

ORIGINAL PAPER

Bakery flour dust exposure causes non-allergic inflammation and enhances allergic airway inflammation in mice

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Clinical and
Experimental
Allergy

Summary

Background Baker's asthma is one of the most commonly reported occupational lung diseases in countries where fresh bread is baked daily in large quantities, and is characterized by rhinitis, bronchial hyperresponsiveness, and reversible airflow obstruction. Epidemiological studies have identified pre-existing atopy as an important risk factor for developing baker's asthma, yet the aetiology and pathogenesis of baker's asthma remain poorly understood.

Objective We sought to develop a mouse model of baker's asthma that could be used to characterize the development and progression of baker's asthma.

Methods We were unable to sensitize mice to bakery flour dust or flour dust extract. We assessed total inflammatory cells, cellular differential, total serum IgE and the pro-inflammatory cytokine response to oropharyngeally instilled bakery flour dust or flour dust extract by itself or in the context of ovalbumin (OVA) sensitization and challenge.

Results Both bakery flour dust and flour dust extract consistently elicited a neutrophilic inflammation in a Toll-like receptor 4-independent manner; suggesting that endotoxin is not playing a role in the inflammatory response to flour dust. Moreover, bakery flour dust and dust extract significantly enhance the inflammatory response in OVA-sensitized and challenged mice.

Conclusions Bakery flour dust and flour dust extract are strongly pro-inflammatory and can cause non-allergic airway inflammation and can enhance allergen-mediated airway inflammation.

Keywords allergic asthma, baker's asthma, endotoxin, flour dust, LPS, occupational airways disease, toll-like receptor 4

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Introduction

Baker's asthma was first described 300 years ago by Bernardino Ramazzini in his pioneering work, *De Morbis Artificum Diatriba* [1]. Today, baker's asthma is one of the most frequently reported occupational respiratory diseases in western countries [2], and represents more than 20% of insurance compensations due to occupational asthma in Finland and Canada [3]. Characterized by rhinitis which often accompanies respiratory symptoms such as airflow obstruction and bronchial hyperresponsiveness, baker's asthma has been shown to develop in

work environments in which there is continued exposure to bakery flour dust. Respiratory symptoms in bakery workers are commonly believed to be attributable to wheat flour proteins and supplemental enzymes to improve dough quality [4] and there is data in the literature to suggest that levels of exposure to flour dust correlate with health end-points such as sensitization and respiratory symptoms [5–7]. Additionally, there are studies that identify pre-existing atopy as an important independent risk factor for the development of baker's asthma [8]. While it is generally believed that sensitization to components of bakery flour dust contribute to the development

of baker's asthma [9], many bakers with airway symptoms are not sensitized to flour allergens as measured by specific IgE, skin prick test, or inhalation tests [10]. Interestingly, normal healthy subjects exposed to bakery flour dust have a significant increase in bronchoalveolar lavage (BAL) cellularity over baseline despite having no detectable allergic reaction to flour allergens [11]. These observations taken together suggest that adaptive immunity may not be the only mechanism leading to the development of baker's asthma.

Bakery flour dust is a complex mixture of proteins and other organic materials including bacterial endotoxin [12]. Bacterial endotoxin, or lipopolysaccharide (LPS), is ubiquitous in the environment [13]; especially so in bioaerosols as seen in grain elevators [14]. LPS by itself can contribute to pulmonary neutrophilic inflammation and decrements in lung function in both humans and rodents [15, 16] and can modify the adaptive immune response found in allergic asthma [17]. Our findings indicate that while flour dust causes non-allergic airway inflammation, this is not primarily driven by LPS contamination. Moreover, we found that flour dust can enhance pre-existing allergen-mediated airway inflammation that may be independent of the effects of LPS contamination of bakery flour.

Materials and methods

Animals

C57BL/6J and BALB/c male mice (6–8 weeks) were obtained commercially (Jackson Laboratories, Bar Harbor, ME, USA). Toll-like receptor 4 (TLR4) deficient animals were generously provided by Dr Akira (Osaka University) and backcrossed onto C57BL/6J for at least eight generations. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee at the National Institute of Environmental Health Sciences, and were carried out in accordance with the standards established by the U.S. Animal Welfare Acts.

Sensitization with bakery flour dust or flour dust extract

In our initial attempt to sensitize mice to bakery flour dust or flour dust extract six BALB/c or in separate experiments C57BL/6 mice per group were injected intraperitoneally (i.p.) on day 0 and day 7 with either 10 µg bakery flour dust (European whole grain Type 1 flour product dust; codex R1 lot 18/4; obtained through the National Italian Bakeries Association) or 10 µg flour dust extract (IHRP 82; the generous gift of Drs Adler Dorli and Daniela Roncarolo of Lofarma SpA Milano, Italy) complexed with 2 mg aluminium hydroxide (Pierce, Rockford, IL, USA). On days 14 and 15, mice received saline alone or 0.1 or 1.0 mg

of bakery flour dust, or 0.1 or 1.0 mg of flour dust extract via oropharyngeal aspiration in a total volume of 50 µL endotoxin-free phosphate-buffered saline (PBS) as previously described [18]. These experiments were then repeated. In a subsequent attempt to sensitize mice to bakery flour dust or flour dust extract based on a protocol used to sensitize mice to cockroach antigen, six BALB/c mice per group were injected with 10 µg bakery flour dust or flour dust extract on day 0 emulsified in incomplete Freund's adjuvant. Mice were then given an initial intranasal (i.n.) administration of either 10 µg bakery flour dust or 10 µg flour dust extract on days 14, 22 and 23. Mice were killed and lavaged on day 24.

