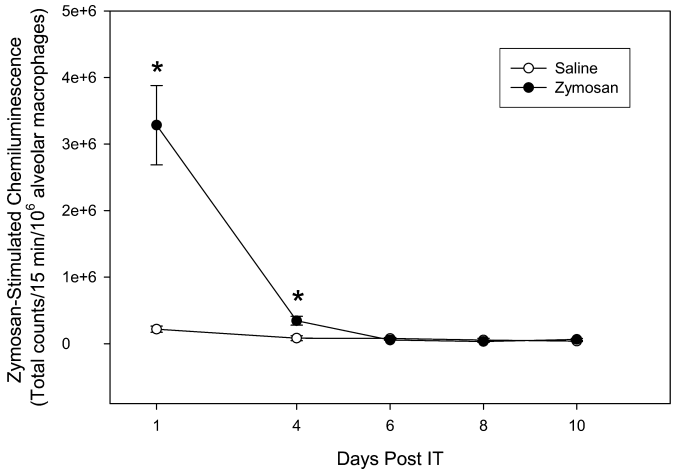

FIG. 4. Macrophage chemiluminescence. Values are means ± SEM of 8–12 rats per exposure group. Asterisk indicates a significant increase versus the saline control level ($p < .05$).

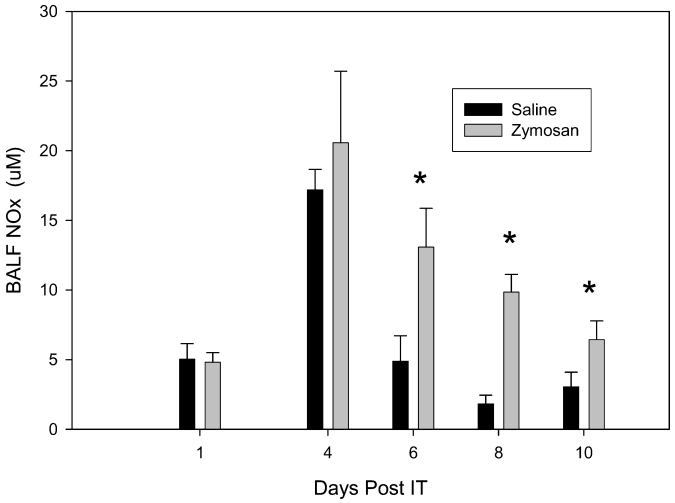

FIG. 5. NOx production in BALF. Asterisk indicates a significant increase versus the saline control level ($p < .05$). Values are means ± SEM of 8–12 rats per exposure group.

levels by day 4 (data not shown). There were no significant differences between the control group and the zymosan-treated group for IL-2 and IL-4 (data not shown).

Lymphocytes Cytokines

Lymphocytes were collected from lung-associated lymph nodes and cultured for 24 h, and media was collected for analysis of lymphokine secretion. Unstimulated baseline expression of IL-2, IL-4, IL-10, IL-12p70, and IFN-γ was not significantly different between the zymosan and saline groups (Figures 7 and 8). Con A stimulation of lymphocytes collected from zymosan-treated rats led to a statistically significant increase in IFN-γ compared to corresponding saline controls at days 4 and 6 (Figure 7). Con A stimulation caused a significant increase in IL-10 secretion by lymphocytes from the zymosan group compared to the corresponding saline group at all time points except day 4 (Figure 8). No significant differences were observed in IL-2 levels at any time point when comparing the zymosan and saline groups (data not shown).

