GRAIN DUST INDUCED LUNG INFLAMMATION IS REDUCED BY *RHODOBACTER SPHAEROIDES* DIPHOSPHORYL LIPID A

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ABSTRACT

To further determine the importance of endotoxin in grain dust-induced inflammation of the lower respiratory tract, we evaluated the efficacy of pentaacylated diphosphoryl lipid A derived from the lipopolysaccharide of Rhodobacter *spheroides* (RsDPLA) as a competitive inhibitor of grain dust-induced airway inflammation. RsDPLA is a relatively inactive compound in comparison to lipid A derived from E. *coli* (LPS) and has been demonstrated to act as a competitive inhibitor of LPS-induced inflammation. To assess the potential stimulatory effect of RsDPLA in relation to LPS, we incubated THP-1 cells with RsDPLA (0.001-100 μg/ml), LPS (0.02 μg endotoxin activity/ml), or CDE (0.02 μg endotoxin activity/ml). Incubation with RsDPLA revealed TNF-α stimulatory effect at 1, 10, and 100 μg/ml. In contrast, incubation with LPS or CDE resulted in TNF-α release at 0.02 μg/ml. Pretreatment of THP-1 cells with varying concentrations of RsDPLA prior to incubation with LPS or CDE (0.02 μg endotoxin activity/ml) resulted in a dose-dependent reduction in the LPS or CDE induced release of TNF-α with concentrations of RsDPLA of up to 1 μg/ml, but not at 10 or 100 μg/ml. To further understand the role of endotoxin in grain dust-induced airway inflammation, we utilized the unique LPS inhibitory property of RsDPLA to determine the inflammatory response to inhaled CDE in mice in the presence of RsDPLA. 10 μg of RsDPLA intratracheally did not cause a significant inflammatory response in comparison to intratracheal saline. However, pretreatment of mice with 10 μg of RsDPLA intratracheally prior to exposure to CDE (5.4 and 0.2 $\mu g/m^3$) or LPS (7.2 and 0.28 $\mu g/m^3$) resulted in significant reductions in the lung lavage concentrations of total cells, neutrophils, TNF-α, and IL-6 in comparison to mice

pretreated with sterile saline. These results confirm the LPS-inhibitory effect of RsDPLA and support the role of endotoxin as the principal agent in grain dust causing airway inflammation.

Key words: grain dust, endotoxin, lung inflammation, Rhodobacter spheroides, LPS inhibition, asthma

Running title: GRAIN DUST INDUCED LUNG INFLAMMATION IS REDUCED BY RsDPLA

INTRODUCTION

Occupational and environmental exposure to grain dust may cause a variety of acute respiratory symptoms, including chest tightness, wheezing, dyspnea, and transient declines in airflow (1). Epidemiologic studies in grain workers have demonstrated that chronic exposure to grain dust may lead to chronic respiratory symptoms, including chronic bronchitis, and progressive, irreversible airflow obstruction (2). Prior studies suggest an association between endotoxin and the physiologic and inflammatory response to grain dust. In grain handlers, work-related respiratory symptoms were found to be strongly associated with the concentration of endotoxin in the bioaerosol (3). Inhalation of endotoxin and corn dust extract (CDE) produce similar symptoms, equivalent changes in airflow, and similar increases in bronchoalveolar lavage (BAL) inflammatory cells and inflammatory cytokines (4).

To further investigate the relative importance of endotoxin in grain-dust induced airway inflammation, we employed methods which attempted removal of endotoxin from CDE, and demonstrated a relationship between the concentration of endotoxin in CDE and the magnitude of inflammation in the lung (5). However, these methods were limited by the inability to completely remove endotoxin from CDE and by the possibility of removing other components in CDE which may promote lung inflammation. In this investigation, we propose to examine the relationship between endotoxin and grain dust-induced lung inflammation by specifically inhibiting endotoxin-mediated inflammatory events. Previous investigations have shown that the pentaacylated diphosphoryl lipid A derived from the lipopolysaccharide of Rhodobacter *spheroides*

(RsDPLA) is a relatively inactive compound in comparison to lipid A derived from E. *coli* (LPS) (6). Moreover, it appears that RsDPLA acts as a competitive inhibitor of LPS-induced inflammation (7, 8). In this investigation, we utilized the unique LPS inhibitory property of RsDPLA to determine the relative stimulatory effect of CDE *in vitro* and the inflammatory response in the lungs of mice following exposure to CDE in the presence of an endotoxin inhibitor. A priori, we hypothesize that that the inflammatory response to CDE will be diminished in the presence of RsDPLA.

METHODS

Chemicals: Lyophilized *E. coli* 0111:B4 lipopolysaccharide (LPS) was purchased from Sigma Chemical Co., St. Louis, MO. Corn dust used in this study was obtained from the air filtration system at an eastern Iowa grain handling facility. Sterile, pyrogen free water (pfw) and sterile, pyrogen free normal saline (pfs) was purchased from Baxter Medical Laboratories, Deerfield, IL. Hank's balanced salt solution (HBSS) was purchased from the University of Iowa tissue culture facility.

LPS and CDE preparation: LPS solution was prepared by mixing lyophilized *E. coli* LPS in pfw. CDE was prepared by mixing 3.0 grams of dust in 30 ml of pfw (0.1% solution) by methods previously described (5). Both LPS and CDE solutions were filter sterilized through a 0.22μm filter (Acrocap Low Protein Binding Filter, Gelman Sciences, Ann Arbor, MI) and then used immediately or stored at -70° C.

RsDPLA preparation: R. *sphaeroides* ATCC 17023 was grown photoheterotrophically for 12-14 days and harvested by centrifugation. LPS was extracted from R. *sphaeroides* by the phenol-chloroform-petroleum ether method of Galanos (9). RsDPLA was prepared by treating R. *sphaeroides* LPS with 0.02 M sodium acetate (pH 2.5) for 70 min at 100° (10). Crude RsDPLA was dissolved in chloroform-methanol and purified by DEAE-cellulose column chromatography. Pentaacyl RsDPLA was eluted with a linear ammonium acetate gradient and characterized by analytical thin-layer chromatography, gas-liquid chromatography, mass spectrometry, and nuclear magnetic resonance spectroscopy (11). The RsDPLA was in the

free acid form, was solubilized in 0.5% triethylamine salt, and was further diluted in saline prior to use in the experiments.