Flour dust extract

Lofarma IHRP 82 is a sterile low-endotoxin aqueous extract of commercial bakery flour that is used both diagnostically and clinically in allergy immunotherapy (A. M. Cirila, Milan, personal communication). This extract contains all the proteins contained in whole bakery flour dust but lacks the insoluble particulate matter, starches and gluten contained in whole flour. Flour dust extract is prepared as follows. Briefly, an 8% solution of Italian bakery flour (Farina Molino Fiocchi) is prepared in PBS/0.15 M NaCl, pH 7.2 and shaken overnight at 4 °C. This solution is lyophilized and stored at –20 °C. For our experiments, the flour dust extract was resuspended in endotoxin-free PBS immediately before instillation as described.

Flour and extract challenge

Six C57BL/6 mice per group were challenged once with saline, low-endotoxin content bovine serum albumin (< 0.01 ng/mg) (Equitech-Bio Inc., Kerrville, TX, USA), at 0.1, 0.5 or 1 mg/mouse, bakery flour dust at 0.1, 0.5 or 1 mg/mouse or flour dust extract at 12.5, 50 or 125 µg/mouse. All solutions were prepared in endotoxin-free PBS and delivered in a final volume of 50 µL via oropharyngeal aspiration as previously described [18].

Ovalbumin sensitization and challenge

Six C57BL/6 mice per group were sensitized on days 0 and 7 by i.p. injections of 10 µg OVA (Sigma, St Louis, MO, USA) complexed with aluminium hydroxide (Pierce). On day 14, mice received either the amount of LPS found in 1 mg of bakery flour dust as determined by limulus amoebocyte lysate (LAL) (from *Escherichia coli* serotype O111:B4; Sigma Aldrich; 1.26 µg/mouse or 3780 EU/mouse), flour dust, endotoxin-free flour dust or flour extract via oropharyngeal aspiration in a final volume of 50 µL prepared in endotoxin-free PBS. Mice were subsequently exposed daily for 40 min on three consecutive

days to an aerosol of 1% OVA (Sigma) generated using a Collision nebulizer (BGI Inc., Waltham, MA, USA). Animals were harvested 24 h after the last aerosol challenge.

Analysis of airway inflammation and cytokines in whole lung lavage

Whole lung lavage and cell differentials were determined as previously described [19]. TNF- α , IL-5, macrophage inflammatory protein (MIP)-2, KC and IL-17 ELISAs were performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Serum IgE levels were determined by ELISA according to the manufacturer's instructions (PharMingen, San Diego, CA, USA). Total protein was determined by the Lowry method according to manufacturer's instructions (Bio-Rad, Hercules, CA, USA).

Endotoxin assay and lipopolysaccharide removal from flour dust

The LPS content of flour was assayed using the chromogenic LAL assay (QCL-1000, Cambrex Bio Science Inc., Walkersville MD, USA). Bacterial LPS was removed from flour dust using the Endo Trap red kit (Profos AG, Germany). Briefly, a pre-packed column was filled twice with 6 mL regeneration buffer (phosphate-buffer pH 7.4, LPS concentration < 0.02 EU/mL) and then drained out completely. Equilibration buffer (phosphate-buffer pH 7.4 with 80 mM NaCl and LPS concentration < 0.02 EU/mL) was also added and drained out. The flour dust suspension was then applied to the column and the eluate collected. The measured LPS in reagents used in this study were as follows: flour 3780 EU/mg, flour-extract 365 EU/mg, and LPS-free flour 9 EU/mg.

Histology

After lavage, the left lung of each animal was inflation fixed with 10% neutral-buffered formalin at 20 cm H₂O, embedded in paraffin and cut in 5 μ m sections. Full face sections cut parallel to the apex of the pleural surface were stained with hematoxylin and eosin. Histological sections presented in Fig. 6 are 200 $\times$ of original magnification.

Statistical analyses

ANOVA was used for multiple comparisons. Specific comparisons were made using the Student's *t*-test and presented as mean $\pm$ SE. Statistical significance was accepted as *P* < 0.05.

Results

Bakery flour dust and flour dust extract elicit pulmonary neutrophilic inflammation in a toll-like receptor 4 independent manner

In our initial experiments, we attempted to develop a mouse model of baker's asthma based on the OVA model. However, we were unable to successfully sensitize either C57BL/6 or BALB/c mice to bakery flour such that subsequent challenge with flour dust elicited an allergic inflammatory response. In one experiment, we injected BALB/c mice with either bakery flour dust or flour dust extract complexed with alum according to the standard OVA sensitization protocol. We then challenged with saline, 0.1 or 1 mg of whole bakery flour via oropharyngeal aspiration (Fig. 1). The inflammatory response to this attempted sensitization and challenge protocol and to all others (Materials and methods) was neutrophilic. We show in Fig. 1d that in this attempted sensitization protocol, there was no increase in total serum IgE as the measurable levels were consistent with sham-sensitized sham-challenged mice in an OVA protocol (Fig. 1d, left). Had the sensitization been successful, we would have expected total serum IgE to be comparable with that seen in OVA-sensitized OVA-challenged mice (Fig. 1d, left). Despite the absence of atopic sensitization, neutrophilic inflammation in response to the challenges was a consistent feature of these initial studies (Fig. 1c). To address the mechanism by which neutrophils were recruited to the lung in these exposures, we measured protein levels of the neutrophil chemoattractant cytokines MIP-2, the arbitrarily named cytokine KC [20] and IL-17 in whole lung lavage fluid. We show in Figs 1e and f that both MIP-2 and KC are significantly increased as a result of bakery flour exposure in mice we attempted to sensitize with either bakery flour dust or flour dust extract. Interestingly, there was no IL-17 present in whole lung lavage in this experiment or in any subsequent experiment.

