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Pre-exposure to Staphylococcal enterotoxin A exacerbates the pulmonary allergic eosinophil recruitment in rats

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ABSTRACT

Gram-positive *Staphylococcus aureus* releases classical enterotoxins which aggravates allergic airway diseases. However, little is known about the mechanisms underlying the cell influx exacerbation in asthmatic individuals under exposure to Staphylococcal enterotoxins. We therefore aimed to investigate the effects of airways exposure to Staphylococcal enterotoxin A (SEA) to pulmonary leukocyte recruitment in rats sensitized and challenged with ovalbumin (OVA). Rats were exposed to SEA at 4 h prior to OVA challenge or at 4 h post-OVA challenge. Bronchoalveolar lavage (BAL) fluid, bone marrow and lung tissue were obtained at 24 h after OVA challenge. Pre-exposure to SEA markedly enhanced the eosinophil counts in both BAL fluid and pulmonary tissue in OVA-challenged rats, whereas neutrophil and mononuclear cell counts remained unchanged. In bone marrow, pre-exposure to SEA alone significantly increased the number of eosinophils, and that was further increased in OVA-challenged rats. Exposure to SEA post-OVA challenge did not affect the number of eosinophils, neutrophils and mononuclear cells in BAL fluid. Pre-exposure to the endotoxin lipopolyssacharide (LPS) in OVA-challenged animals rather enhanced the neutrophil number in BAL fluid. In rats pre-exposed to SEA and OVA-challenged, a marked elevation in the levels of TNF-α and eotaxin (but not of IL-10) in BAL fluid was observed. The eotaxin levels increased by about of 3-fold in alveolar macrophages treated with SEA in vitro. In conclusion, airways pre-exposure to SEA causes a selective increase in eosinophil number in BAL fluid and bone marrow of OVA-challenged rats by mechanisms involving enhancement of TNF-α and eotaxin synthesis.

1. Introduction

Staphylococcus aureus is a gram-positive bacterium that produces and secretes the so-called staphylococcal enterotoxins, which are a family of structurally related heat-stable 25 to 30 kDa proteins. These enterotoxins comprise several serological types, namely the classical types A to E, and the newly characterized types G to Q [1]. Two novel genes coding for enterotoxins types S and T have been recently described [2]. Among the Staphylococcal enterotoxins, toxins A and B can be easily obtained in relatively large amounts and purity [3], and therefore has been used to a great extent in experimental investigations. In addition to the well-known ability to cause food poisoning [4], Staphylococcal enterotoxins often lead to multiorgan dysfunction [5] that may be a consequence of the release of several inflammatory mediators such as cytokines (IL-1, IL-2, IL-6, IL-8, IFN-γ, TNF-α), mast cell-derived amines (histamine and 5-hydroxytriptamine), neuropeptides (substance P), lipid mediators (PGE_2 and PAF), amongst others [6–12]. Moreover, Staphylococcal enterotoxins are considered prototypical superantigens, which interacts with the outside of the MHC molecule and V-beta chain of T-cell-receptor without the need for internalization, processing, or presentation within the confines of a major histocompatibility complex [13].

S. aureus, which often asymptomatically colonize the upper respiratory tract, has been associated with allergic diseases. A correlation between levels of IgE antibodies to staphylococcal enterotoxins and the severity of eosinophilic inflammation in upper airway disease has been found [14–16]. Polyvalent sensitization with Staphylococcal enterotoxins A and B has been associated with increased IgE reactivity leading to exacerbation of allergic responses in humans [17]. However, little is known about the mechanisms underlying such pulmonary inflammatory exacerbation in asthmatic individuals under exposure to Staphylococcal enterotoxins. An experimental study showed that mice cutaneous sensitization with Staphylococcal enterotoxin B cause allergic skin inflammation and increased serum IgE levels [18]. Later, nasal and bronchial exposure to Staphylococcal enterotoxin B was shown to enhance the allergic pulmonary eosinophilic inflammation in ovalbumin (OVA)-sensitized mice, which was associated with increased mRNA expression of IL-5, IL-4, IFN-γ, IL-12 p40, eotaxin-1 and TGF-β as well as IgE titers in the serum [19]. However, no studies exist

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investigating the potential interactions between airways exposure to Staphylococcal enterotoxin A (SEA) and pulmonary allergic inflammation in experimental animals to help in the comprehension of the mechanisms underlying the exacerbation of allergic diseases by Staphylococcal enterotoxin exposure. Therefore, this study initially tested the hypothesis that exposure to SEA enhances the airways cell recruitment (with emphasis in eosinophil influx) in rats actively sensitized and challenged with ovalbumin (OVA), and next investigated the contribution of Th-cytokines in the resulting cell infiltration. The effects of airways exposure to the endotoxin lipopolyssacharide (LPS), the main structural component of the outer membrane of Gram-negative bacteria, have been compared with those of SEA.

