Experimental Lung Research

ISSN: 0190-2148 (Print) 1521-0499 (Online) Journal homepage: http://www.tandfonline.com/loi/ielu20

SINGLE PRE-EXPOSURE TO A HIGH DOSE OF ZYMOSAN ENHANCES LUNG DEFENSE MECHANISMS AND ACCELERATES THE PULMONARY CLEARANCE OF A BACTERIAL PATHOGEN IN RATS

Shih-Houng Young, James M. Antonini & Jenny R. Roberts

To cite this article: Shih-Houng Young, James M. Antonini & Jenny R. Roberts (2008) SINGLE PRE-EXPOSURE TO A HIGH DOSE OF ZYMOSAN ENHANCES LUNG DEFENSE MECHANISMS AND ACCELERATES THE PULMONARY CLEARANCE OF A BACTERIAL PATHOGEN IN RATS, Experimental Lung Research, 34:9, 559-578, DOI: 10.1080/01902140802372426

To link to this article: https://doi.org/10.1080/01902140802372426

Published online: 02 Jul 2009.

Experimental Lung Research, 34:559–578, 2008

ISSN: 0190-2148 print / 1521-0499 online
DOI: 10.1080/01902140802372426

informa
healthcare

SINGLE PRE-EXPOSURE TO A HIGH DOSE OF ZYMOSAN ENHANCES LUNG DEFENSE MECHANISMS AND ACCELERATES THE PULMONARY CLEARANCE OF A BACTERIAL PATHOGEN IN RATS

Shih-Houng Young, James M. Antonini, and Jenny R. Roberts □ *Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia, USA*

□ *The present study examines the effects of pre-exposure to zymosan (a 1→3-β-glucan from baker yeast) on lung defense against bacterial infection. Rats received a single dose of zymosan A (0.6, 1.2, or 2.5 mg/kg body weight [bw]) or vehicle control (saline) via intratracheal instillation 3 days prior to intratracheal inoculation with* 5×10^5 Listeria monocytogenes. *Left lungs were homogenized and cultured to assess bacterial clearance, and bronchoalveolar lavage was performed on the right lungs to monitor lung inflammation and injury. Prior to bacterial infection, zymosan exposure resulted in elevated inflammation and oxidant production in the lungs. Zymosan treatment followed by infection led to an accelerated pulmonary clearance of bacteria when compared to the saline control group in a dose-dependent fashion. In addition, lower levels of injury and inflammation were associated with the enhanced bacteria clearance observed in zymosan-infected rats. Our findings suggest that zymosan exposure may enhance the lung immune response by activating alveolar macrophages prior to infection, and stimulating T cells involved in the adaptive immune response early after infection, thus resulting in a heightened pulmonary immune response.*

Keywords 1→3-β-glucans, *Listeria monocytogenes* lung clearance, pulmonary inflammation, zymosan

Fungi and bacteria have been identified as important etiological agents in indoor air pollution [1]. It is well known that exposure to endotoxin (a cell wall product from gram-negative bacteria) is strongly associated with lung disease [2]. However, higher endotoxin levels in dust sample alone do not always correlate well with inflammatory indicators [3]. Young and colleagues [4] demonstrated that 1→3-β-glucan (a major cell wall component of fungi) is also an important inducer of pulmonary inflammation. Exposure to fungi

Received 11 June 2008; accepted 28 July 2008.

Disclaimer. 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.

Address correspondence to Shih-Houng Young, PhD, Health Effects Laboratory Division, National Institute for Occupational Safety and Health, 1095 Willowdale Road (M/S 2015), Morgantown, WV 26505, USA. E-mail: sby5@cdc.gov

has been shown to have a strong correlation to both upper (throat irritation and sinus symptoms) or lower (wheeze, chest tightness, shortness of breath, and cough) respiratory symptoms [5]. The goal of this study was to examine the effects of pre-exposure to zymosan, a 1→3-β-glucan, on lung defense against bacterial (*Listeria monocytogenes*) infection using a rodent model.

Different components of yeast cell wall have been examined for their inflammatory potential [6–9]. The main components of yeast cell wall are 1→3-β-glucan, mannan, and chitin, which comprise 90% of the cell wall [10]. Fungal cell walls typically are 80% polysaccharide and 3% to 20% protein by dry weight [11]. In their native environment, 1→3-β-glucans are cross-linked with mannan and chitin [10]. However, there is very limited information in regards to the inflammatory potential of other non-1→3-β-glucans components and their ability to alter pulmonary infection. Young and colleagues [12] compared the pulmonary inflammatory potential of a cell wall component partially enriched in either 1→3-β-glucan, mannan, or chitin and concluded that 1→3-β-glucans and chitin were the major components that cause pulmonary inflammation. However, chitin is not as abundant as 1→3-β-glucan in fungal cell wall [13]. Therefore, study of 1→3-β-glucan is relevant to inflammatory responses due to fungi exposure.

1→3-β-Glucan and endotoxin have been considered to be markers for the presence of fungi and gram-negative bacteria, respectively. There is epidemiological evidence of a strong interaction between fungi and bacteria [5]. However, the nature of this interaction is still largely unknown. A synergistic effect of 1→3-β-glucan and endotoxin has been suggested from an experiment with guinea pigs [14], which found increases of lung lavage cells after exposure to curdlan and endotoxin. However, results from a previous study by our laboratory have shown that there may not always be synergy between the 2 components. There were no synergy in inflammatory responses after exposure to endotoxin first then zymosan A or to both simultaneously [15]. Furthermore, less than additive pulmonary responses (lower lactate dehydrogenase [LDH], albumin, and tumor necrosis factor [TNF]-α levels) were observed in animals that were pretreated with 1→3-β-glucan (zymosan A) then exposed to endotoxin [15]. The discrepancies in the studies may have resulted because of different 1→3-β-glucans that were used. In the present study, we used live bacteria (*L. monocytogenes)* instead of endotoxin to further examine the effects of zymosan A pretreatment on inflammatory responses.

L. monocytogenes is a gram-positive, intracellular bacteria [16, 17], which induces a different response from endotoxin (a gram-negative bacteria). *L. monocytogenes* activates not only the innate (nonspecific) but also the cell-mediated (antigen-specific) immune response upon infection. The well-defined immune response to *L. monocytogenes* allows it to serve as an

experimental probe to evaluate the affects of an immunotoxic xenobiotic on both innate and cell-mediated host immunity [18–23]. *L. monocytogenes* was used here as a model pathogen to investigate lung defense mechanism in response to pulmonary exposure to zymosan A.