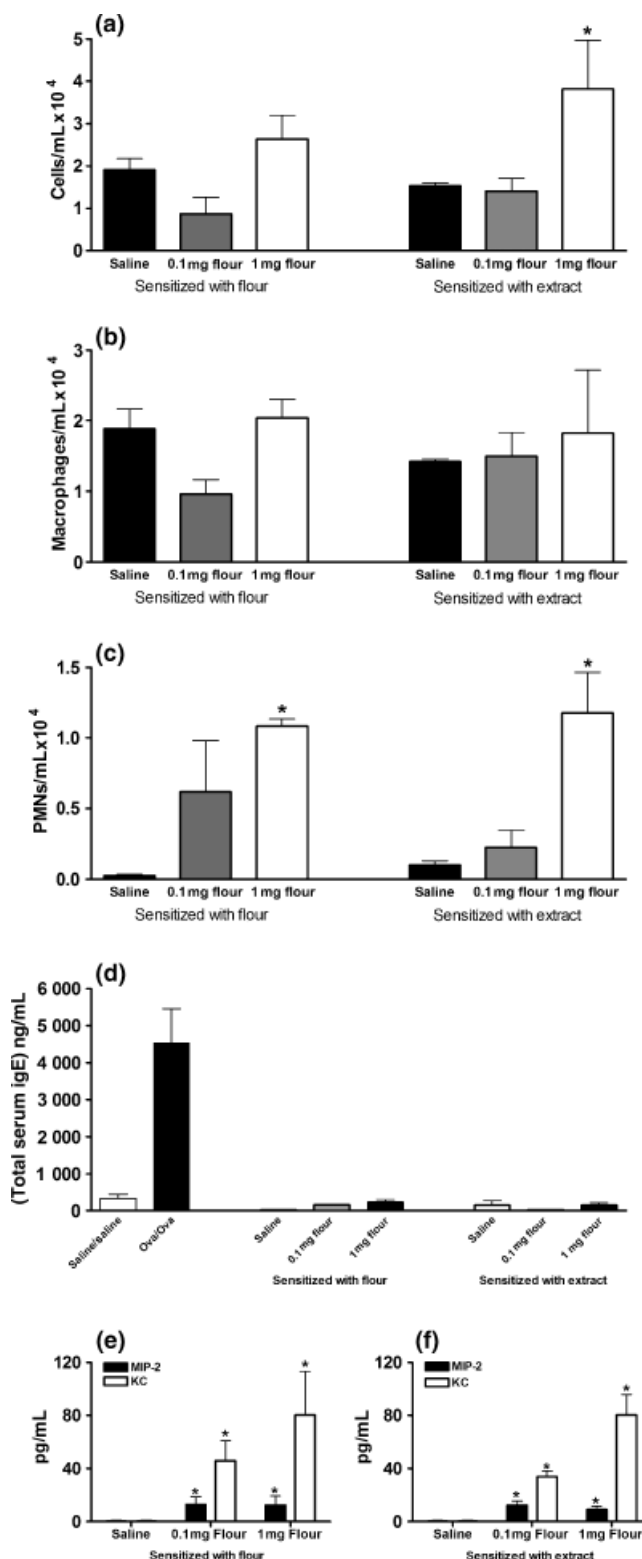
To further define and characterize the inflammatory response to bakery flour dust or flour dust extract, mice were challenged with saline; low, medium or high concentrations of bakery flour dust, flour extract or bovine serum albumin (BSA) as a negative control (Fig. 2). Both bakery flour dust and flour dust extract caused a significant, dose-dependent increase in pulmonary neutrophilic inflammation 4 h after exposure (Fig. 2b). Both MIP-2 and KC protein were induced by instillation of bakery flour dust or flour dust extract as shown in Figs 2e and f. Neither cytokine was induced by instillation of BSA as a negative control. Additionally, both flour and flour dust extract caused a significant increase in the overall number of macrophages present in the lung at the high dose, while bakery flour also caused an increase in macrophages at the medium dose (Fig. 2c). The concentration of TNF- α

protein in whole lung lavage fluid after bakery flour dust instillation was significantly elevated when compared with control groups. TNF- α protein was also significantly elevated at the high dose of extract when compared with control groups. BSA instilled as a control did not elicit

significant pulmonary cellular inflammation or TNF- α protein in whole lung lavage fluid (Fig. 2d).

To address the kinetics of, and to further define the overall inflammatory response to bakery flour dust we exposed C57BL/6 mice to a single instillation of bakery flour and examined the inflammatory response 4 and 24 h afterwards (Fig. 3). Consistent with our earlier observations, both MIP-2 and KC were significantly induced by bakery flour dust when compared with saline controls both 4 and 24 h after instillation. We observed that the absolute number of macrophages in whole lung lavage was increased in both flour and saline instillation groups (Fig. 3b). However, only bakery flour caused an increase in the absolute number of lavageable polymorphonuclear leucocyte or neutrophil. This inflammatory response was more pronounced 4 h after the exposure and both the macrophage and the neutrophilic inflammatory responses were still elevated at 24 h after instillation (Fig. 3b and c).