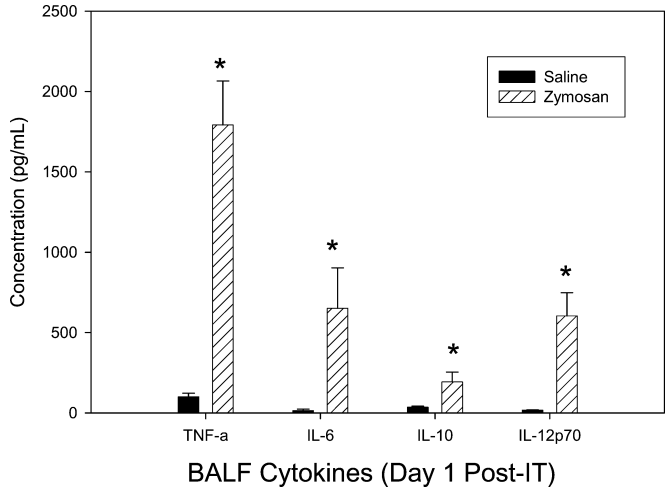

FIG. 6. Cytokine (TNF-a, IL-6, IL-10, and IL-12p70) production in BALF 1 day post IT. Asterisk indicates a significant increase versus the saline control level ($p < .05$). Values are means ± SEM of 8–12 rats per exposure group.

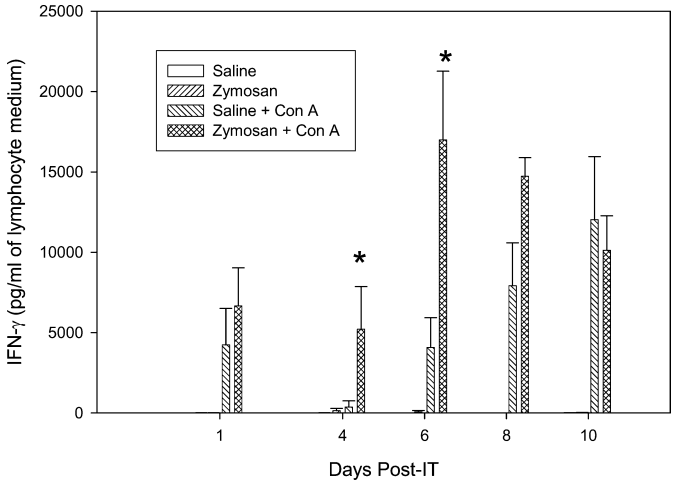

FIG. 7. IFN-γ level in lymphocyte culture medium. Lymphocytes from lung-associated lymph nodes were cultured for 24 h in RPMI 1640 medium with and without Con A (3.3 μg/ml). Asterisk indicates a significant increase versus the saline control + Con A level ($p < .05$). Values are means ± SEM of 8–12 rats per exposure group.

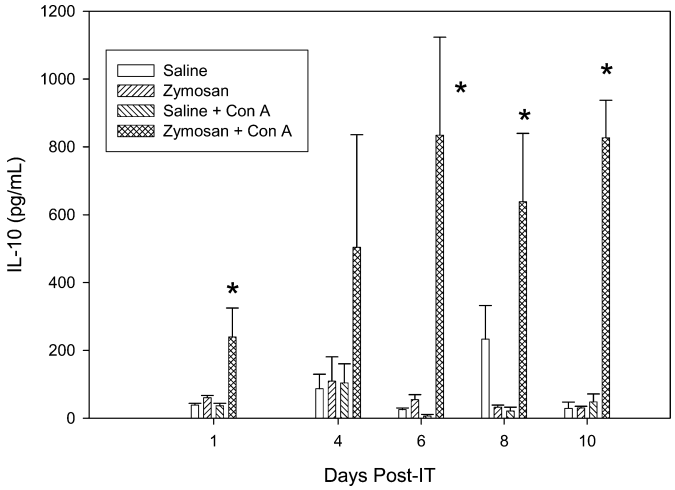

FIG. 8. IL-10 production in lymphocyte culture medium. Lymphocytes from lung-associated lymph nodes were cultured for 24 h in RPMI 1640 medium with or without Con A (3.3 μg/ml). Asterisk indicates a significant increase versus the control + Con A level ($p < .05$). Values are means ± SEM of 8–12 rats per exposure group.

DISCUSSION AND CONCLUSIONS

Dales et al. (1998) observed an increase in T-cell proliferation and a reduction in CD4+/CD8+ ratio in children who lived in fungi-contaminated homes compared to children who lived in less contaminated homes when monitored over a 12-m. period. In the present study, we used zymosan (a 1 → 3-β-glucan from *Saccharomyces cerevisiae*) to model environmental fungal exposure and to investigate the mechanism by which immune defense responses may be altered after fungal exposure in rats. The results show that a one-time zymosan exposure induced an early pulmonary inflammatory response followed by a proliferation of lymphocytes and alteration of the CD4+/CD8+ ratio, which persisted over the 10-day study period. The animal results appear to be consistent with the epidemiology findings by Dales et al. (1998).