MATERIALS AND METHODS

Animals

Specific-pathogen-free male Sprague-Dawley [Hla: (SD) CVF] rats, approximately 7 to 8 weeks old at arrival (~ 225 to 250 g) were purchased from Hilltop Lab Animals (Scottdale, PA). The animals were housed under temperature and humidity controlled conditions in cages with HEPA-filtered air. Rats were allowed to acclimate in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved animal facility for 1 week 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

Three days before infection with *L. monocytogenes*, male Sprague-Dawley rats, ~8 to 9 weeks old, were intratracheally instilled with either saline (control) or zymosan at a dose of 0.6, 1.2, or 2.5 mg/kg body weight (bw). On day 0 the animals were inoculated with 5×10^5 *L. monocytogenes*. On day 0 (before infection), and days 3, 5, and 7 after infection, rats were euthanized and bronchoalveolar lavage (BAL) was performed on the right lungs ($n = 10$–14 per group, except for non–*L. monocytogenes*-treated animals where $n = 4$–6). The cells recovered by BAL were differentiated, and chemiluminescence, a measure of macrophage oxidant production, was determined. Albumin and lactate dehydrogenase (LDH) activity, indicators of lung injury, were measured in BAL fluid. The lung-associated lymph nodes were removed and lymphocytes were recovered. Immunophenotyping was performed on the lymphocyte suspension and BAL cells via flow cytometry. The cytokine levels in the BAL fluid were also determined.

Zymosan Treatment

Zymosan A was purchased from Sigma Chemical (St. Louis, MO) and was suspended in sterile phosphate-buffered saline (PBS). Three days before *L. monocytogenes* infection, 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 the indicated doses or saline as a vehicle control.

Intratracheal Bacteria Inoculation

L. monocytogenes was cultured overnight in brain-heart infusion broth (Difco Labratories, Detroit, MI) at 37°C in a shaking incubator. Following incubation, the bacterial concentration was determined spectrophotometrically at an optical density of 600 nm. The sample was diluted with sterile saline to the concentration of 5×10^5 *L. monocytogenes* in 500 μL sterile saline. *L. monocytogenes* was intratracheally instilled 3 days post zymosan instillation. The dose of *L. monocytogenes* chosen was found to give a uniform infection and did not result in mortality in untreated naïve Sprague-Dawley rats in a previous study [24].

Pulmonary Clearance of *L. monocytogenes*

At days 3, 5, and 7, the left lungs were removed from all rats in each treatment group. The excised tissues were suspended in 10 mL sterile water, homogenized using a PowerGen 700 homogenizer (Fisher Scientific, Pittsburgh, PA), and cultured quantitatively on brain-heart infusion agar plates (Becton Dickinson, Cockeysville, MD). The viable colony-forming units (CFU) were counted after an overnight incubation at 37°C.

Bronchoalveolar Lavage and Cell Differentials

At 0 (before infection), 3, 5, and 7 days post bacterial instillation, the rats were deeply anesthetized with 0.4 mL of Sleepaway (26% sodium pentobarbital, 7.8% isopropyl alcohol, and 20.7% propylene glycol; Fort Dodge Animal Health, Fort Dodge, IA) and then exsanguinated by severing the abdominal aorta. The left bronchus was clamped off, and BAL was performed on the right lungs of rats. The lungs were first lavaged with 1 mL/100 g bw of Ca^{2+}- and Mg^{2+}-free PBS at pH 7.4. The first fraction of lavage fluid remained in the lungs for 30 seconds, with constant massaging of the lungs, and was repeated twice. This first fraction of BAL fluid was centrifuged at $500 \times g$ for 10 minutes and supernatant used for analysis of LDH, albumin, and cytokines. The lung was further lavaged with 6-mL aliquots of PBS until a total of 30 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 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 minutes at 800 rpm and pelleted onto a slide using a cytospin (Shandon Cytospin II; Shandon, Pittsburgh, PA), and 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

Lung-associated lymph nodes were harvested into a 15-mL conical tube that contained 1 mL of RPMI 1640 medium (Sigma) with 2 mM glutamine, 100 μg/mL streptomycin, 100 U/mL penicillin, 5×10^{-5} M 2-β-mercaptoethanol, 5 mM HEPES, and 10% heat-inactivated fetal bovine serum (FBS) (Sigma). A Teflon fluorocarbon resin and stainless steel shaft pestle (Fisher Scientific, Pittsburgh, PA; catalog no. 05-559-26) were used to gently release lymphocytes into medium. Total number of lymphocytes was determined using a Coulter Multisizer II.

Immunophenotyping and Flow Cytometry

Immunophenotyping was performed on lymphocyte suspensions via flow cytometry. Briefly, 50 μL of lymphocyte suspensions ($\sim 0.5 \times 10^6$ cells) were 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 minutes. Anti-CD3 and anti-CD45R were used for T and B cells enumeration, respectively. 7-Aminoactinomycin D (7-AAD) and the monoclonal antibodies, anti-CD3, anti-CD4, anti-CD8a, anti-CD45R, and NKR-P1A, were purchased from Becton Dickinson (San Diego, CA). Antibodies for different cell membrane surface markers were prepared in fluorescence-activated cell sorting (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 minutes on ice. Each antibody was titrated for optimum amount of antibody. Each tube was then washed 1 time with 1 mL FACS buffer to remove excess of unbound antibody. The cells were then fixed with 1% paraformaldehyde (concentration at final), and analyzed on FACSCalibur (flow cytomter; BD Biosciences Immunocytometry Systems, San Jose, CA). Two panels of 3 or 4 colors analysis were set up for immunophenotyping: CD45R-FITC/CD3-PE/7-AAD and CD4-FITC/NKR-P1A-PE/CD8-PerCP/CD3-APC. The live lymphocyte population was selected based on forward scatter and side scatter light signal intensity, and was identified by 7-AAD staining. A total of 10,000 events were collected per sample.

Biochemical Parameters of Injury

The albumin content and LDH activity in the acellular first fraction of BAL fluid 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). 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 System, Montclair, NJ).

Alveolar Macrophage Chemiluminescence (CL)

Luminol-dependent CL was performed on BAL cells as a measure of the light generated by reactive species produced by activated alveolar macrophages (AMs) and polymorphonuclear neutrophils (PMNs). CL was performed with an automated Berhold Autolumat LB 953 luminometer (Wallac, Gaitherburg, MD) at 390 to 620 nm for 15 minutes. Nonopsonized, insoluble zymosan (2 mg/mL; Sigma) 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 minutes in 0.008 mg/dL (weight/volume) luminol in a total of 0.5 ml of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer followed by the measurement of CL for 15 minutes. Zymosan-stimulated CL was calculated as the counts per minute (cpm) in the zymosan-stimulated assay minus the cpm in the resting assay, and this was normalized to the total number of macrophages recovered from the BAL.

BAL Fluid Cytokines

Cytokine protein concentrations were determined with enzyme-linked immunosorbent assay (ELISA) kits from Biosource International (Camarillo, CA). The results of this colormetric assay were obtained with a Spectramax 250 plate spectrophotometer using Softmax Pro 2.6 software (Molecular Device, Sunnyvale, CA). The following cytokine levels from the first fraction of BAL were determined: interleukin (IL)-2, IL-4, IL-6, IL-10, tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-12p70, and IL-18 on days 0, 3, 5, and 7 post bacteria inoculation.