Because we observed endotoxin contamination in the bakery flour used in these experiments (3780 EU/mg) we asked whether LPS contamination was eliciting the pulmonary inflammatory response. To address this question, we challenged mice deficient for TLR4, the primary LPS receptor, with bakery flour dust. We show in Fig. 3 that the biologic response to flour dust is TLR4-independent as both wild-type and TLR4-deficient mice responded similarly to flour dust instillation. These responses are consistent with previous results in which we show significant increases in total cells, macrophages, neutrophils, TNF- α , MIP-2 and KC when compared with the saline-exposed animals (Fig. 4).



Bakery flour dust and flour dust-extract enhance pre-existing allergic inflammation

Epidemiologic data suggest that bakery flour dust exposure can contribute to the severity of atopy and allergic asthma. To determine the role of bakery flour dust exposure in the context of pre-existing allergic sensitization, we sensitized Th2 pre-disposed BALB/c mice to OVA and subsequently challenged them to OVA after saline, flour dust, flour extract or LPS-free flour instillation. We observed a significant increase in total cells with pre-

Fig. 1. Inflammatory response of BALB/c mice injected intraperitoneally (i.p.) with alum and either bakery flour dust (sensitized with flour) or flour dust extract (sensitized with extract) according to the ovalbumin protocol, and then challenged with either saline or flour as indicated. Total pulmonary inflammatory response (a), macrophages in whole lung lavage (b), neutrophilic inflammatory response (c), total serum IgE (d), macrophage inflammatory protein (MIP)-2 and KC in mice injected i.p. with alum and flour before flour challenge (e) and MIP-2 and KC in mice injected i.p. with alum and extract before flour challenge (f). Data presented are mean $\pm$ SEM. * P < 0.05 vs. saline. PMN, polymorphonuclear leucocyte or neutrophil.