2. Material and methods

2.1. Animals

The experimental protocols were approved by the Ethical Principles in Animal Research adopted by the Brazilian College for Animal Experimentation (COBEA). All experimental procedures were approved by the Animal Care and Use Committee of the State University of Campinas (UNICAMP). Male Wistar rats (200–250 g) were housed in temperature-controlled rooms and received water and food *ad libitum* until used.

2.2. Sensitization and challenge with ovalbumin (OVA)

We actively sensitized the animals by subcutaneous injections of 200 μg OVA dissolved in 0.15 ml of aluminum hydroxide (200 mg/ml in 0.9% NaCl), according to our previous experience [20]. Non-sensitized animals were injected with aluminum hydroxide alone. Fourteen days later, sensitized (and non-sensitized) rats were intranasally challenged with 1 mg of OVA (0.2 ml) or instilled with phosphate-buffered saline (PBS; 0.2 ml). Bronchoalveolar lavage (BAL) fluid, bone marrow and lung tissue were obtained at selected times thereafter, and processed as detailed below.

2.3. Experimental protocols

In the first series of experiments, at 4 h prior to OVA challenge (or PBS instillation), rats were pre-exposed (intranasal administration) to 3 ng of SEA (Sigma Chem. Co., St Louis, MO, USA) or PBS (0.2 ml). At 24 h thereafter, rats were anaesthetized, and BAL fluid obtained. In some protocols, bone marrow was also obtained. The exposure time (4 h) and dose of SEA (3 ng) was based on our previous experience in rats [21].

In the second series of experiments, at 4 h after OVA challenge (or PBS instillation), rats were intranasally exposed to SEA (3 ng) or PBS (0.2 ml). At 24 h thereafter, rats were anaesthetized, and BAL fluid obtained.

In the third series of experiments, at 4 h prior to OVA challenge (or PBS instillation), rats were pre-exposed (intranasal administration) to either *Salmonella abortus equi* LPS (0.1 and 1.0 μg/rat; Sigma Chemical Co., St Louis, MO, USA) or PBS (0.2 ml). At 24 h thereafter, rats were anaesthetized with halothane inhalation, and BAL fluid obtained.

2.4. Bronchoalveolar lavage (BAL) fluid and leukocyte counts

The BAL fluid was obtained at 12, 24 or 48 h after OVA challenge. The lungs were washed by flushing with PBS solution containing heparin (20 UI/ml). Briefly, the trachea was cannulated with a polyethylene (1 mm in diameter) connected a syringe. The lungs were washed by flushing with PBS solution containing heparin (20 UI/ml). The PBS buffer was instilled through a tracheal cannula as one 10-ml aliquot followed by three 5-ml aliquots. The first 10 ml aliquot was centrifuged (450 ×*g* for 10 min at 20 °C), and supernatant recovered and stored at −80 °C for measurement of inflammatory mediators. The three 5-ml aliquots were pooled and centrifuged (450 ×*g* for 10 min at 20 °C). The cell pellet obtained from all aliquots (the 10-ml together with those of the 5-ml aliquots) were combined and resuspended in 2 ml of PBS solution. Total leukocyte counts were performed in Neubauer chamber, while differential counts were carried out on a minimum of 200 cells using cytospin preparation stained with Diff-Quick. The leukocytes were classified as neutrophils, eosinophils and mononuclear cells based on normal morphological criteria.

2.5. Leukocyte counts in bone marrow

After OVA (or PBS) intranasal instillation, femurs were removed immediately after killing. The epiphyses were cut transversely and bone marrow cells were flushed out with PBS containing heparin (20 IU/ml). Total leukocyte counts were performed in Newbauer chamber, while differential counts were carried out on a minimum of 200 cells using cytospin preparation stained with Diff-Quick. The leukocytes were classified as immature and mature neutrophils or eosinophils based on normal morphological criteria. Results are expressed as the number of leukocytes per femur.