Pulmonary damage and inflammation were observed early after zymosan treatment. Elevations in LDH activity, albumin content, and neutrophil infiltration were found in the lungs of the zymosan-treated group. BAL macrophage activation also was enhanced, as evidenced by increases in CL and NOx. This increase in macrophage activation may account for the elevation in injury and inflammation that was observed in the zymosan-treated animals. In addition, BALF cytokine expression (TNF-α, IL-6, IL-10, and IL-12 p70) was increased by zymosan treatment.

TNF-α is a proinflammatory cytokine released from macrophages or activated T cells in response to microbes or other agents. TNF-α plays a key role in the initiation of inflammation in the lung and other tissues (Driscoll et al., 1997). Zymosan-induced TNF-α production occurs through the activation of nuclear factor κB (Young et al., 2001b). The BALF cytokine profile is consistent with a previous epidemiology study that reported an increased level of TNF-α, IL-6, and NOx in the nasal lavage fluid of school staff in a mold-contaminated school (Hirvonen et al., 1999). A similar increase of TNF-α and IL-6 was reported in the BALF of mice exposed to fungal spore *Aspergillus versicolor* (Jussila et al., 2002). This pattern of cytokine expression and macrophage activation may influence the development of the adaptive immune response toward a cell-mediated response, characterized by CD4+ Th1 and CD8+ T cells, or a humoral response involving Th2 T cells and B cells. Although the mechanism controlling this differentiation is not fully understood, it is believed that specific cytokines influence the initial proliferation phase of T-cell activation.

Cytokines are known to play a role in communication between innate immune cells, such as dendritic cells, macrophages, neutrophils, and NK cells, and adaptive immune cells (Janeway, 1999). Zymosan exposure resulted in a significant increase in IL-12p70, a cytokine produced by macrophages and dendritic cells, which has been shown to be involved in promoting a cell-mediated immune response. Activation of these innate responders by glucan may determine the direction of the adaptive immune response.

Most adaptive immune responses are a combination of both cell-mediated and humoral immunity. IL-12 and IL-4 play a role in inducing the differentiation of Th1 and Th2 cells, respectively (O'Garra & Arai, 2000). The cell-mediated response, including Th1 and CD8+ cells, is driven by IL-12p70 (O'Garra & Arai, 2000), which is produced by macrophages and dendritic cells. Activated Th1 and CD8+ T cells produce IL-2, and NK cells and activated CD8 T cells produce IFN-γ. On the other hand, the Th2 response is driven by IL-4 (Muraille & Leo, 1998) and is characterized by production of IL-10 and IL-4. The cytokines from one subset of T lymphocytes can inhibit the other. The present study showed a 18-fold increase of CD4+ T helper cells in BALF of zymosan-treated rats on day 1. Th1 cells and Th2 cells release different sets of cytokines, which define their distinct actions in immunity. From the BALF cytokine analysis in the present study, there was a 35-fold increase in IL-12p70, a 4.5-fold increase in IL-10, and a 46.5-fold increase of IL-6 in the zymosan-treated group on day 1. BALF cytokine results showed that IL-4 was not significantly elevated, while IL-12p70 was dramatically increased, suggesting that the cell-mediated immune response may be favored. An increase in IFN-γ when costimulated with con A was observed in the lymphocyte cultured medium of zymosan-treated rats, which also supports a Th1-directed response. A similar Th1 pathway was reported in human lymphocytes exposed to α-D-glucans (Raveendran Nair et al., 2004). Glucan treatment of a patient with allergic rhinitis, who typically presents with production of Th2 cytokines, has been shown to increase IL-12 production and decrease IL-4 production (Kirmaz et al., 2005). Thus, glucan exposure may promote more of a cell-mediated immune response; however, this does not exclude the ability of glucan to stimulate cells involved in humoral immunity.