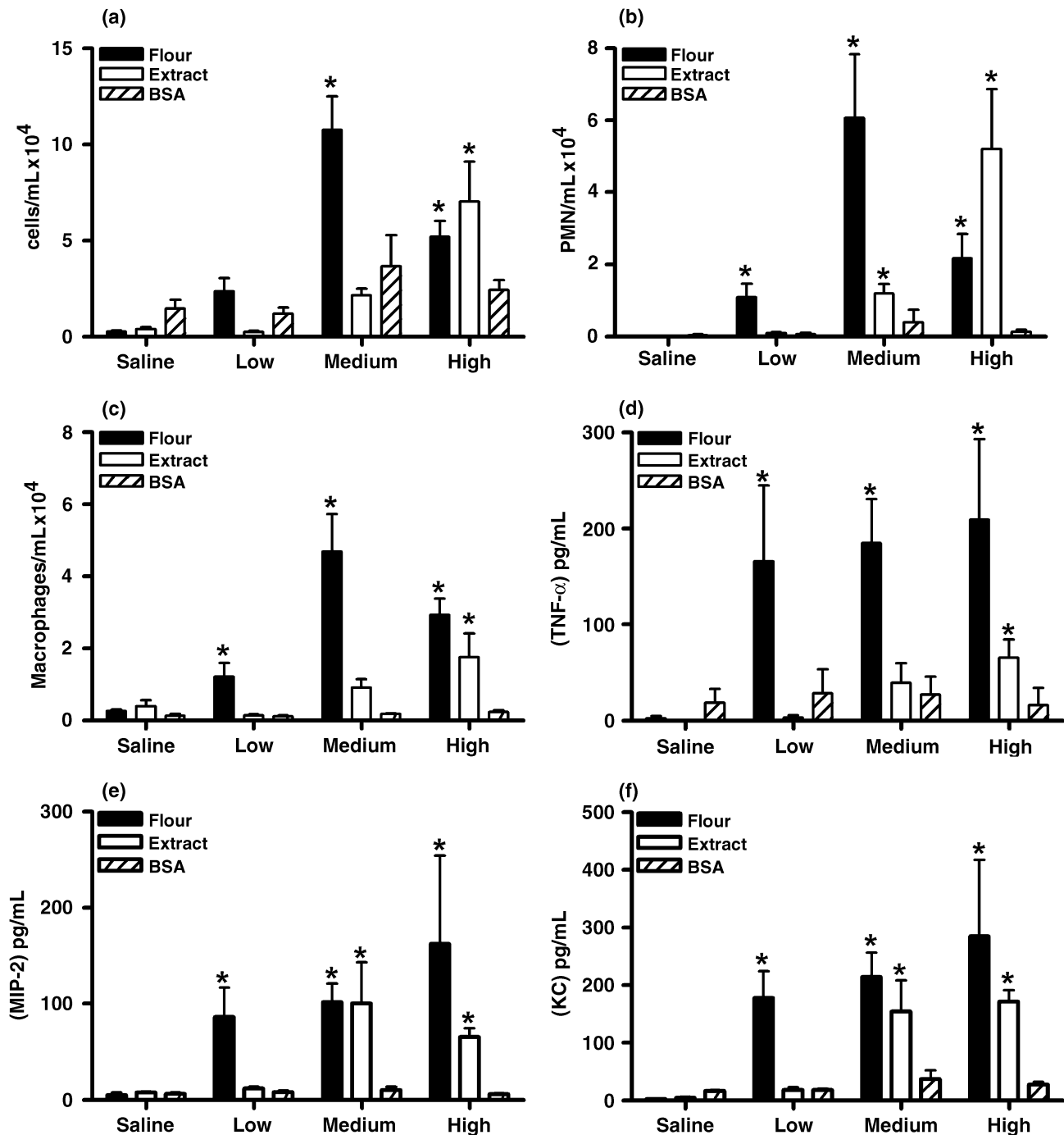


Fig. 2. Inflammatory response to bakery flour dust, flour dust extract or bovine serum albumin (BSA). Total pulmonary inflammatory response (a), neutrophilic inflammatory response (b), macrophage inflammatory response (c), lavageable TNF- α response (d), lavageable macrophage inflammatory protein (MIP)-2 (e) and lavageable KC (f) in response to low, medium or high (as described in 'Materials and methods') concentrations of bakery flour dust, flour dust extract or BSA. Data presented are mean $\pm$ SEM. * P < 0.05 vs. saline. PMN, polymorphonuclear leucocyte or neutrophil.

exposure to flour or flour-extract (Fig. 5a). Mice pre-treated with LPS in the same amounts found in 1 mg of bakery flour dust were not different in total number of inflammatory cells (Fig. 5a) when compared with mice pre-treated with saline. Mice pre-exposed to either flour or LPS-free flour had significantly enhanced macrophage

(Fig. 5b) and eosinophilic (Fig. 5d) inflammation consequent to OVA challenge. Additionally, there was a trend towards a significant increase in eosinophils with flour-extract pre-exposure. We also show significantly elevated TNF- α (Fig. 5e) and IL-5 (Fig. 5f) protein in whole lung lavage in mice pre-exposed to LPS, flour or LPS-free flour

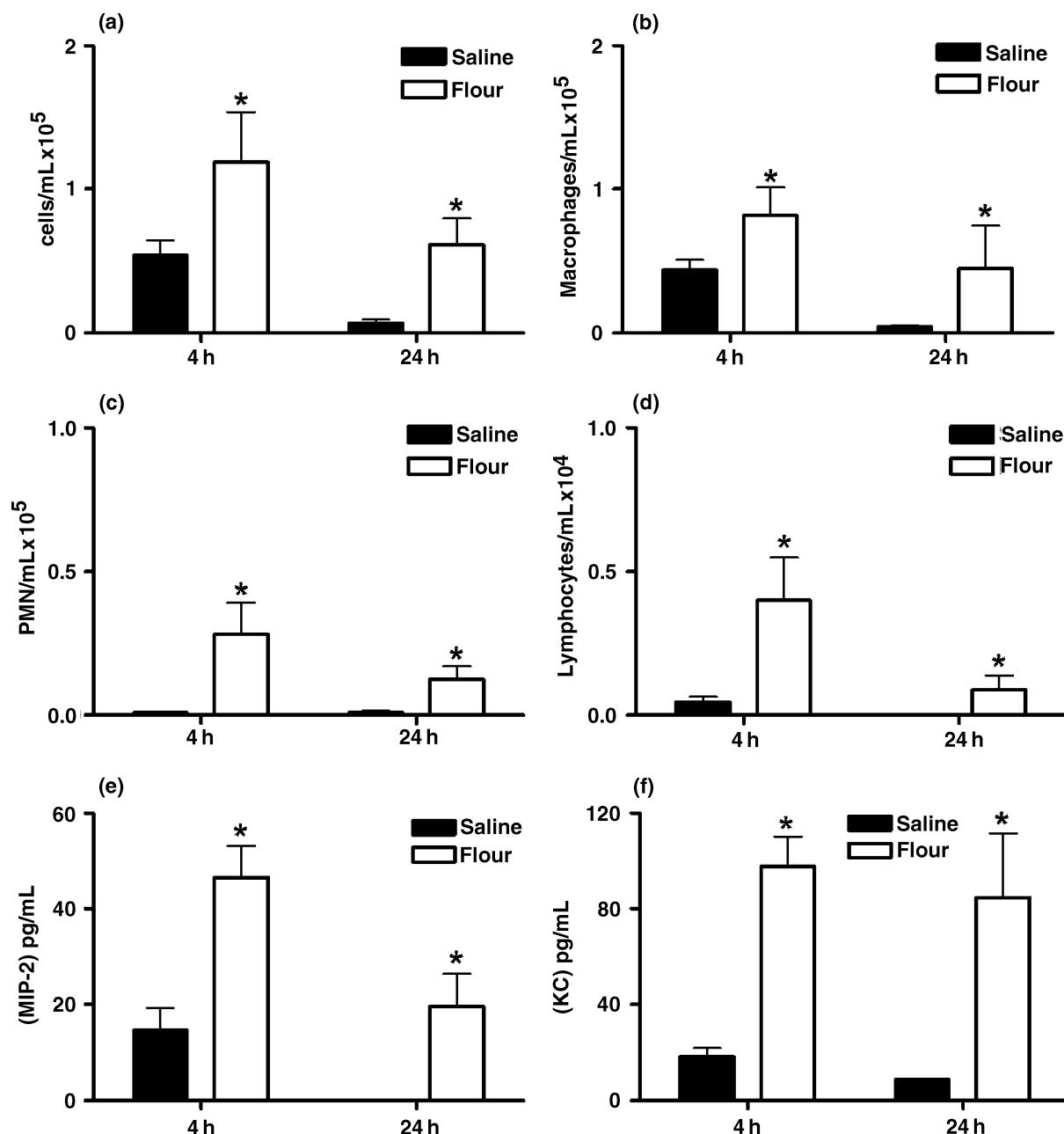


Fig. 3. Time course of pulmonary inflammatory response to high dose flour exposure (as described in 'Materials and methods'). Total pulmonary inflammatory response (a), macrophage inflammatory response (b), neutrophilic inflammatory response (c), lymphocytic inflammatory response (d), total lavageable macrophage inflammatory protein (MIP)-2 (e) and KC (f) are presented. Data presented are mean $\pm$ SEM. * P < 0.05 vs. saline. PMN, polymorphonuclear leucocyte or neutrophil.