Statistical Analysis

All data are presented as means $\pm$ standard error of measurement (SEM). Statistical analysis was performed using SigmaStat v3.11 software (Systat Software). For multiple comparisons, a 2-way analysis of variance (ANOVA) was performed and significant differences between groups were determined by using the Student-Newman-Keuls method. The significance was set at $P \leq .05$.

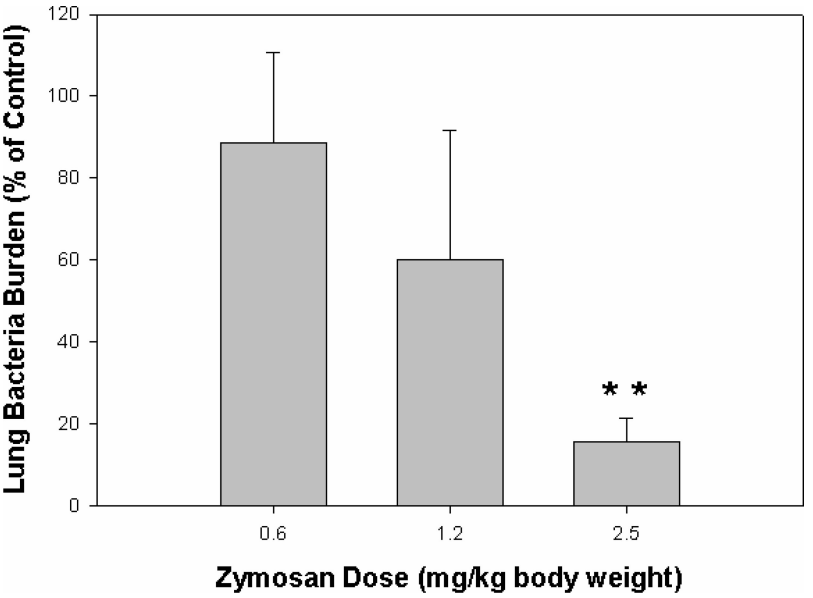

FIGURE 1 Dose-dependent increase in pulmonary bacterial clearance in rats 3 days post infection that were preexposed to zymosan A (0.6, 1.2, or 2.5 mg/kg bw) or saline 3 days prior to intratracheal inoculation with 5×10^5 *L. monocytogenes*. Values are expressed as percentage of bacteria counts in control rats ± SEM of 4 to 12 rats per exposure group. **Significantly different from all groups ($P < .05$).

RESULTS

Pulmonary Clearance of *L. monocytogenes*

Pulmonary clearance of bacteria after pretreatment with 0.6, 1.2, or 2.5 mg/kg bwt of zymosan was assessed on day 3 (Figure 1). There was a dose-dependent enhancement in the clearance of bacteria from the lungs of rats treated with zymosan prior to infection. There was significantly less bacteria in the lungs of rats pretreated with the highest dose of zymosan when compared to all other groups. Table 1 shows the time course of lung bacteria clearance after prior instillation with 2.5 mg/kg bw zymosan. Instillation with zymosan prior to infection resulted in an increase in bacterial clearance from the lungs for up to 7 days post infection.

TABLE 1 Bacteria Clearance From Lung for 2.5 mg/kg bw Zymosan Dose at Days 3, 5, and 7

Days	CFU from saline group	Standard error	CFU from zymosan group	Standard error
3	17,088,000	2,271,904	2,707,733*	958,970
5	350,906	231,670	122,379	29,908
7	4,240	3,207	433*	220

*Significantly different from saline control rats ($P < .05$). $n = 10$–14 per group.

Lung Injury: LDH Activity and Albumin

Zymosan treatment induced elevated LDH levels at day 0 when compared to control rats (Figure 2*A*). However, LDH levels in rats treated with zymosan (2.5 mg/kg bw) were significantly lower than saline rats post bacteria infection at days 3 and 5. Albumin levels followed the same pattern as LDH. At day 0 (pre-infection), higher albumin levels were observed in the zymosan-treated group (Figure 2*B*). The increase in albumin post-infection on days 3 and 5 that was observed in the saline group did not occur in the zymosan-treated group. There were no significant differences in albumin or LDH at day 7 between the zymosan and saline groups.

BAL Cell Differentials

Prior to infection, zymosan treatment resulted in a significant influx of both PMNs (Figure 3*A*) and AMs (Figure 3*B*) into the lungs, indicating increased inflammation in the treated group prior to infection. On days 3 and 5 post infection, the PMN number in the zymosan-treated rats decreased compared to the saline-treated rats (Figure 3*A*). The number of AMs in the lungs of zymosan-treated rats did not change after infection from the levels observed on day 0. However, there was a continuous increase in AM number in the saline-treated rats over the course of the infection. By day 7, there were no significant differences between the zymosan and saline groups.

Immunophenotyping: Lung-Associated Lymphocytes and BAL Cells

Table 2 shows the immunophenotyping of lung-associated lymphocytes. Zymosan treatment induced a significant ($P < .05$) increase in CD3+CD4+ and CD3+CD8+ T cells, B cells (CD45R+), and natural killer (NK) cells (NKR-P1A+CD3−) in the lung-associated lymph node at day 0 preinfection, and in most cases, the increase continued post *L. monocytogens* infection when compared to the saline-treated rats. The saline group showed a proliferation of lymphocytes post infection; however, the increase was not as great as that of the zymosan group. A similar trend was observed in BAL recovered cells as shown in Table 3. The saline group showed a continuous proliferation of CD3+CD4+ T cells, CD3+CD8a+ T cells, CD3+ T cells, CD45R B cells, and NK cells, which was not observed in zymosan group. Zymosan treatment induced a significant increase in lymphocytes at days 0 and 3 when compared to the saline group. However at days 5 and 7, lymphocyte levels in the BAL of saline group were no different from the zymosan pretreated group in most cases.