2.6. Histological analysis

Rats were anesthetized with overdose of inhaled halothane and their abdominal cavities exposed by a midline incision. By cutting the abdominal aorta, the animals were allowed to exsanguinate. Their chest cavities were then opened by means of a standard Y-shaped incision, so that the anterior chest plate could be removed. With the lungs *in situ*, the trachea was cannulated and perfused with 10% buffered formalin at a constant pressure of 20 cmH_2O until both lungs were completely inflated. The trachea was then suture ligated, and the lungs and heart were removed *en bloc* and post-fixed by immersion for at least 24 h in the same fixative solution. Next, both lungs were macroscopically examined and cut transversally into slices of approximately 3 mm. Only the middle third of the caudal aspects of both lungs were sent to embedding in paraffin. Sections of these portions, 4 to 5 μm thick, were stained with hematoxylin-eosin and evaluated for bronchiolitis, under a Nikon Eclipse E200 microscope adapted to a Nikon Coolpix 995 camera (3 Mpixel). For each animal, using the 40 × objective, three random digital images were taken within areas of overt bronchiolar inflammation. Total and differential inflammatory cell counts were determined from these images, using the Imagelab Analysis software (version 2.4).

2.7. Measurement of eotaxin, TNF-α and IL-10 levels in BAL fluid

Levels of eotaxin, TNF-α, and IL-10 were measured in the first 10-ml aliquot of BAL fluid supernatant obtained at 24 h after OVA challenge (or PBS instillation), using commercially available enzyme linked immunosorbent assay (ELISA) according to the manufactured instructions (R&D Systems, MN, USA).

2.8. Alveolar macrophage isolation

Alveolar macrophages (2×10^6/ml) were isolated from BAL fluid of naïve rats ($n = 8$, each sample done in quadruplicate), after which were harvested with Krebs medium (pH 7.4) and allowed to adhere to plastic tissue culture dishes for 2 h at 37 °C in atmosphere of air containing 5% CO_2. Non-adherent cells were removed by washing the dishes three times with sterile PBS. The adherent population was incubated for 2 h with 1 ml of Krebs (control) or SEA (1 μg/ml). Subsequently, the supernatants were recovered and stored at −80 °C for further measurement of eotaxin levels.

2.9. Statistical analysis

Data were presented as the mean values ± SEM and were analyzed by analysis of variance (ANOVA) for multiple comparisons followed by

Bonferroni post-test, using a program package for statistical analysis (GraphPad software, version 3.00; San Diego, USA). Data obtained from histological analysis are expressed as median and average ± SD per group, and were analyzed by the Kruskal–Wallis test, using the software SPSS for Windows (release 8.0). Significance was accepted at $p < 0.05$.

3. Results

3.1. Leukocyte counts in BAL fluid after OVA challenge

In non-sensitized rats instilled with PBS (0.2 ml; $n = 7$), leukocytes consisted mainly of mononuclear cells in BAL fluid (>95%), with few neutrophils (2.2 to 3.2%) and eosinophils nearly absent. Similarly, in non-sensitized rats instilled with OVA (1 mg/0.2 ml; $n = 7$), leukocytes consisted mainly of mononuclear cells in BAL fluid (91%), with few neutrophils (9%), and eosinophils nearly absent. However, instillation of OVA in previously sensitized animals caused a marked eosinophil influx in BAL fluid at 12, 24 and 48 h post-OVA challenge, with maximal responses obtained at 24 h (Table 1). A significant increase in the neutrophil counts in BAL fluid was observed at 12 and 24 h (but not 48 h) post-OVA challenge compared with PBS-instilled animals. The number of mononuclear cells in BAL fluid was not significantly changed by OVA challenge at 12 and 24 h, but a slight increase was observed at 48 h (Table 1). Since eosinophil peaked at 24 h after OVA challenge, further experimental protocols were routinely carried out at this time-period.

3.2. Effects of pre-exposure to SEA on leukocyte counts in BAL fluid

In the first series of experiments, rats were exposed to SEA (3 ng/0.2 ml) at 4 h prior to OVA challenge, and leukocyte number evaluated in BAL fluid at 24 h after OVA challenge. Pre-exposure to SEA (3 ng) in OVA-challenged rats enhanced by 56% ($p < 0.05$) the eosinophil counts compared with control animals (Fig. 1A). The number of neutrophils and mononuclear cells in BAL fluid were not significantly affected by SEA pre-exposure in OVA-challenged rats (Table 2).