but not flour extract. Total serum IgE for all exposures (Fig. 5g) was also significantly elevated over sham-sensitized sham-challenged (saline/saline) controls although the flour pre-exposed mice did not have enhanced total serum IgE compared with sensitized mice pre-exposed to saline before OVA challenge (Fig. 5g, black bar; saline). Consistent with these observations, we show in Fig. 6 that vascular cuffing is increased in OVA-sensitized and challenged mice challenged with bakery flour dust instillation (Fig. 6b) when compared with OVA-

sensitized and challenged mice instilled with saline alone (Fig. 6a).

Discussion

A more clear understanding of the biological response to organic dust exposure in the work environment has the potential to be of considerable clinical significance. We show here for the first time, that exposure to flour dust can exacerbate allergen-mediated airway inflammation and

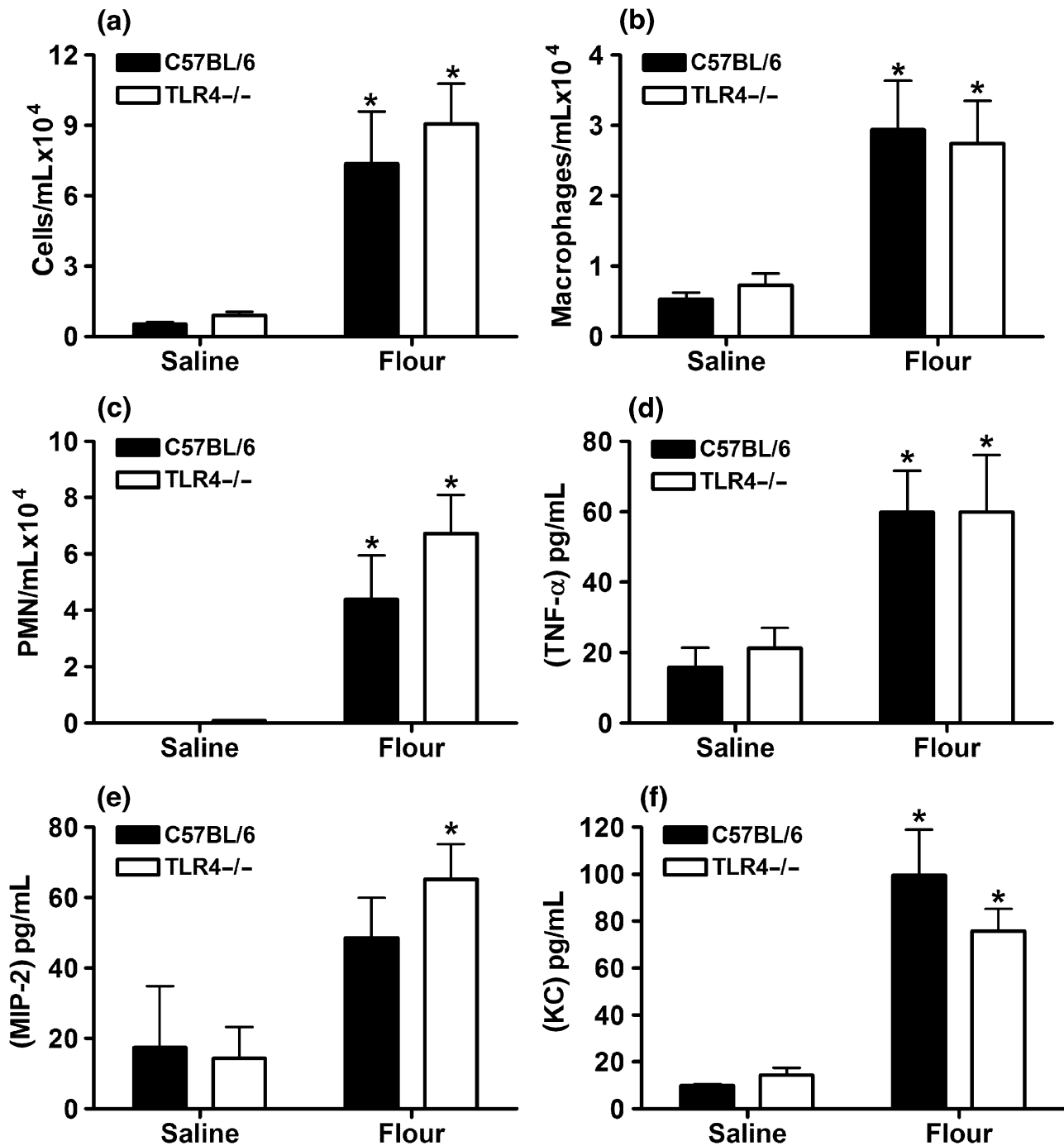


Fig. 4. Inflammatory response of C57BL/6 and toll-like receptor (TLR) 4-deficient mice to high dose bakery flour dust (as described in 'Materials and methods'). Total pulmonary inflammatory response (a), macrophage inflammatory response (b), neutrophil inflammatory response (c), lavageable TNF- α (d), macrophage inflammatory protein (MIP)-2 (e) and KC (f) are presented. Data presented are mean $\pm$ SEM. * P < 0.05 vs. saline. PMN, polymorphonuclear leucocyte or neutrophil.

can lead to acute airway inflammation mediated by increased MIP-2 and KC, yet independent of sensitization to flour dust and independent of the endotoxin content of the dust.