The immunophenotyping results showed that both CD4+ T helper cells and CD8+ cytotoxic T cells significantly increased in the lymph node of the zymosan-treated group. The CD8+ population increased at a high rate, resulting in a decrease of the CD4+/CD8+ ratio. There was also a significant increase in the CD8+ T cell population in the BALF. These data suggest that the increase in CD8+ T cells is one of the prominent effects of zymosan-induced inflammation. The increase in CD8+ T cells may be related to an elevated IL-6 level in BALF. Zymosan-induced a 46.5-fold greater increase in IL-6 production compared to the saline-treated rats. IL-6 has been shown to induce cytotoxic T-cell differentiation. IL-6 has also been shown to play a role in T-cell activation (Lotz et al., 1988). In the presence of IL-1, IL-6 controls the initial step of T-cell activation, with CD8+ T cells being the target cell (Akira et al., 1990). 1 → 3-β-Glucans have been shown to induce differentiation of precursor T cells to cytotoxic effector cells in vitro within 5 days (Hamuro et al., 1978). The current study demonstrated that CD8+ cell differentiation can also be induced in vivo. A similar report of an increase in CD8 antigen expression was observed in intraepithelial lymphocytes via oral administration of β-glucan (Tsukada et al., 2003). In immune-suppressed mice lacking CD4+ T cells,

CD8+ T cells were shown to compensate and induce vaccine immunity against fungi *Blastomyces dermatitidis* and *Histoplasma capsulatum* (Wuthrich et al., 2003). This suggests that CD8+ T cells may play a more important role than CD4+ T cells in response to fungal infection.

Interestingly, in addition to the data indicating activation of the cell-mediated response in the zymosan-treated group, there was a small, but significant, elevation in IL-10 in the BALF on day 1. However, this response did not persist, and IL-10 was only elevated in lymphocyte culture after it was stimulated with con A. This increase may be due to stimulation of the B-cell population, which was increased in the zymosan-treated lymphocytes. This finding may indicate a small role for the humoral immune response in fungal exposure, although the primary response appears to be cell mediated. Alternately, IL-10 is a known anti-inflammatory cytokine and may be produced early after exposure as a feedback mechanism in response to the increased inflammation caused by the zymosan group.

In conclusion, our present study suggests that exposure to zymosan, which contains mostly 1 $\rightarrow$ 3-β-glucan, increased T-cell proliferation, reduced the CD4+/CD8+ ratio, and altered cytokine production in a way that indicates zymosan exposure may favor a cell-mediated immune response. Questions exist as to what role these fungal-induced effects have in increasing the susceptibility to infection. A further evaluation of lung immune responses to fungal exposure before infection with a bacterial pathogen using an animal infectivity model is currently underway.

REFERENCES

Akira, S., Hirano, T., Taga, T., and Kishimoto, T. 1990. Biology of multifunctional cytokines: IL 6 and related molecules (IL 1 and TNF). *FASEB J.* 4:2860–2867.

Beijer, L., Thorn, J., and Rylander, R. 2003. Mould exposure at home relates to inflammatory markers in blood. *Eur. Respir. J.* 21:317–322.

Dales, R., Miller, D., White, J., Dulberg, C., and Lazarovits, A. I. 1998. Influence of residential fungal contamination on peripheral blood lymphocyte populations in children. *Arch. Environ. Health* 53:190–195.

Douwes, J., Wouters, I., Dubbeld, H., van Zwieten, L., Steerenberg, P., Doekes, G., and Heederik, D. 2000. Upper airway inflammation assessed by nasal lavage in compost workers: A relation with bioaerosol exposure. *Am. J. Ind. Med.* 37:459–468.

Driscoll, K. E., Carter, J. M., Hassenbein, D. G., and Howard, B. 1997. Cytokines and particle-induced inflammatory cell recruitment. *Environ. Health Perspect.* 105 (suppl. 5):1159–1164.

Fogelmark, B., Goto, H., Yuasa, K., Marchat, B., and Rylander, R. 1992. Acute pulmonary toxicity of inhaled beta-1,3-glucan and endotoxin. *Agents Actions* 35(1–2):50–56.