Endotoxin assay: Endotoxin concentrations were measured in the LPS and CDE solutions using the Limulus Amebocyte Lysate (LAL) assay (QCL-1000, Whittaker Bioproducts, Inc., Walkersville, MD). Measurement of airborne endotoxin concentrations generated from the various LPS and CDE solutions during animal exposure studies were carried out, using methods previously described (12).

Mice: Male, 6 week old, inbred mice (C3H/HeBFEJ) purchased from Jackson Laboratory (Bar Harbor, ME) were used in all exposure studies. They were housed in our institution's rodent vivarium, fed a normal diet (Formulab Chow #5008, PMI, Inc., Richmond, IN) provided water *ad libitum*, maintained on wood chip bedding (Northeastern Product Corp., Warrensberg, NY), and used within 2 weeks. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the University of Iowa.

Exposure apparatus and exposure protocol: A series of four mouse inhalation exposure experiments were performed to aerosols of LPS and CDE, the methods of which we have previously described (12). Exposures were performed in a 20 L exposure chamber, using a PITT#1 nebulizer with solutions for inhalation exposure supplied by a syringe pump. In each experiment, groups of 20 mice underwent inhalation exposure to LPS or CDE solutions over 4 hours. Prior to inhalation challenge, each mouse underwent general anesthesia (Metofane, Pitman-Moore, Mundelein, IL) followed by instillation of equivalent volumes (40 µl) of RsDPLA (10µg) or PFS into the hypopharynx through a 24 gauge Jelco intravenous catheter (Johnson and Johnson, Arlington TX). This dose of RsDPLA was

chosen after performing dose-response experiments with mice which demonstrated that 10 μg of RsDPLA does not cause changes in lung lavage cellularity in comparison to PFS. Following exposure, animals were euthanized by cervical dislocation, the diaphragm was punctured to deflate the lungs, and whole lung lavage was performed.

Lung lavage: Mice trachea were isolated and cannulated with PE-90 tubing, and whole lung lavage was performed *in situ*. One ml aliquots of pfs were infused into the trachea at 25 cm H_20 pressure head, collected, and the process was repeated six times. Lung lavage fluid was processed by our standard method (13).

In vitro endotoxin biologic activity: To measure the *in vitro* cellular response to the RsDPLA, LPS, and CDE, THP-1 cells (ATCC, Rockville, MD), a human monocytic leukemia cell line, were used (14). Cells (passage 20-24) were grown in pyrogen free 10% RPMI media in a 24 well cell culture plate (Costar, Inc., Cambridge, MA) at a density of 1 x 10^6 cells/well at 37°C, 5% CO_2. For dose-response curve experiments, the cells were incubated with RsDPLA at varying concentrations (0, 0.001, 0.01, 0.1, 1.0, 10, 100 μg/ml) in HBSS. For coincubation studies with LPS or CDE, RsDPLA (0-100 μg/ml) was introduced 30 minutes prior to treatment with LPS or CDE (endotoxin concentration 0.02 μg/ml). After 24 hours, the cultures then underwent freeze-thaw cycling twice at -20°C, followed by centrifugation (2500 x g) for five minutes. The supernatants were collected and assayed for TNF-α.

TNF-α Analysis: TNF-α bioactivity was measured using the TNF-α sensitive L929 mouse fibroblast cell assay (15). L929 cells were grown to confluence in a 96 well microtiter plate at a density of 4 x 10^4 cells/well. Fresh 5% DMEM containing actinomycin-D (5 μg/ml) was added

to each well, and each well was pulsed with 100 µl of sample or reference recombinant human TNF-α (Cetus Corporation, Emeryville, CA). Plates were incubated for 20 hours (37°C, 5% CO_2), the media was removed, and L929 cells were stained with 0.5% crystal violet in 20% methanol for 5 minutes. The optical density (OD) of the L929 cells was determined on a microplate reader (Bio-Tek EL311). Sample TNF-α values (units of activity/ml) were determined by comparing the sample OD to a standard curve generated with serial dilutions of reference TNF-α.

Statistical Analysis: Two primary comparisons were made in this study: 1) comparison of the *in vitro* TNF-α production of THP-1 cells exposed to LPS and CDE solutions after pretreatment with RsDPLA; and 2) comparison of the *in vivo* inflammatory response as assessed by both whole lung lavage fluid in mice exposed to LPS or CDE after pretreatment with RsDPLA or sterile saline intratracheally. Statistical comparisons for continuous data were made using the Mann-Whitney U test (16).

RESULTS

The *in vitro* stimulatory effect of RsDPLA on THP-1 cells was determined by performing dose-response experiments in which THP-1 cells were incubated with increasing concentrations of RsDPLA (0.001 to 100 μg/ml) for 24 hours. The supernatant fluid was assayed for TNF-α bioactivity (fig. 1). At relatively low concentrations of RsDPLA (<1 μg/ml), very little TNF-α stimulatory effect was observed, in comparison to higher concentrations of RsDPLA (≥1 μg/ml), which caused substantially greater stimulatory release of TNF-α in a dose-dependent fashion. Although higher concentrations of RsDPLA caused a stimulatory effect on THP-1 cells, this effect was achieved at considerably higher concentration compared to LPS, which stimulates TNF-α release at concentrations as low as 0.02 μg/ml (fig. 2a).

Because of its relative non-stimulatory effect at low concentrations, further studies were carried out to determine whether RsDPLA could display inhibitory properties when incubated with cells prior to LPS or CDE stimulation. Incubation of THP-1 cells with LPS (fig. 2a) or CDE (fig. 2b) alone (endotoxin activity 0.02 μg/ml) resulted in the stimulation of TNF-α production and release from these cells. When THP-1 cells were pretreated with 10 fold increasing concentrations of RsDPLA (0.001-100 μg/ml) prior to stimulation with LPS or CDE, there was a similar pattern in the magnitude of TNF-α release from THP-1 cells for each concentration of RsDPLA. At lower concentrations of RsDPLA (0.001-1 μg/ml), there was a dose-dependent reduction in LPS and CDE stimulatory release of TNF-α, which was maximal in both instances at 1 μg/ml. However, this inhibitory effect

was not apparent at higher concentrations (10 and 100 μg/ml), where RsDPLA stimulates THP-1 cells to produce and release TNF-α (figure 1).