Previous hypotheses have focused on increased levels of ambient flour dust that may be associated with enhanced sensitization to flour proteins [21, 22]. This is

supported by the detection of specific IgE antibodies against wheat in some studies [23, 24], although wheat protein specific IgE is not observed in all cohorts of baker's asthma [25]. Ambient flour dust exposure is exceedingly high in most bakeries, ranging from 1 to 10 mg/m³ of respirable air [8, 26] with frequent peaks of

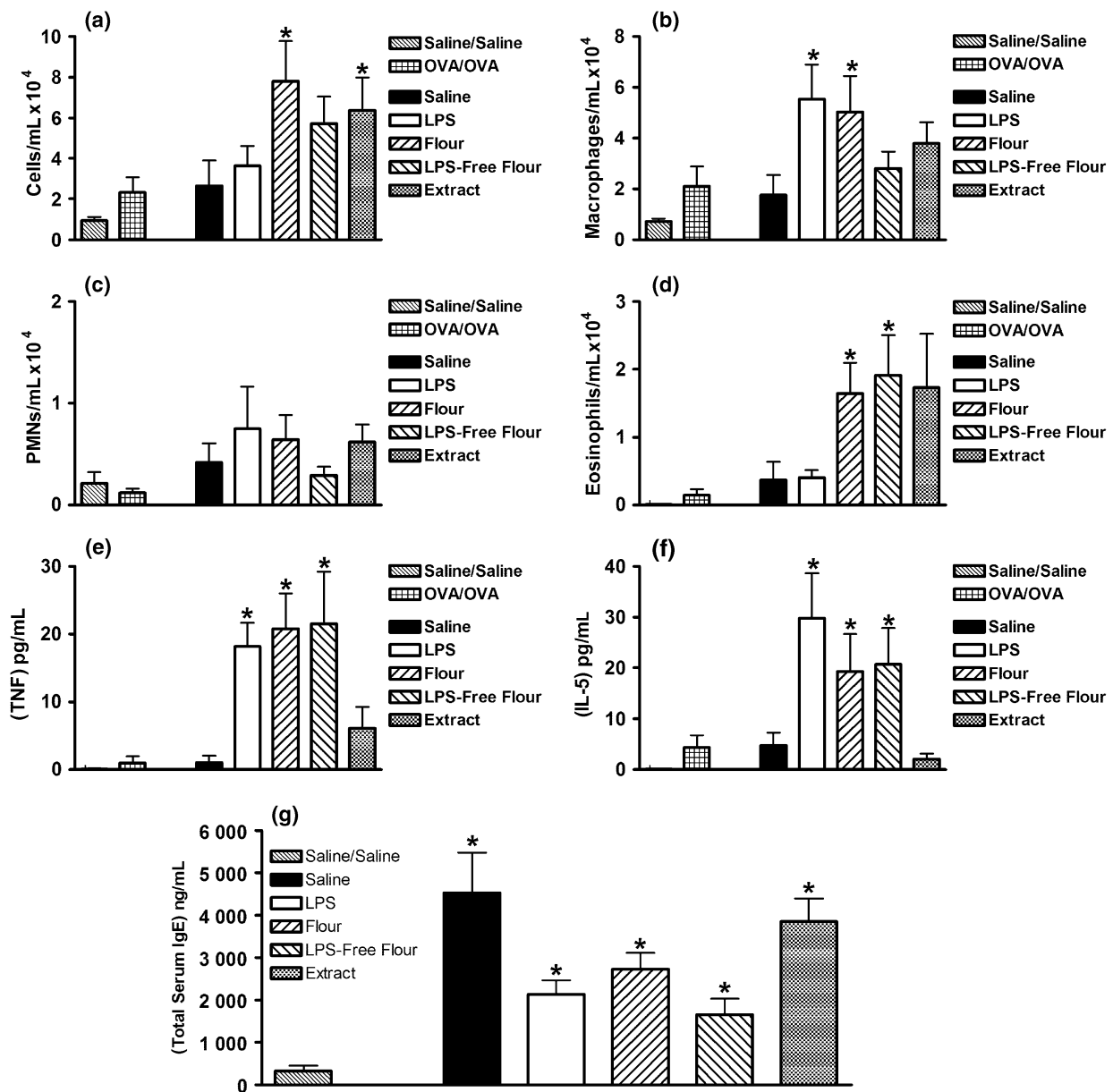


Fig. 5. Pulmonary inflammatory response to high dose bakery flour dust, lipopolysaccharide-free bakery flour dust, or flour dust extract (as described in 'Materials and methods') in the context of ovalbumin(OVA) sensitization and challenge. Total pulmonary inflammatory response (a), macrophage inflammatory response (b), neutrophils (c), eosinophilic inflammatory response (d), lavageable TNF- α (e), lavageable IL-5 (f) and total serum IgE (g) are presented. Data presented are mean $\pm$ SEM. * $P < 0.05$ vs. saline. LPS, lipopolysaccharide; PMN, polymorphonuclear leucocyte or neutrophil.