Green, L. C., Wagner, D. A., Glogowski, J., Skipper, P. L., Wishnok, J. S., and Tannenbaum, S. R. 1982. Analysis of nitrate, nitrite, and [^{15}N]nitrate in biological fluids. *Anal. Biochem.* 126:131–138.

Hamuro, J., Wagner, H., and Rollinghoff, M. 1978. beta(1-3)Glucans as a probe for T cell specific immune adjuvants II. Enhanced in vitro generation of cytotoxic T Lymphocytes. *Cell. Immunol.* 38:328–335.

Hirvonen, M. R., Ruotsalainen, M., Roponen, M., Hyvarinen, A., Husman, T., Kosma, V. M., Komulainen, H., Savolainen, K., and Nevalainen, A. 1999. Nitric oxide and proinflammatory cytokines in nasal lavage fluid associated with symptoms and exposure to moldy building microbes. *Am. J. Respir. Crit. Care Med.* 160:1943–1946.

Janeway, C. 1999. *Immunobiology: The immune system in health and disease*. New York: Current Biology, Garland.

Jussila, J., Komulainen, H., Kosma, V. M., Nevalainen, A., Pelkonen, J., and Hirvonen, M. R. 2002. Spores of A*spergillus versicolor* isolated from indoor air of a moisture-damaged building provoke acute inflammation in mouse lungs. *Inhal. Toxicol.* 14:1261–1277.

Kirmaz, C., Bayrak, P., Yilmaz, O., and Yuksel, H. 2005. Effects of glucan treatment on the Th1/Th2 balance in patients with allergic rhinitis: A double-blind placebo-controlled study. *Eur. Cytokine Netw.* 16:128–134.

Lotz, M., Jirik, F., Kabouridis, P., Tsoukas, C., Hirano, T., Kishimoto, T., and Carson, D. A. 1988. B cell stimulating factor 2/interleukin 6 is a costimulant for human thymocytes and T lymphocytes. *J. Exp. Med.* 167:1253–1258.

Muraille, E., and Leo, O. 1998. Revisiting the Th1/Th2 paradigm. *Scand. J. Immunol.* 47:1–9.

O'Garra, A., and Arai, N. 2000. The molecular basis of T helper 1 and T helper 2 cell differentiation. *Trends Cell. Biol.* 10:542–550.

Raveendran Nair, P. K., Rodriguez, S., Ramachandran, R., Alamo, A., Melnick, S. J., Escalon, E., Garcia, P. I., Jr., Wnuk, S. F., and Ramachandran, C. 2004. Immune stimulating properties of a novel polysaccharide from the medicinal plant *Tinospora cordifolia. Int. Immunopharmacol.* 4:1645–1659.

Rylander, R. 1985. Organic dusts and lung reactions—Exposure characteristics and mechanisms for disease. *Scand. J. Work Environ. Health* 11(3):199–206.

Rylander, R. 1994. Organic dusts–from knowledge to prevention. *Scand. J. Work Environ. Health* 20 (special issue):116–122.

Rylander, R. 1997. Investigations of the relationship between disease and airborne (1 $\rightarrow$ 3)-beta-D-glucan in buildings. *Mediators Inflamm* 6:275–277.

Rylander, R. 1999a. Health effects among workers in sewage treatment plants. *Occup. Environ. Med.* 56:354–357.

Rylander, R. 1999b. Indoor air-related effects and airborne (1 $\rightarrow$ 3)-beta-D-glucan. *Environ. Health Perspect.* 107. (Suppl. 3):501–503.

Rylander, R., and Fogelmark, B. 1994. Inflammatory responses by inhalation of endotoxin and (1-3)-beta-D-glucan. *Am. J. Ind. Med.* 25:101–102.

Rylander, R., Persson, K., Goto, H., Yuasa, K., and Shigenori, T. 1992. Airborne beta-1,3-glucan may be related to symptoms in sick buildings. *Indoor Environm.* 1:263–267.

Rylander, R., Goto, H., Yuasa, K., Fogelmark, B., and Polla, B. 1994. Bird droppings contain endotoxin and (1-3)-beta-d-glucan. *Int. Arch. Allergy Immunol.* 103(1):102–104.