RsDPLA was tested for its *in vivo* ability to stimulate lung inflammation. Mice underwent intratracheal instillation of RsDPLA at doses of 0.1, 1, 10, and 100 μg or PFS and these doses of RsDPLA (except 100 μg) were not found to stimulate neutrophil recruitment in the lung (data not shown). Because of the lack of neutrophil recruitment in the lung lavage fluid after intratracheal instillation of RsDPLA at 10 μg, this dose was chosen for subsequent experiments to determine whether RsDPLA had inhibitor properties when given intratracheally prior to inhalation exposure to LPS or CDE.

Following intratracheal instillation of RsDPLA or pyrogen-free saline (PFS), groups of 10 mice underwent inhalation challenge to LPS or CDE at various airborne endotoxin levels. At high airborne levels of endotoxin (LPS: 7.2 $\mu g/m^3$, CDE: 5.4 $\mu g/m^3$), mice pretreated with PFS developed increasing concentrations of total cells, predominantly neutrophils, as well as elevated concentrations of TNF-α and IL-6 in lung lavage fluid following exposure to both LPS (fig 3a) and CDE (fig 3b). In mice pretreated with RsDPLA, statistically significant reductions in both total cell, neutrophil, and IL-6 concentrations were observed after exposure to LPS or CDE in comparison to PFS pretreated mice exposed to the same bioaerosols ($p < 0.05$ for all comparisons made). At high airborne levels of endotoxin, no differences in lung lavage TNF-α were detected between RsDPLA and PFS treated mice following exposure to either LPS or CDE.

Mice pretreated with PFS followed by exposure to low airborne levels of endotoxin (LPS: 0.28 $\mu g/m^3$, CDE 0.2 $\mu g/m^3$), developed

increased lung lavage concentrations of total cells, neutrophils, TNF-α, and IL-6 (figs. 4a and 4b), but these increases in cellularity and cytokines were not as great in comparison to the high endotoxin exposures (figs. 3a and 3b). Following exposure to LPS, mice pretreated with RsDPLA demonstrated significant reductions in lavage concentrations of total cells, neutrophils, TNF-α, and IL-6 in comparison to control mice pretreated with PFS. Following exposure to CDE, mice pretreated with RsDPLA also demonstrated significant reductions in lavage concentrations of total cells, neutrophils, and TNF-α, but no differences in lavage concentrations of IL-6 were detected between treatment groups.

DISCUSSION

Our study results confirm previous observations that RsDPLA is less potent than LPS at stimulating TNF-α release, but is capable of inhibiting LPS-mediated TNF-α release(7). Likewise, RsDPLA is capable of inhibiting CDE-mediated production and release of TNF-α, suggesting a similar mechanism by which LPS and CDE stimulate THP-1 cells to produce and release TNF-α. Furthermore, when administered to mice intratracheally at a dose of 10 μg, RsDPLA alone does not cause significant lung inflammation. However, when 10 μg of RsDPLA was administered prior to inhalation of either LPS or CDE, this dose was capable of inhibiting LPS and CDE-mediated lung inflammation, again suggesting a common mechanism by which LPS and CDE cause lung inflammation. Our results suggest that CDE causes lung inflammation through endotoxin-mediated inflammatory events, which are inhibited by RsDPLA.

The results of this study add further support to the growing evidence that endotoxin is the principal component in grain dust causing airway inflammation. In epidemiologic studies, we have found that the concentration of endotoxin in the bioaerosol is strongly associated with respiratory symptoms, airflow obstruction, and longitudinal declines in airflow among grain handlers (3). We have also found that higher concentrations of endotoxin in the bioaerosol are associated with accelerated longitudinal declines in airflow (17). In addition, we have investigated the relationship between endotoxin and grain dust induced airway disease through distinct lines of investigation, using a murine model of acute grain dust inhalation challenge. We have demonstrated a clear dose-response relationship between the concentration of endotoxin

in the CDE bioaerosol and the magnitude of lung inflammation and cytokine release in lung lavage fluid (12). A second approach involved determining whether host susceptibility to endotoxin affected the inflammatory response to CDE (12). Utilizing strains of mice which are genetically endotoxin-resistant (C3H/HeJ) or endotoxin-sensitive (C3H/HeBFEJ), we demonstrated that when exposed to an identical CDE exposure, mice which are endotoxin-resistant develop significantly less lung lavage inflammatory cells and cytokines in comparison to endotoxin-sensitive mice. Furthermore, when endotoxin-sensitive mice were made endotoxin tolerant (acquired endotoxin hyporesponsiveness) through serial injections with LPS, these mice developed significantly less airway inflammation in comparison to control mice following the same CDE exposure. A third approach examining the relationship between endotoxin and grain dust induced lung inflammation involved determining whether endotoxin removal from CDE altered the magnitude of CDE-induced lung inflammation. In these experiments, we demonstrated that using three different methods of endotoxin removal from biological solutions, the magnitude of the inflammatory response to CDE was related to the endotoxin concentration in the CDE (5). Finally, in the present investigation, by utilizing the unique properties of RsDPLA which act as a specific inhibitor of endotoxin, we have demonstrated that the inflammatory response to CDE is substantially reduced when this agent is given intratracheally prior to exposure to CDE. Thus, we have demonstrated convincing evidence linking endotoxin with the acute inflammatory response to CDE; by demonstrating a dose-response relationship between endotoxin concentration in the CDE bioaerosol and the magnitude of lung inflammation, by altering the host's ability to

respond to endotoxin, by altering the concentration of endotoxin in CDE, and by selectively inhibiting endotoxin-mediated inflammatory events.