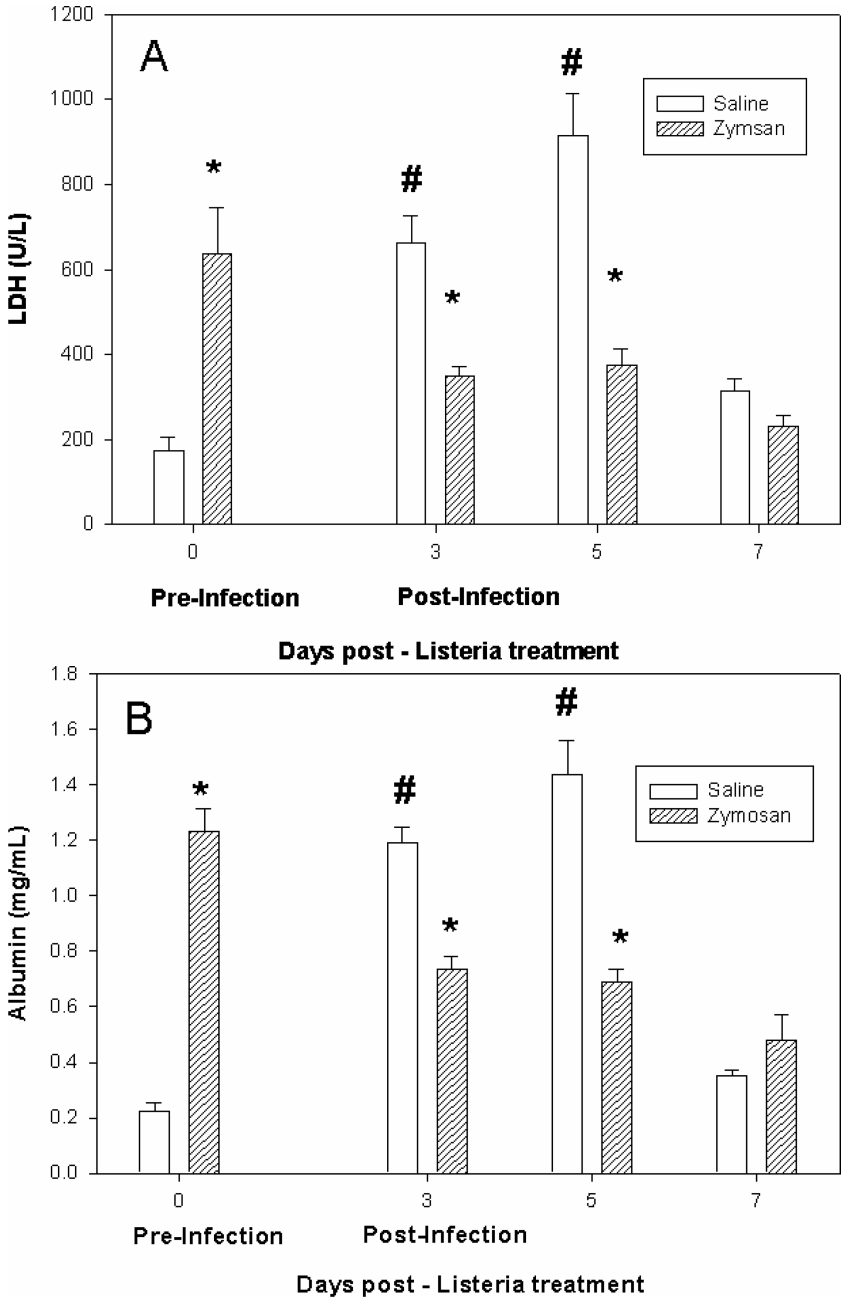

FIGURE 2 Lactate dehydrogenase (LDH) (*A*) and albumin (*B*) in the BAL fluid of rats that were pre-exposed to zymosan A (2.5 mg/kg bw) or saline 3 days prior to intratracheal inoculation with 5×10^5 *L. monocytogenes* ($n = 10$–14 per group). Values are means ± SE. *Significantly different from saline group ($P < .05$). #Significatly higher than pre–infection day level ($P < .05$).

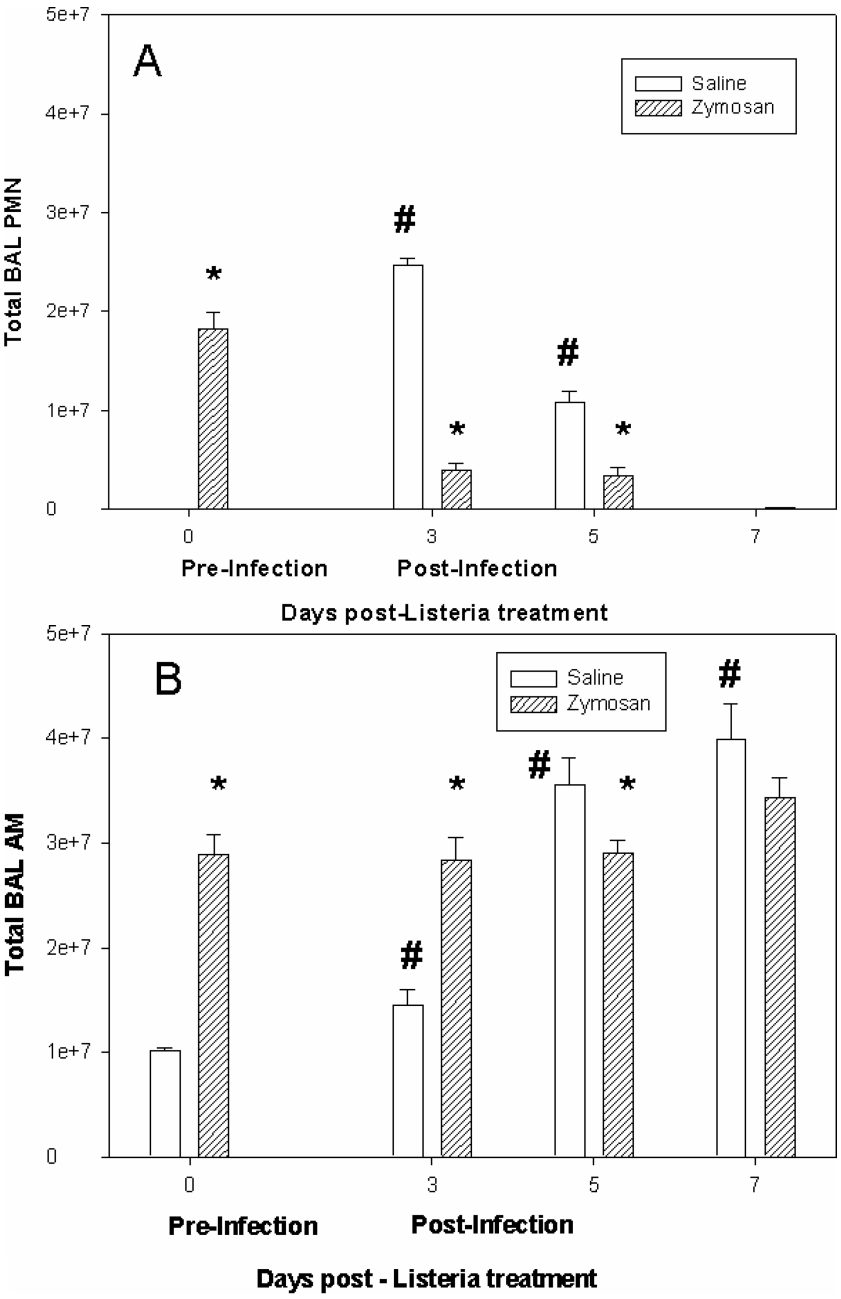

FIGURE 3 Total number of PMNs (*A*) and AMs (*B*) in the BAL fluid of rats preexposed to zymosan A (2.5 mg/kg bw) or saline 3 days prior to intratracheal inoculation with 5×10^5 *L. monocytogenes* ($n = 10$–14 per group). Values are means ± SE. *Significantly different from saline group ($P < .05$). #Significatly higher than pre–infection day level ($P < .05$).