Rylander, R., Thorn, T., and Attefors, R. 1999. Airways inflammation among workers in a paper industry. *Eur. Respir. J.* 13:1151–1157.

Shin, S. H., Ponikau, J. U., Sherris, D. A., Congdon, D., Frigas, E., Homburger, H. A., Swanson, M. C., Gleich, G. J., and Kita, H. 2004. Chronic rhinosinusitis: An enhanced immune response to ubiquitous airborne fungi. *J. Allergy Clin. Immunol.* 114:1369–1375.

Thorn, J., Beijer, L., and Rylander, R. 1998. Airways inflammation and glucan exposure among household waste collectors. *Am. J. Ind. Med.* 33:463–470.

Tsukada, C., Yokoyama, H., Miyaji, C., Ishimoto, Y., Kawamura, H., and Abo, T. 2003. Immunopotentiation of intraepithelial lymphocytes in the intestine by oral administrations of beta-glucan. *Cell. Immunol.* 221:1–5.

Urbain, B., Mast, J., Beerens, D., N'Guyen, T., Goddeeris, B., Ansay, M., and Gustin, P. 1999. Effects of inhalation of dust and endotoxin on respiratory tracts of pigs. *Am. J. Vet. Res.* 60(9):1055–1060.

Wan, G. H., Li, C. S., Guo, S. P., Rylander, R., and Lin, R. H. 1999. An airbone mold-derived product, beta-1,3-D-glucan, potentiates airway allergic responses. *Eur. J. Immunol.* 29:2491–2497.

Wuthrich, M., Filutowicz, H. I., Warner, T., Deepe, G. S., Jr., and Klein, B. S. 2003. Vaccine immunity to pathogenic fungi overcomes the requirement for CD4 help in exogenous antigen presentation to CD8+ T cells: Implications for vaccine development in immune-deficient hosts. *J. Exp. Med.* 197:1405–1416.

Young, S.-H., and Castranova, V. 2005. Animal Model of (1 → 3)-beta-Glucan-induced pulmonary inflammation in rats. In *Toxicology of* 1 → 3-*Beta-glucans, Glucans as a marker for fungal exposure* eds. (S.-H. Young and V. Castranova), pp. 65–93. New York: Taylor & Francis.

Young, S.-H., Robinson, V. A., Barger, M., Porter, D. W., Frazer, D. G., and Castranova, V. 2001a. Acute inflammation and recovery in rats after intratracheal instillation of a 1 → 3 -beta -glucan (zymosan A). *J. Toxicol. Environ. Health A* 64:311–325.

Young, S.-H., Ye, J., Frazer, D. G., Shi, X., and Castranova, V. 2001b. Molecular Mechanism of TNF-α Production in 1 → 3-β-Glucan (Zymosan) Activated Macrophages. *J. Biol. Chem.* 276:20781–20787.

Young, S.-H., Robinson, V. A., Barger, M., Zeidler, P., Porter, D. W., Frazer, D. G., and Castranova, V. 2002. Modified endotoxin responses in rats pre-treated with 1 → 3-beta -glucan (zymosan A). *Toxicol. Appl. Pharmacol.* 178:172-179.

Young, S.-H., Robinson, V. A., Barger, M., Frazer, D. G., Castranova, V., and Jacobs, R. R. 2003a. Partially opened triple helix is the biologically active conformation of 1 → 3-beta-glucans that induces pulmonary inflammation in rats. *J. Toxicol. Environ. Health A* 66:551–563.

Young, S.-H., Robinson, V. A., Barger, M., Whitmer, M., Porter, D. W., Frazer, D. G., and Castranova, V. 2003b. Exposure to particulate 1 → 3-beta-glucans induces greater pulmonary toxicity than soluble 1 → 3-beta-glucans in rats. *J. Toxicol. Environ. Health A* 66:25–38.

Zejda, J. E., and Dosman, J. A. 1993. Respiratory disorders in agriculture. *Tuber. Lung Dis.* 74(2):74–86.

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Pulmonary Exposure to 1 # 3-β-Glucan Alters Adaptive Immune Responses in Rats

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