The results of our study are the first to demonstrate the ability of RsDPLA to inhibit endotoxin mediated lung inflammation using an inhalational model of acute lung inflammation. Previous investigations have demonstrated that RsDPLA is capable of blocking the induction of TNF-α from a macrophage cell line treated with LPS in a concentration dependent manner (7). Pretreatment of mice with RsDPLA intravenously blocked the rise in serum TNF-α normally observed in mice following intravenous injection with LPS (18). Furthermore, RsDPLA was protective in preventing endotoxin-mediated death in mice, possibly due to induction of early endotoxin tolerance (19). Our present investigation supports the role of RsDPLA as an inhibitor of CDE-induced lung inflammation through inhibition of endotoxin mediated TNF-α induction *in vitro*. The mechanism by which RsDPLA acts to reduce lung inflammation due to grain dust in our study is not entirely clear, but may be due to direct inhibition of endotoxin binding to cell receptors in the lung or possibly secondary to the induction of early endotoxin tolerance.

Interestingly, mice pretreated with RsDPLA followed by inhalation challenge with either LPS or CDE at high endotoxin concentrations did not develop significant reductions in lung lavage concentrations of TNF-α despite reductions in lavage cellularity and IL-6 levels (fig 3a and 3b). In contrast, TNF-α concentrations in lung lavage fluid were significantly reduced in mice pretreated with RsDPLA exposed to lower concentrations of endotoxin in LPS or CDE (fig 4a and 4b). This observation may be attributed to the possibility that the airborne

levels of endotoxin in the high endotoxin LPS or CDE exposures exceeded the inhibitory capacity of RsDPLA used in the study. In addition, because RsDPLA was administered intratracheally and not by aerosolization, the distribution of RsDPLA in the lung may have differed between these routes of administration, affecting the efficacy of RsDPLA in inhibiting LPS or CDE induced TNF-α release at the higher endotoxin concentrations.

In conclusion, our result confirm prior observations demonstrating the relative non-toxic properties of RsDPLA, including intratracheal lung administration. In addition, we have demonstrated that RsDPLA inhibits grain dust induced lung inflammation, supporting the hypothesis that endotoxin is the primary component in grain dust responsible for the development of airway inflammation.

LEGEND

Figure 1: The mean concentration of TNF-α in the cell lysate of THP-1 cells is presented following incubation with varying concentrations of RsDPLA. Error bars represent the SE.

Figure 2: The mean concentration of TNF-α in the cell lysate of THP-1 cells is shown for cells incubated with LPS (2a) and CDE (2b) alone or pretreated with varying concentrations of RsDPLA. Error bars represent the SE.

Figure 3: The lung lavage mean concentrations of total cells, neutrophils, TNF-α, and IL-6 are presented following inhalation exposure to LPS (3a) and CDE (3b) for mice pretreated intratracheally with RsDPLA (white bars) or sterile saline (black bars). (Airborne endotoxin concentrations measured during each exposure: LPS:7.2 $\mu g/m^3$; CDE:5.4 $\mu g/m^3$) Error bars represent the SE. Asterisk represents a p value < 0.01.

Figure 4. The lung lavage mean concentrations of total cells, neutrophils, TNF-α, and IL-6 are presented following inhalation exposure to LPS (4a) and CDE (4b) for mice pretreated intratracheally with RsDPLA (white bars) or sterile saline (black bars). (Airborne endotoxin concentrations measured during each exposure: LPS:0.28 $\mu g/m^3$; CDE:0.2 $\mu g/m^3$) Error bars represent the SE. Asterisk represents a p value < 0.03.

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figure 1

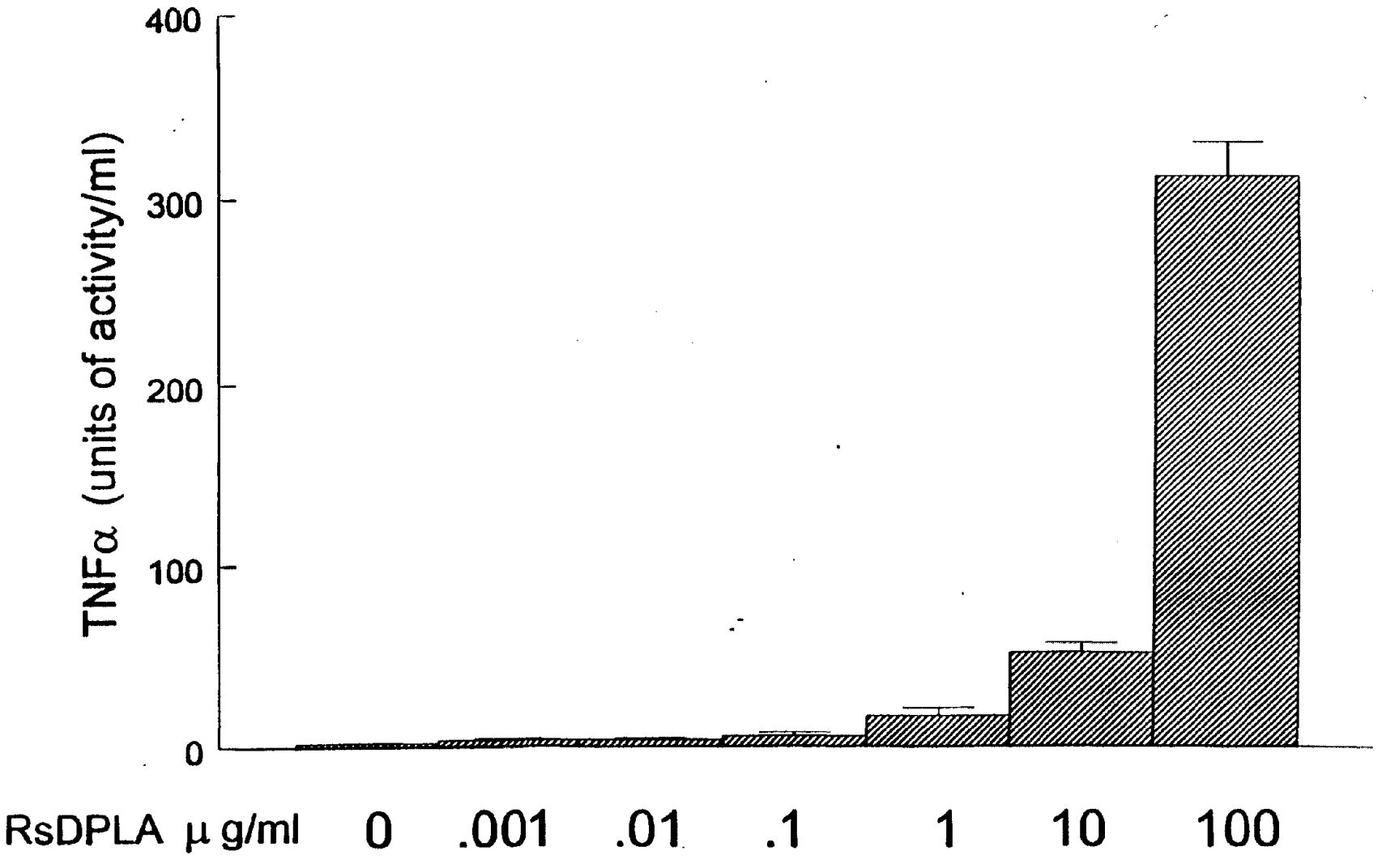
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figure 2a