greater than 50 mg/m^3 [10]. Importantly, however, ambient levels of wheat proteins in the air have not reproducibly been associated with the frequency of atopy and asthma [27]. Thus, it is unclear whether patients with baker's asthma represent a distinct population of individuals sensitized to wheat proteins. To address this question experimentally, we first tested the possibility that bakery flour dust could act as a specific allergic antigen. We attempted to develop a mouse model of baker's asthma based on previously published allergic sensitization and challenge models (Materials and methods) with bakery

flour dust or flour dust extract. In at least four separate experiments, we failed to observe an allergic inflammatory (eosinophilic) response to airway challenge with either bakery flour dust or dust extract consequent to i.p. sensitization. However, we consistently observed enhanced neutrophilic inflammation in response to subsequent challenge with flour dust or dust extract in those experiments. Those observations led us to hypothesize that inhalation of flour dust may contribute directly to airways disease in a non-allergen sensitization dependent manner. We reasoned that endotoxin contamination of the

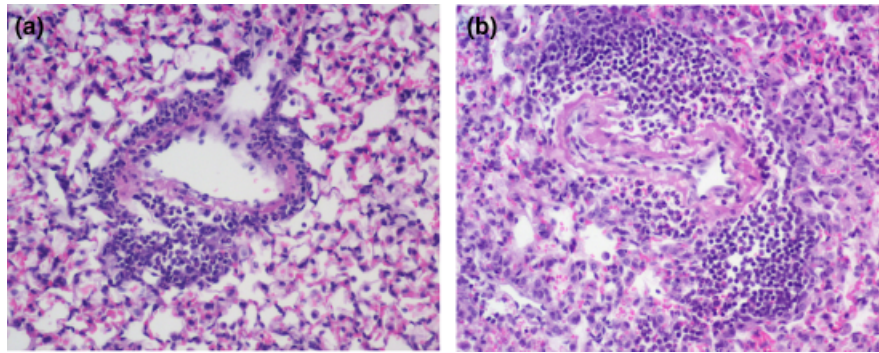


Fig. 6. Vascular cuffing in BALB/c mice sensitized with ovalbumin (OVA) and pre-challenged with either saline (a) or high dose flour (b) (as described in 'Materials and methods') before OVA challenge.

bakery flour dust or the dust extract might be causing the observed neutrophilic inflammation and that this might in turn explain the lack of a clear correlation between sensitization to wheat allergens and baker's asthma.

While we found that commercial bakery flour dust contains high concentrations of bacterial endotoxin, our results show surprisingly, that the pro-inflammatory activity of bakery flour dust is not dependent on the ability of the experimental animals to respond to endotoxin. This is supported by the observation that bakery flour dust containing significant amounts of endotoxin causes acute increases in the presence of MIP-2 and KC and neutrophilic inflammation in the lungs of TLR4-deficient mice. We additionally show that flour dust extract, which contains dramatically reduced amounts of endotoxin compared with whole bakery flour dust, also elicits the same response. Thus, the role that allergic sensitization to flour proteins may play in the development of baker's asthma remains an area of debate.

Because we were unsuccessful in sensitizing mice to flour proteins, we considered the possibility that the acute inflammatory response to flour might exacerbate pre-existing allergic asthma in bakers. To address this possibility experimentally, we examined the effects of exposure to bakery flour dust in a murine model of pre-existing allergic sensitization. Exposure to flour dust, endotoxin-free flour dust, or flour dust extract, but not LPS by itself, before allergen challenge significantly enhanced allergic inflammation, supporting our hypothesis that bakery flour could enhance existing asthma in atopic/allergic individuals. Our observation that bakery flour dust is more potent than flour dust extract in stimulating an inflammatory response, taken with the lack of a clear correlation between sensitization to wheat allergens and baker's asthma suggest that it is not only sensitization to wheat antigens in bakery flour that may determine pre-disposition to baker's asthma. Other non-protein components of the flour may contribute to the development of this disease. Additionally, some patients diagnosed with baker's asthma may in fact be pre-disposed to allergic

asthma independent of sensitization to wheat allergens and the occupational exposure may reveal this pre-disposition. An alternative hypothesis is that the inflammatory response observed in both C57BL/6 and BALB/c mice is a mild form of contact dermatitis or the early stages of hypersensitivity pneumonitis. We are not aware of any data that suggests that bakers have an increased incidence of extrinsic allergic alveolitis or hypersensitivity pneumonitis although recent case reports show that yeasts can be associated with this pathology [28, 29]. These hypotheses remain to be tested.

We have shown that flour dust is strongly pro-inflammatory and can cause non-allergic airway inflammation and can enhance allergen-mediated airway inflammation. Our results suggest that neither endotoxin in the flour dust nor allergic sensitization to wheat flour proteins is pathogenically related to the airway inflammatory response in our experimental system. Nevertheless, closer regulation of levels of bakery flour dust exposure could have a substantial impact on preventing of baker's asthma in the workplace; preventive measures and health surveillance of workers with higher risk, such as atopy or previous respiratory symptoms, are strongly recommended.

Acknowledgements

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