Fig. 2 shows histology sections of connective tissue surrounding the bronchial and bronchiolar segments in all groups. Histological examination of the lungs from sensitized rats intranasally instilled with PBS showed normal tissue, with no inflammatory cells throughout the pulmonary parenchyma (Panel A). Pulmonary inflammatory infiltrates of increased intensity were observed in SEA-exposed rats alone (instilled with PBS instead of OVA; Panel B), OVA-challenged rats under no SEA exposure groups (Panel C) and OVA-challenged rats pre-exposed to SEA (Panel D). In SEA-exposed rats alone, cell infiltrate was usually mild and located predominantly within peri-bronchiolar and peri-vascular regions (Panel B), whereas in OVA-challenged rats under no SEA exposure, peri-bronchiolar and peri-vascular infiltrates were moderate to severe, and occasionally extensive to alveoli (Panel C). Finally, OVA-challenged rats pre-exposed to SEA (Panel D) presented multiple confluent inflammatory foci, consisting of profound peri-bronchiolar and peri-vascular infiltrates often accompanied by exuberant intra-alveolar and intra-bronchiolar exudates. In all of these groups, the inflammatory process was composed by a mixed cell population, including lymphocytes, macrophages, neutrophils, and specially eosinophils. With regard to eosinophils in particular, the number increased from 4.8 ± 0.8 in SEA-exposed rats alone to 12.5 ± 2.9 cells/high power field in SEA-exposed rats challenged with OVA ($p < 0.001$, $n = 7$ each group).

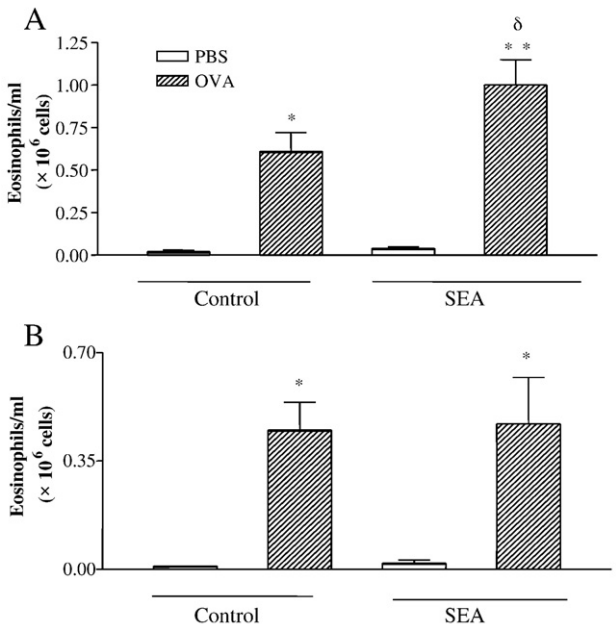

Fig. 1. Effect of rat airways exposure to Staphylococcal enterotoxin A (SEA) in the eosinophil recruitment in bronchoalveolar lavage (BAL) fluid of ovalbumin (OVA)-challenged rats. Animals were exposed to SEA (3 ng) at 4 h prior to OVA challenge (pre-exposure protocols; panel A) or 4 h post-OVA challenge (post-exposure protocols; panel B). Control animals were instilled with phosphate-buffered saline (PBS; 0.2 ml) instead of SEA. The BAL fluid was obtained 24 h thereafter. Results are mean values ± S.E.M. from 5 to 14 rats for each group. $^{*}p < 0.05$, $^{**}p < 0.05$ compared with respective control group; $^{\delta}p < 0.05$ compared with OVA group in PBS-instilled rats.

3.3. Effects of post-exposure to SEA on leukocyte counts in BAL fluid

In a second series of experiments, rats were exposed to SEA (3 ng/0.2 ml; $n = 5$) at 4 h after OVA challenge, and leukocyte number evaluated in BAL fluid at 24 h thereafter. Post-exposure to SEA did not significantly affect the number of eosinophils in BAL fluid of OVA-challenged rats compared with control group (Fig. 1B). The number of neutrophils and mononuclear cells in BAL fluid of OVA-challenged rats were also unchanged by the SEA post-exposure (Table 2).

Table 1
Leukocyte counts in bronchoalveolar lavage (BAL) fluid after intranasal challenge with ovalbumin (OVA) in previously sensitized rats.