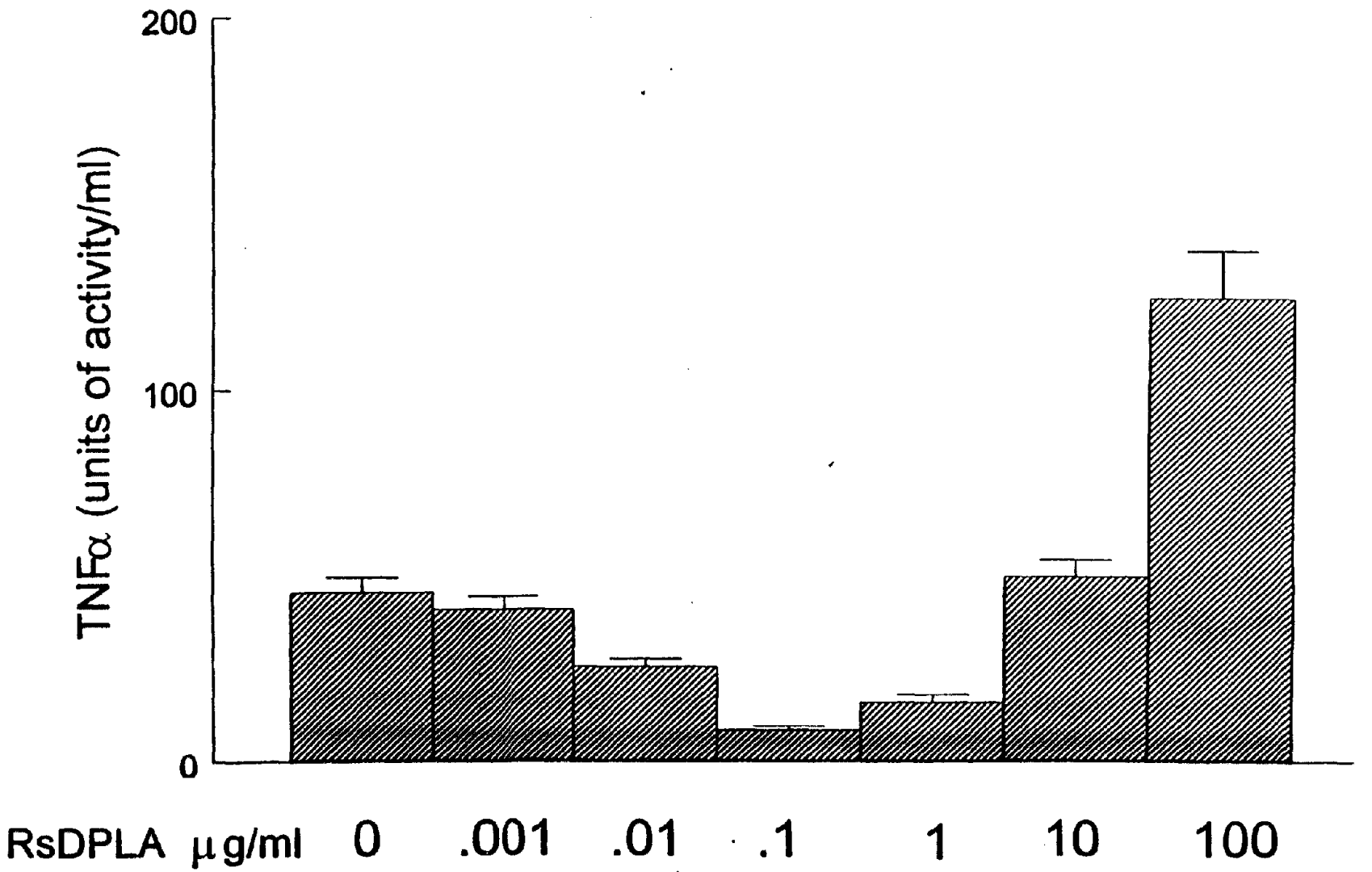
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figure 2b