TABLE 2 Immunophenotyping of Lung-Associated Lymph Node 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 0 preinfection	8.85 ± 0.35	24.22 ± 3.19*	4.48 ± 0.7	19.13 ± 1.92*	13.14 ± 0.85	44.14 ± 5.07*	3.21 ± 0.22	16.38 ± 2.01*	0.14 ± 0.01	0.48 ± 0.06*
Day 3 post infection	20.88 ± 1.47#	39.55 ± 2.84*	13.68 ± 1.02#	28.43 ± 1.88*	38.01 ± 2.98#	74.98 ± 6.19*	15.96 ± 1.97#	35.62 ± 2.94	0.19 ± 0.03	0.68 ± 0.12*
Day 5 post infection	30.03 ± 3.26#	37.73 ± 4.35	19.61 ± 2.35	25.34 ± 2.46	55.23 ± 5.21#	73.68 ± 8.49	14.88 ± 2.37	22.62 ± 2.24*	0.65 ± 0.08#	1.21 ± 0.14*
Day 7 post infection	24.35 ± 2.18	46.75 ± 5.38*	17.16 ± 1.80	29.50 ± 2.80*	41.99 ± 3.5	80.17 ± 9.33*	10.43 ± 1.38	20.82 ± 3.99*	4.06 ± 0.50#	5.92 ± 0.49*

Note. Values are means ± SE. $n = 12–16$; for day 0, $n = 4–6$.

*Significantly greater than mean value of saline control rats. #Significantly greater than previous day level in saline control rats.

Each phenotype cell number was calculated by cell number = (% positive) × (total cell count).

TABLE 3 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 0 Pre-infection	5.83 ± 1.80	132.88 ± 27.74*	3.05 ± 0.72	471.58 ± 111.03*	8.64 ± 1.33	655.14 ± 139.41*	17.36 ± 4.50	278.33 ± 45.04*	4.37 ± 0.92	219.35 ± 16.47*
Day 3 Post-infection	19.10 ± 3.78#	273.16 ± 69.60*	23.46 ± 5.91#	309.21 ± 86.24*	87.64 ± 17.16#	885.44 ± 168.15*	111.38 ± 21.39#	323.77 ± 52.87*	22.40 ± 6.23#	199.92 ± 32.02*
Day 5 Post-infection	285.45 ± 38.77#	326.93 ± 26.25	362.52 ± 48.44#	249.29 ± 34.47	987.66 ± 206.43#	682.99 ± 95.60	243.69 ± 54.01#	174.27 ± 19.12	157.78 ± 37.40#	146.07 ± 18.92
Day 7 Post-infection	1131.19 ± 173.65#	667.89 ± 66.98*	487.88 ± 137.28#	691.16 ± 83.87	2165.72 ± 459.86#	1808.28 ± 248.36	216.71 ± 29.52	209.92 ± 34.81	179.91 ± 17.59	175.67 ± 19.29

Note. Values are means ± SE. $n = 12$–16; for day 0, $n = 4$–6.

*Significantly greater than mean value of saline control rats. #Significantly greater than previous day level in saline control rats.

Each phenotype cell number was calculated by cell number = (% positive) × (total cell count).

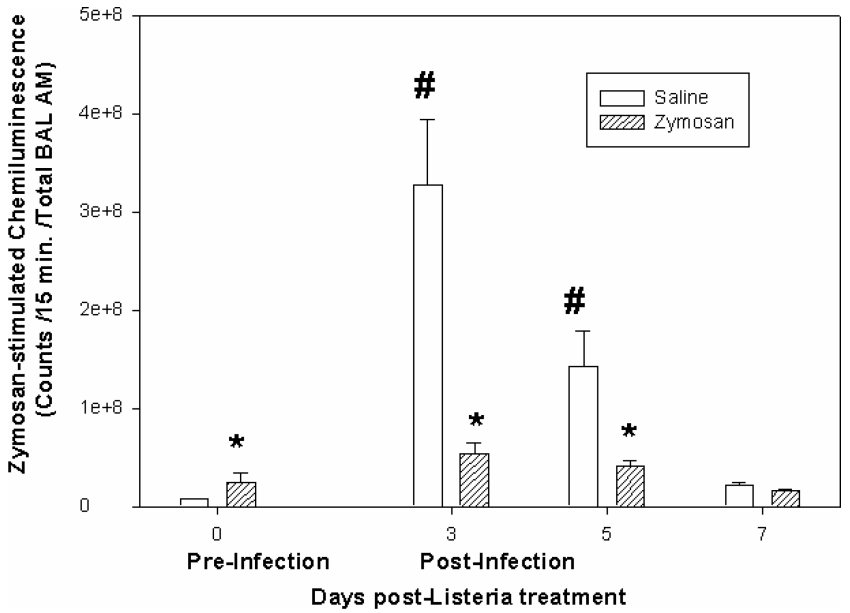

FIGURE 4 BAL cellular chemiluminescence (CL) depicted in total counts per 15 minutes for total BAL AMs after stimulation with nonopsonized zymosan in rats that were pre-exposed to zymosan A (2.5 mg/kg bw) or saline 3 days prior to intratracheal inoculation with 5×10^5 *L. monocytogenes* ($n = 10$–14 per group).Values are means ± SE. *Significantly different from all groups ($P < .05$). #Significatly higher than pre–infection day level ($P < .05$).

Production of Reactive Oxygen Species

On day 0, prior to infection, CL in the zymosan-treated rats was significantly higher than saline-treated rats, indicating increased oxidant production by AMs in this group (Figure 4). Saline-treated rats exhibit a normal increased of CL level post infection on day 3; however, oxidant production in zymosan-treated rats did not vary greatly from preinfection levels, and was significantly lower than that of control levels. There were no significant differences between saline and zymosan groups on day 7.