Groups	OVA challenge	Cell number (×10⁶/ml)			
		Total cells	Neutrophils	Eosinophils	Mononuclear cells
PBS	12 h	2.2 ± 0.3	0.05 ± 0.01	0.01 ± 0.01	2.2 ± 0.3
OVA		3.7 ± 0.08*	0.5 ± 0.1*	0.2 ± 0.06*	2.9 ± 0.2
PBS	24 h	3.1 ± 0.2	0.1 ± 0.03	0.01 ± 0.01	3.0 ± 0.2
OVA		6.1 ± 0.7*	1.9 ± 0.6*	0.6 ± 0.1*	3.5 ± 0.5
PBS	48 h	1.6 ± 0.06	0.04 ± 0.02	0.00 ± 0.00	1.6 ± 0.04
OVA		2.6 ± 0.1*	0.05 ± 0.01	0.3 ± 0.07*	2.2 ± 0.2*

In control groups, phosphate-buffered saline (PBS) was instilled instead of OVA. Bronchoalveolar fluid was collected at the indicated time-periods after OVA challenge. Results are mean values ± S.E.M. from 7 rats for each group.
* $p < 0.05$ compared with respective PBS group.

Table 2
Lack of effect of Staphylococcal enterotoxin A (SEA) exposure in the number of neutrophils and mononuclear cells in bronchoalveolar lavage (BAL) fluid after ovalbumin (OVA) challenge in rats.

	Groups	Neutrophils (10⁶/ml)	Mononuclear (10⁶/ml)
Control	PBS + PBS	0.12 ± 0.02	3.2 ± 0.4
Control	PBS + OVA	1.87 ± 0.36*	3.7 ± 0.3
Pre-exposure	SEA + PBS	0.18 ± 0.02	3.1 ± 0.3
Pre-exposure	SEA + OVA	1.86 ± 0.32*	4.5 ± 0.7
Post-exposure	PBS + SEA	0.23 ± 0.03	5.2 ± 0.6
Post-exposure	OVA + SEA	2.07 ± 0.42*	5.2 ± 0.4

In pre-exposure protocols, animals were exposed to SEA (or instilled with PBS) at 4 prior OVA challenge, whereas in post-exposure protocols, animals were exposed to SEA (or instilled with PBS) at 4 h post-OVA challenge. The BAL fluid was obtained at 24 h thereafter. The data represent the mean values ± S.E.M. of 5–7 rats for each group.
* $p < 0.05$ compared with the group which received PBS instillation instead of OVA.