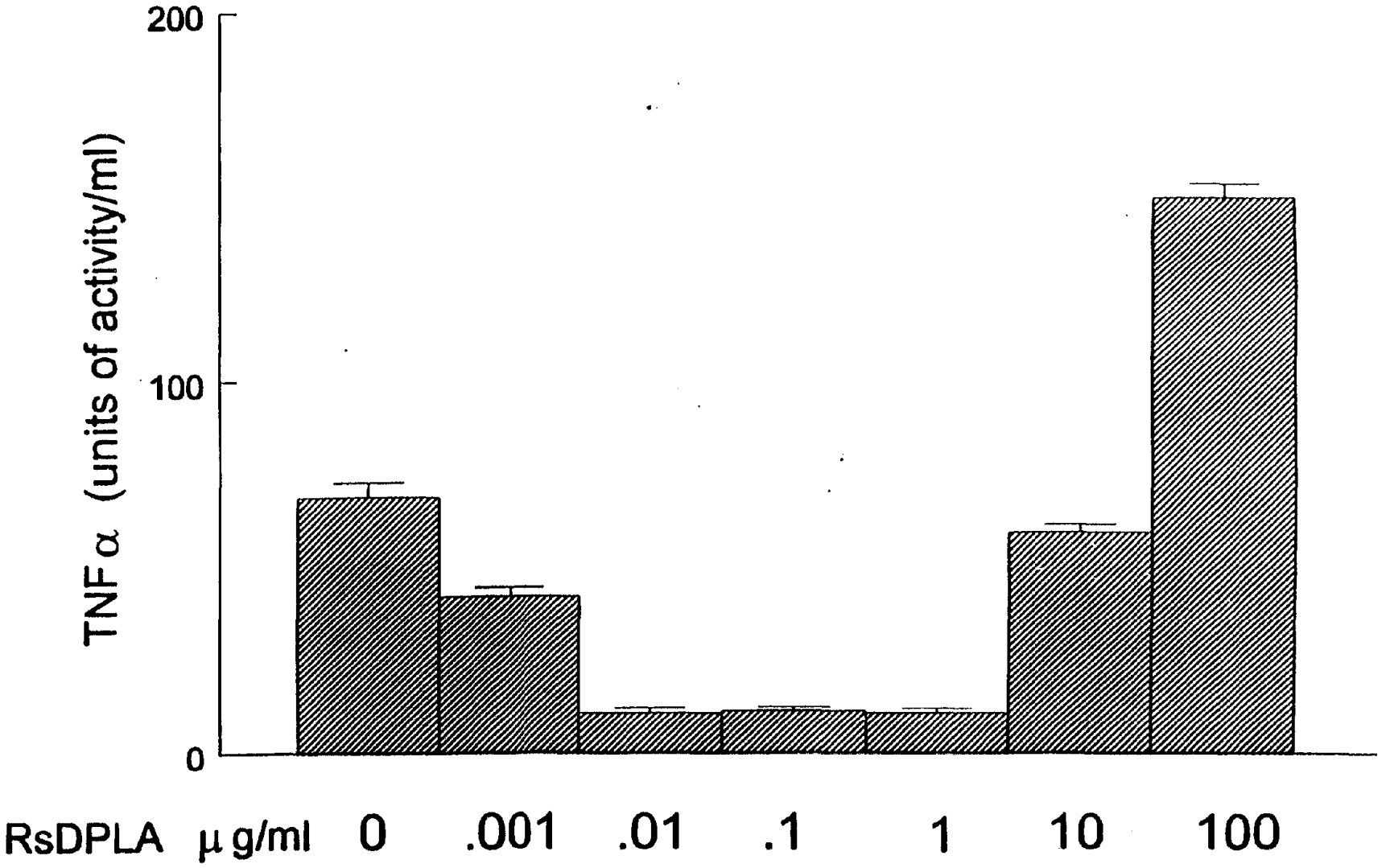
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figure 3a

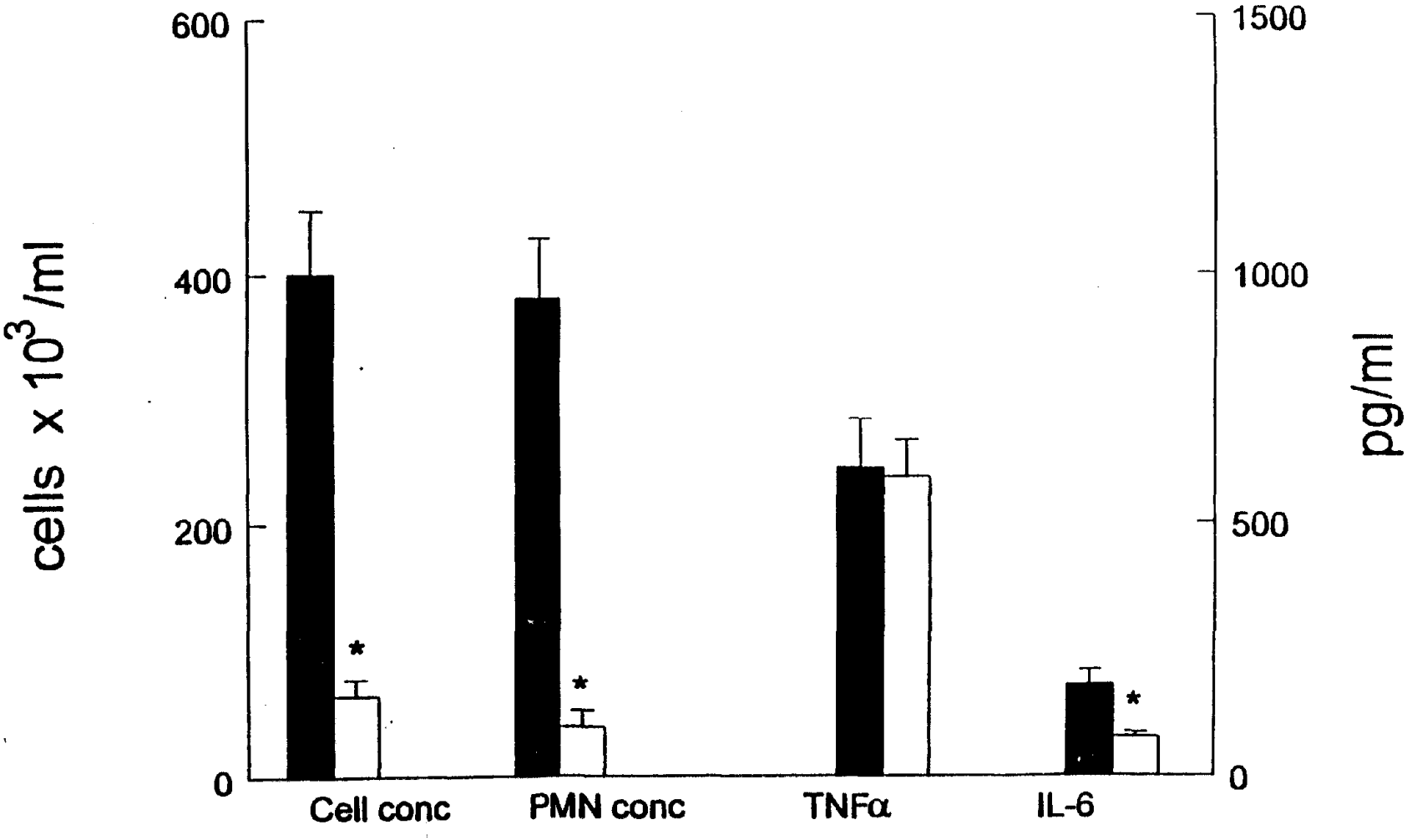
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figure 3b