BAL Fluid Cytokines

Table 4 shows the increase of proinflammatory cytokines TNF-α which has a similar pattern as in LDH and albumin. Pre-treatment with zymosan induced a high TNF-α level at day 0. However, subsequent exposure to *L. monocytogenes* did not further induce TNF-α in the zymosan group. Another proinflammatory cytokine, IL-6 (Table 4), also showed similar trends as TNF-α. IL-6 level in zymosan group was significantly lower than saline group on days 3 post infection. These data demonstrate zymosan-treated rats have a lower level of proinflammatory cytokines response post bacteria infection. IL-10 is an anti-inflammatory cytokine. IL-10 level (Table 4) was lower in

TABLE 4 Cytokines Production in First BALF

	TNF-α		IL-6		IL-10		IL-12p70		IL-18	
	Saline	Zymosan	Saline	Zymosan	Saline	Zymosan	Saline	Zymosan	Saline	Zymosan
Day 0 Pre-infection	128.12 ± 11.08	334.32 ± 34.69*	0± 0	13.30 ± 5.70	185.74 ± 10.28	120.09 ± 8.01*	0± 0	11.04 ± 3.64*	24.41 ± 1.95	78.86 ± 11.84*
Day 3 Post-infection	429.24 ± 28.08#	165.73 ± 31.95*	584.29 ± 44.78#	158.06 ± 23.39*	171.37 ± 9.90	133.16 ± 9.57*	125.84 ± 17.11#	16.87 ± 3.82*	126.28 ± 11.65#	91.96 ± 14.84
Day 5 Post-infection	88.27 ± 11.09	124.15 ± 20.34	97.31 ± 29.89#	81.10 ± 25.11	106.65 ± 7.03	133.10 ± 7.08	1.00 ± 0.66	4.81 ± 1.71	405.57 ± 66.33#	168.84 ± 26.85*
Day 7 Post-infection	123.88 ± 22.35	153.06 ± 34.75	1.82 ± 1.75	1.62 ± 1.62	129.59 ± 8.41	139.27 ± 7.28	2.19 ± 1.54	3.60 ± 1.12	88.79 ± 12.83#	72.4 ± 10.43

Note. Values are means ± SE (pg/mL). $n = 12$–16; for day 0, $n = 4$–6.

*Significantly greater than mean value of saline control rats of same days ($P < .05$). #Significantly greater than pre–infection day saline control rats' level ($P < .05$).

TABLE 5 Cytokines Production in First BALF

	IL-2		IL-4		IFN-γ	
	Saline	Zymosan	Saline	Zymosan	Saline	Zymosan
Day 0 Pre-infection	117.71 ± 20.75	81.77 ± 9.79	29.27 ± 3.05	45.48 ± 8.59	1.08 ± 0.63	1.96 ± 1.09
Day 3 Post-infection	95.94 ± 7.73	122.55 ± 12.35	61.51 ± 8.86$^{\#}$	82.1 ± 13.37	37.45 ± 5.18$^{\#}$	2.53 ± 2.1*
Day 5 Post-infection	47.31 ± 5.61	107.29 ± 8.51*	23.45 ± 4.19	74.25 ± 11.39*	2.79 ± 1.12	0.61 ± 0.35*
Day 7 Post-infection	89.61 ± 8.69	97.68 ± 9.46	46.0 ± 7.39$^{\#}$	76.01 ± 11.16*	0.88 ± 0.57	1.47 ± 0.94

Note. Values are means ± SE (pg/mL). $n = 12$–16; for day 0, $n = 4$–6.

*Significantly greater than mean value of saline control rats of same days ($P < .05$). $^{\#}$Significantly greater than pre–infection day saline control rats' level ($P < .05$).

zymosan-treated rats on day 0, which corresponded to a significantly higher TNF-α level in zymosan-treated rats. However, a significant lower IL-10 level in zymosan-treated rats was observed on day 3, which also had significantly lower TNF-α and IL-6 levels.

Pre-treatment with zymosan also reduced immunomodulating cytokines IL-12 p70 and IL-18 productions post bacteria infection compared to saline group (Table 4). Cytokines IL-12 p70 were higher in zymosan group on day 0, but was significantly lower in zymosan group post infection on day 3 (Table 4). IL-18 had a similar trend as IL-12p70 but peaked at day 5 before returning to control levels on day 7 (Table 4). These results strongly suggest pre-treatment with zymosan reduces subsequent immunomodulating cytokine production.

The responses of cytokines IL-2, IL-4, and IFN-γ were shown in Table 5. There were no significant differences between zymosan- or saline-induced IL-2 or IL-4 response on days 0 and 3; however, a higher IL-2 response was measured on day 5 and higher IL-4 response was measured on days 5 and 7 for zymosan rats. IFN-γ levels were not different at day 0, but significantly decreased in the zymosan group at days 3 and 5.

SUMMARY AND DISCUSSION

1→3-β-Glucans have been reported to have antibacterial activity [25, 26], either via intraperitoneal injection [27], or via oral administration [28]. α-Glucan has also been reported to enhance host defense against bacteria infection through intraperitoneal injection [27]. The authors suggest that the underlying mechanism for defense against pathogens was the ability of 1→3-β-glucans to activate macrophages and PMNs. An example of these stimulatory properties was also reported recently with

anthrax infection via oral administration [29]. Here we demonstrated, via intratracheal instillation, that 1→3-β-glucan can also enhance bacterial clearance from the lungs.

The pulmonary immune response to infection after pre-exposure to zymosan was examined using *L. monocytogenes* as an infectivity model. We found that pre-exposure to zymosan resulted in a dose-dependent increase in pulmonary bacterial clearance. The significant increase in bacterial clearance was evident 3 days after infection, and on day 10 there were also fewer bacteria in the lungs of rats that had been pretreated with the highest dose of zymosan. Prior to infection, lung injury and inflammatory markers, such as LDH, neutrophil influx, and inflammatory cytokines, were elevated in rats pretreated with zymosan. However, after infection these parameters were all lower in zymosan-treated rats when compared to controls, which may correspond to the lower level of bacteria in the lungs of the treated rats. These data suggest the treatment with zymosan prior to infection may result in the priming of the responses necessary for bacterial killing and clearance.

Alveolar macrophages are responsible for the clearance of inhaled particles and microorganisms from the distal airways and alveolar space. Zymosan has been shown to stimulate and activate AMs [30–32]. It has also been well documented that TNF-α, an AM-derived proinflammatory cytokine, is involved in innate resistance to bacterial infection and in recruitment of neutrophils into the airspace [33]. In the current study, we found that there were significantly more AMs in zymosan-exposed rats prior to bacterial infection, and that zymosan pre-treatment induced a significantly higher TNF-α level in the lung. Neutrophil numbers were also elevated in the lungs of zymosan-treated rats prior to infection. In addition, IL-10, an anti-inflammatory cytokine that inhibits macrophage activation, was lower in zymosan-treated rats. These data support the hypothesis that early inflammation and recruitment/activation of lung phagocytes induced by the zymosan treatment may result in priming of the responses required to resolve bacterial infection.