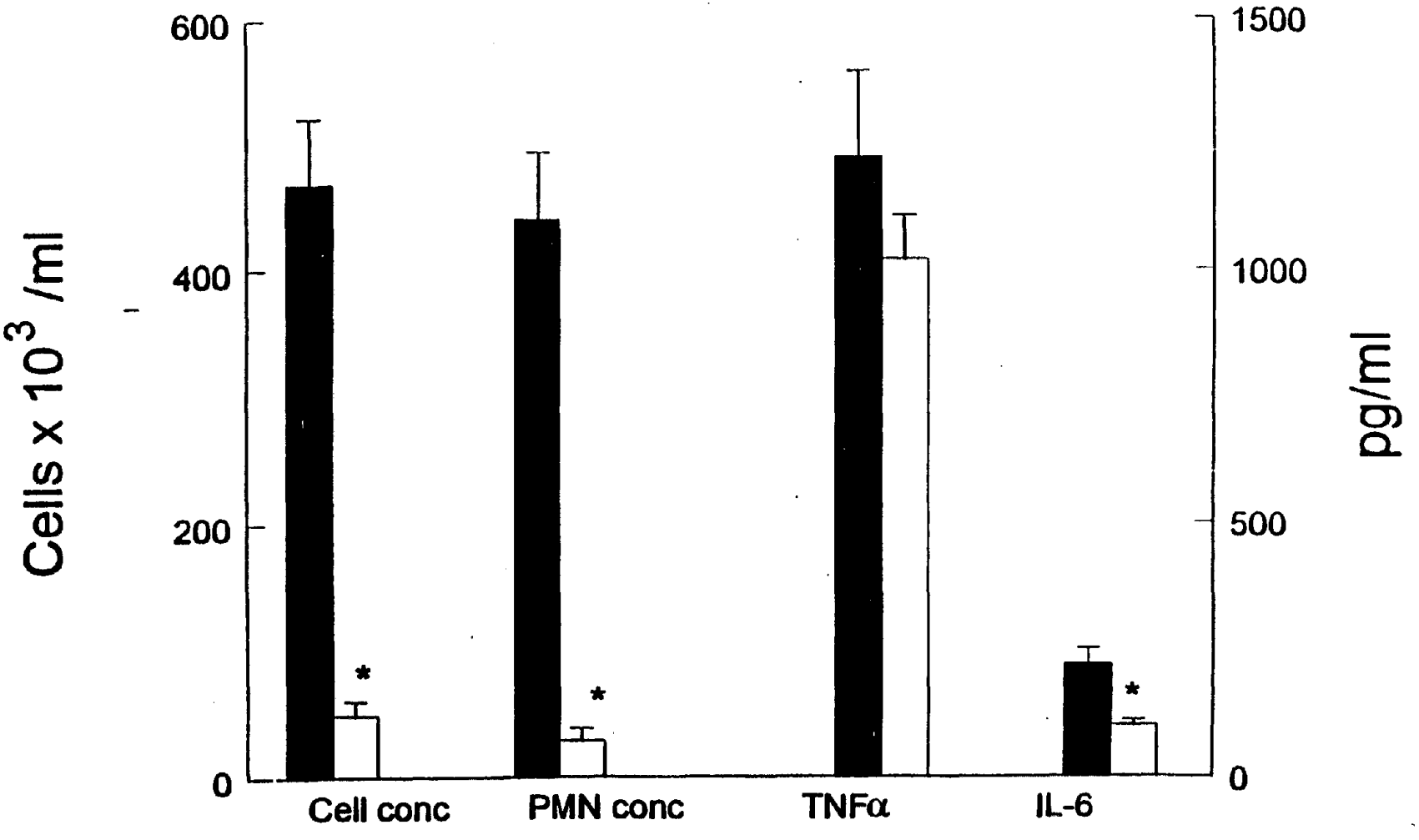
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figure 4a

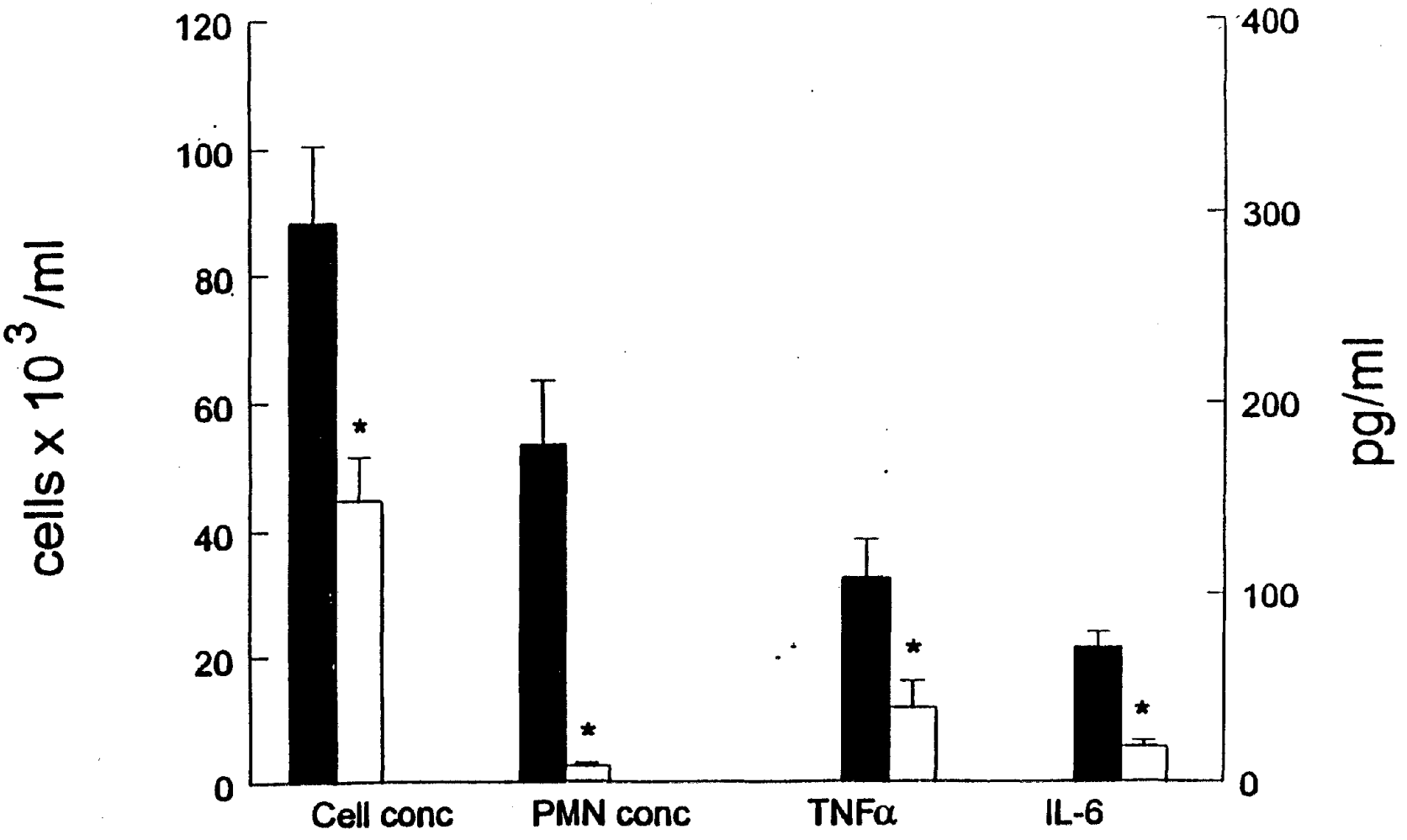
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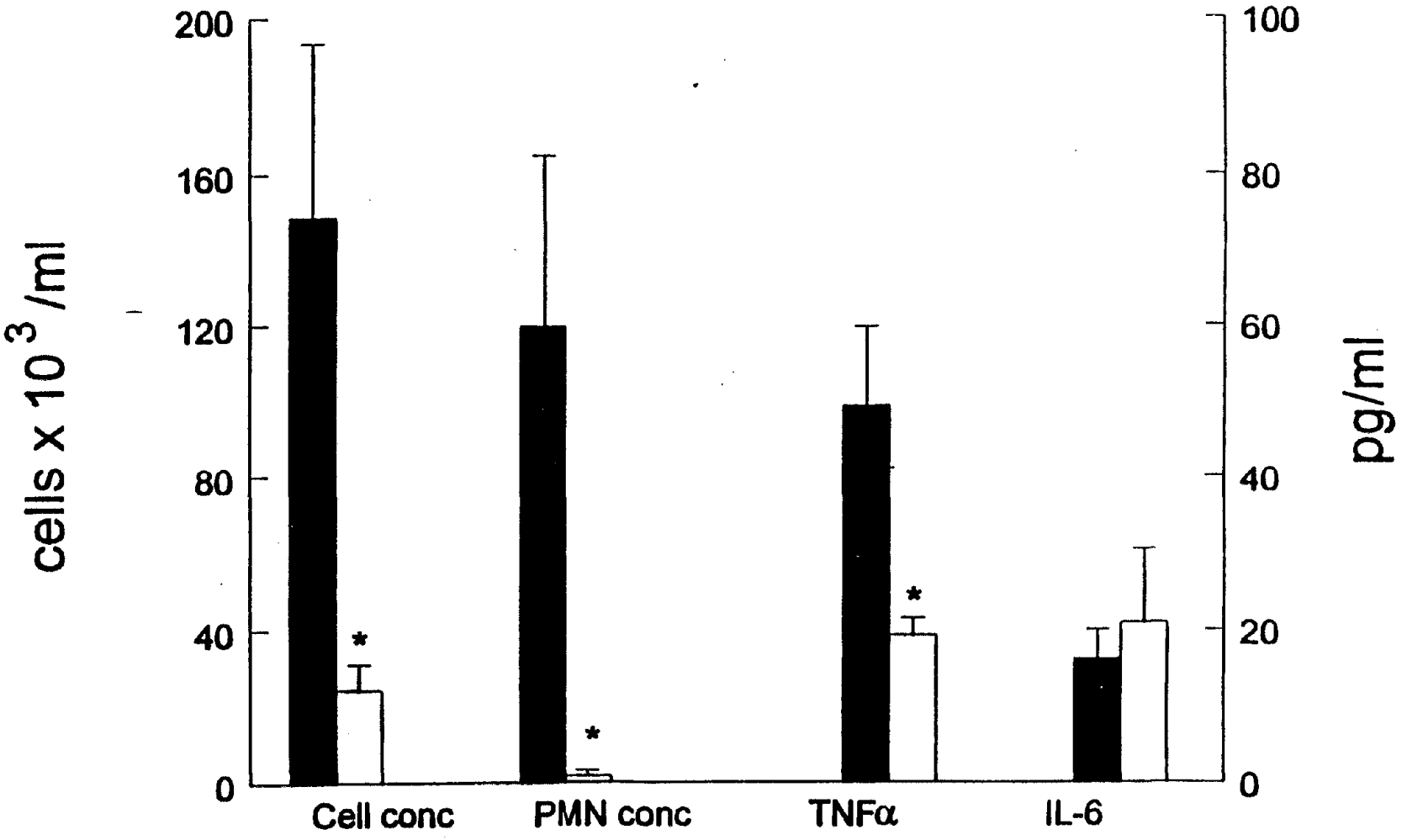
figure 4b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