Reactive oxygen species (ROS) generation by AMs is also an important factor in intracellular killing and clearance of *L. monocytogenes* [21]. In our previous study [34], we demonstrated that pulmonary exposure to zymosan induced an elevation in ROS generation by AMs indicated by increased CL levels 1 to 4 days post zymosan exposure. In the current study, we also observed significantly higher CL levels in zymosan-treated rats prior to infection, further supporting the idea that macrophages may be primed to begin killing and clearing the bacteria when compared to control. In control rats, there was a significant oxidative burst in AMs post infection, as would be expected. Interestingly, there was no further increase in production of ROS post infection in the zymosan-treated rats. The reason for that is unclear, but may be related to the 10-fold lower bacterial numbers in the lungs of

zymosan pretreated rats and the on-going production of ROS. Therefore, there may be less of a need for a large oxidative burst post infection.

The T cell–mediated adaptive response is critical for immune defense against *L. monocytogenes* [35]. By activating type 1 CD4+ T-helper (T_H1) cells, AMs are further stimulated to kill intracellular bacteria and clear the infection, and CD8+ cytotoxic T cells T (T_C) cells are also activated to target infected cells. Changes in lymphocyte profiles in the lung-associated lymph nodes and in the lung were evaluated by immunophenotyping of lymphocytes cells and BAL cells, as shown in Tables 2 and 3. Zymosan-treated rats induced proliferation of lymphocytes, which resulted in a significantly higher numbers of CD3+CD4+ (T_H cells), CD3+CD8+ ($T_C$cells), B cells, and NK cells in both the lymph nodes and the lung prior to infection. CD8+ T cells have been shown to be an important factor in clearance of *L. monocytogenes* [35]. We observed that there was an elevation in CD8+ T cells in zymosan pretreated rats on day 0 (Table 2). Previously, we have shown that zymosan treatment alters adaptive immune response in rat and the greatest proportional increase was in CD8+ T cells [34]. A similar report in the literature showed that $1\rightarrow3$-β-glucans induced differentiation of precursor T cells to cytotoxic effector cells in vitro within 5 days [36]. Although we did not perform functional tests on CD8+ cells, we speculate that the ability of zymosan to enhance a T_C cell response may likely be a contributing factor in the increased bacterial clearance observed in the zymosan-treated group.

The development of a cell-mediated response, as opposed to a humoral response, is influenced by mediators such as cytokines and lymphokines. As mentioned above, the cell-mediated adaptive immune response is characterized primarily by T_H1 and T_C cells, rather than a humoral response, which involves type 2 T-helper (T_H2) cells and B cells. IL-12 and IL-4 play critical roles in inducing the differentiation of T_H1 and T_H2 cells, respectively [37]. IL-12, a cytokine produced by macrophages and dendritic cells, has been described as a T_C cell maturation factor, and is a known promoter of the cell-mediated immune response. Rats pre-treated with zymosan prior to infection had a significantly higher IL-12 response prior to bacteria infection. In addition, IL-18, a T_H1 promoter [38, 39], was also elevated prior to infection in the zymosan-treated rats. Furthermore, the IL-4 level in zymosan-treated rats was also higher, although this difference was not significant. This may suggest that the T_H1 response may be initiated in zymosan-treated prior to infection, enhancing the overall response to the infection. Interestingly, IL-4, a cytokine that favors the humoral immune response and suppresses the response to *Listeria* [40, 41], was significantly elevated in zymosan-treated rats when compared to saline-treated rats at days 5 and 7 post infection. Despite this increase, and a cytokine profile that appears to favor a T_H2 response at the later time points post infection, bacterial clearance was still increased in the zymosan-treated rats. The reason for this is unclear; however, there is evidence that IL-4 can play a role in the early initiation of

macrophage recruitment and activation during a *Listeria* infection [42], and the increase observed here, at days 5 and 7, may not affect the clearance due to a very early and very strong cell-mediated response in zymosan-treated rats.

IFN-γ is also an important mediator in the defense against *L. monocytogenes* [43]. IFN-γ is produced by NK cells and activated CD8+ cells, and is an activator of macrophages ROS production and bactericidal activity. The BAL IFN-γ level was significantly elevated in saline-treated rats on day 3 post infection; however, IFN-γ levels in zymosan-treated rats did not increase significantly post infection. These data correspond well with the pattern of ROS production observed post infection, where there is also a significant increase in the saline group, but not in the zymosan group. The reason for this response is unclear. It is possible that because zymosan treatment induced an early activation of both macrophages and T cells prior to infection, this group of rats was primed to begin killing and clearing the bacteria earlier, and a significantly stronger activation of the adaptive immune response was not as necessary as compared to the saline-treated rats.

There are other examples of exposures to environmental pollutants that exhibit similar priming effects as zymosan. For example, both acute and chronic exposure to silica has been shown to enhance bacteria clearance in rats [44]. However, unlike zymosan, exposure to silica also induced a higher lung injury and inflammation throughout the treatment before and after infection than saline. The differences in these parameters suggest that there may be different mechanisms involved in the priming effect.

In summary, zymosan pre-exposure accelerated the clearance of bacteria from the lungs compared to the control group. Although it is not clear what is the exact underlying mechanism of this enhanced clearance, evident suggests that zymosan may activated macrophages and induced the T cell–mediated immune response prior to infection. The "priming" effect may play an important role in the accelerated clearance of the bacterial infection.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES

[1] Young RS, Jones AM, Nicholls PJ: Something in the air: endotoxins and glucans as environmental troublemakers. J Pharm Pharmacol. 1998;50:11–17.

[2] Jacobs RR: Airborne endotoxins: An association with occupational lung disease. Appl Ind Hygiene. 1989;4:50–56.

[3] Castranova V, et al: Efficacy of acid/base washes in removing the bioactive agent from cotton dust: response of the guinea pig model to inhalation of treated dust. In: Proceedings of the 15th Cotton Dust Research Conferences. San Antonio, TX: National Cotton Council; 1991:252–255.

[4] Young S-H, et al: Acute inflammation and recovery in rats after intratracheal instillation of a 1→3 -beta -glucan (zymosan A). J Toxicol Environ Health A. 2001;64:311–325.

[5] Park JH, et al: Fungal and endotoxin measurements in dust associated with respiratory symptoms in a water-damaged office building. Indoor Air. 2006;16:192–203.
[6] Domer J, Garner R: Fungal wall components and immunostimulation. In: Latge J-P, Boucias, eds. Fungal Cell Wall and Immune Response. 1990; Berlin: Springer-Verlag. 1990;415–430.
[7] Limper AH, et al: Pneumocystis carinii: cell wall beta-glucan-mediated pulmonary inflammation. J Eukaryot Microbiol. 2003. 50(Suppl):646.
[8] Sorenson WG, Shahan TA, Simpson J: Cell wall preparations from environmental yeasts: effect on alveolar macrophage function in vitro. Ann. Agric. Environ. Med. 1998;5:65–71.
[9] Schuyler M, Gott K, Cherne A: Effect of glucan on murine lungs. J Toxicol Environ Health A. 1998;53:493–505.
[10] Ballou CE, Yeast cell wall and cell surface. In: Strathern JN, Jones EW, Broach JR, eds. The Molecular Biology of the Yeast *Saccharomyces*, Metabolism and Gene Expression. 1982, New York: Cold Spring Harbor Laboratory. 1982;335–360.
[11] Ruiz-Herrera J, Fungal Cell Wall: Structure, Synthesis, and Assembly. Ann Arbor: CRC Press. 1991;248.
[12] Young S-H, et al: A comparison of the pulmonary inflammatory potential of different components of yeast cell wall. J Toxicol Environ Health A. 2007;70:1114–1124.
[13] DiCarlo FJ, Fiore JV: On the composition of zymosan. Science. 1957;127:756–757.
[14] Fogelmark B, Sjostrand M, Rylander R: Pulmonary inflammation induced by repeated inhalations of beta-(1,3)-D-glucan and endotoxin. Int J Exp Pathol. 1994;75:85–90.
[15] Young S-H, et al: Modified endotoxin responses in rats pre-treated with 1→3-beta -glucan (zymosan A). Toxicol Appl Pharmacol. 2002;178:172–179.
[16] Southwick FS and Purich DL: Intracellular pathogenesis of listeriosis. N Engl J Med, 1996;334:770–776.
[17] Shen H, Tato CM, Fan X: Listeria monocytogenes as a probe to study cell-mediated immunity. Curr Opin Immunol, 1998;10:450–8.
[18] Antonini JM, et al: Residual oil fly ash increases the susceptibility to infection and severely damages the lungs after pulmonary challenge with a bacterial pathogen. Toxicol Sci. 2002;70:110–9.
[19] Antonini JM, et al: Suppression in lung defense responses after bacterial infection in rats pretreated with different welding fumes. Toxicol Appl Pharmacol, 2004;200:206–18.
[20] Roberts JR, et al: Soluble metals in residual oil fly ash alter innate and adaptive pulmonary immune responses to bacterial infection in rats. Toxicol Appl Pharmacol, 2007;221:306–319.
[21] Yin XJ, et al: Roles of reactive oxygen species and heme oxygenase-1 in modulation of alveolar macrophage-mediated pulmonary immune responses to Listeria monocytogenes by diesel exhaust particles. Toxicol Sci. 2004;82:143–153.
[22] Yin, XJ, et al: Sustained effect of inhaled diesel exhaust particles on T-lymphocyte-mediated immune responses against *Listeria monocytogenes*. Toxicol Sci. 2005;88:73–81.
[23] Van Loveren H, et al: Effects of ozone on the defense to a respiratory Listeria monocytogenes infection in the rat. Suppression of macrophage function and cellular immunity and aggravation of histopathology in lung and liver during infection. Toxicol Appl Pharmacol. 1988;94:374–393.
[24] Antonini JM, Roberts JR, Clarke RW: Strain-related differences of nonspecific respiratory defense mechanisms in rats using a pulmonary infectivity model. Inhal Toxicol. 2001;13:85–102.
[25] Di Luzio NR, et al: Comparative tumor-inhibitory and anti-bacterial activity of soluble and particulate glucan. Int J Cancer. 1979;24:773–779.
[26] Kokoshis PL, et al: Increased resistance to *Staphylococcus aureus* infection and enhancement in serum lysozyme activity by glcuan. Science. 1978;199:1340–1342.
[27] Takeda Y, et al: Augmentation of host defense against bacterial infection pretreated intraperitoneally with an alpha-glucan RBS in mice. Immunopharmacol Immunotoxicol. 1990;12:457–477.
[28] Itoh W, Augmentation of protective immune responses against viral infection by oral administration of schizophyllan. Mediat Inflamm. 1997;6:267–269.
[29] Vetvicka V, et al: Pilot study: orally-administered yeast beta-1,3-glucan prophylactically protects against anthrax infection and cancer in mice. *J Am Nutraceut Assoc.* 2002;5:1–5.
[30] Kadish JK, Choi CC, Czop JK: Phagocytosis of unopsonized zymosan particles by trypsin-sensitive and beta-glucan-inhibitable receptors on bone marrow-derived murine macrophages. Immunol Res. 1986;5:129–138.

[31] Daum T, Rohrbach MS: Zymosan induces selective release of arachidonic acid from rabbit alveolar macrophage via stimulation of a beta-glucan receptor. FEBS Lett. 1992;309:119–122.
[32] Suzuki T, et al: Serum components induce beta-d-glucan-inhitable uptake of zymosan particles by murine peritoneal macrophages. Biol Pharmaceut Bull. 1993;16:223–227.
[33] Laskin DL, and Pendino KJ: Macrophages and inflammatory mediators in tissue injury. Annu Rev Pharmacol Toxicol. 1995;35:655–677.
[34] Young S-H, Roberts JR, Antonini JM: Pulmonary exposure to 1 → 3-beta-glucan alters adaptive immune responses in rats. Inhal Toxicol. 2006;18:865–74.
[35] Busch DH, Pamer EG: T lymphocyte dynamics during *Listeria monocytogenes* infection. Immunol Lett. 1999;65:93–98.
[36] Hamuro J, Wagner H, Rollinghoff M: Beta(1-3)glucans as a probe for T cell specific immune adjuvants II. Enhanced in vitro generation of cytotoxic T lymphocytes. Cell Immunol. 1978;38:328–335.
[37] O'Garra A, and Arai N: *The molecular basis of T helper 1 and T helper 2 cell differentiation.* Trends Cell Biol. 2000;10:542–550.
[38] Berg RE, Cordes CJ, Forman J: Contribution of CD8+ T cells to innate immunity: IFN-gamma secretion induced by IL-12 and IL-18. Eur J Immunol. 2002;32:2807–2816.
[39] Neighbors M, et al: A critical role for interleukin 18 in primary and memory effector responses to *Listeria monocytogenes* that extends beyond its effects on Interferon gamma production. J Exp Med, 2001;194:343–354.
[40] Kaufmann SH, et al: *Interleukin-4 and listeriosis.* Immunol Rev, 1997;158:95–105.
[41] Nakane A, et al: Endogenous interleukin-4, but not interleukin-10, is involved in suppression of host resistance against *Listeria monocytogenes* infection in interferon-depleted mice. Infect Immun. 1996;64:1252–1258.
[42] Flesch IE, Wandersee A, Kaufmann SH: IL-4 secretion by CD4+ NK1+ T cells induces monocyte chemoattractant protein-1 in early listeriosis. J Immunol. 1997;159:7–10.
[43] Dunn PL, North RJ: Early gamma interferon production by natural killer cells is important in defense against murine listeriosis. Infect Immun. 1991;59:2892–2900.
[44] Antonini JM, et al: Subchronic silica exposure enhances respiratory defense mechanisms and the pulmonary clearance of *Listeria monocytogenes* in rats. Inhal Toxicol. 2000;12:1017–1036.