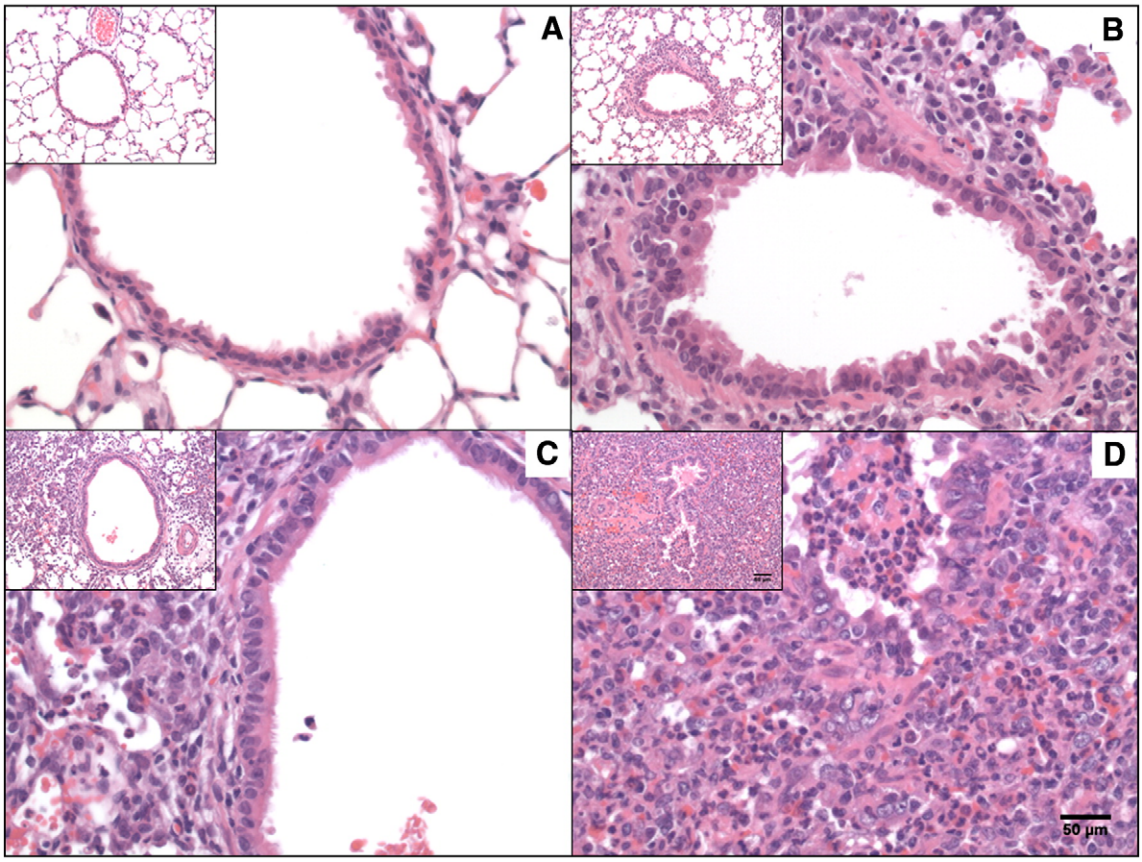

Fig. 2. Representative images from PBS/PBS (A), PBS/SEA (B), PBS/OVA (C) and OVA/SEA (D) animals. Note the marked peri-bronchiolar infiltrate in OVA-challenged rats pre-exposed to SEA (panel D). Hematoxylin and eosin; scale bars = 50 µm. PBS, phosphate-buffered saline; SEA, Staphylococcal enterotoxin; OVA, ovalbumin.

3.4. Effects of pre-exposure to LPS on leukocyte counts in BAL fluid

In a third set of experiments, rats were exposed to LPS (0.1 and 1 µg) at 4 h prior to OVA challenge, and leukocyte number evaluated in BAL fluid at 24 h after OVA challenge. Pre-exposure to LPS in PBS-instilled rats caused a dose-dependent increase in the neutrophil counts, without affecting the eosinophil and mononuclear cell counts compared with control group (Fig. 3). Pre-exposure to LPS in OVA-challenged animals further enhanced the neutrophil number in an additive manner (Fig. 3). The number of eosinophils and mononuclear cells in OVA-challenged rats was not changed in LPS-exposed rats (not shown, $n = 5$–11).

3.5. Effects of pre-exposure to SEA on leukocyte counts in bone marrow

As showed above, pre-exposure of rats to SEA (but not to LPS) enhances the eosinophil influx in BAL fluid. Therefore, we examined the leukocyte number in bone marrow of SEA pre-exposed rats at 24 h post-OVA challenge. A significant increase in eosinophil counts was observed in OVA-challenged rats. Pre-exposure to SEA alone also significantly increased the number of eosinophils compared with non-exposed rats ($n = 7$; Fig. 4). A further increase in eosinophil counts in bone marrow was seen in SEA-exposed and OVA-challenged rats. The number of neutrophils in bone marrow was neither significantly changed by OVA challenge nor by SEA pre-exposure (Fig. 4).

3.6. Levels of eotaxin, TNF-α and IL-10 in BAL fluid

Rats were pre-exposed to SEA at 4 h prior to OVA challenge, and measurements of cytokines carried out in BAL fluid at 24 h post-OVA challenge, as showed in Fig. 5.

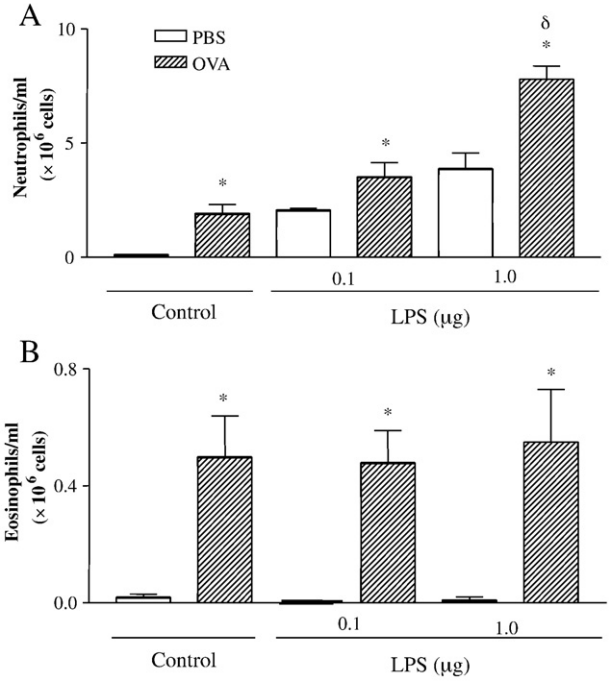

Fig. 3. Effect of rat airways pre-exposure to lipopolyssacharide (LPS) in the neutrophil and eosinophil recruitment in bronchoalveolar lavage (BAL) fluid of ovalbumin (OVA)-challenged rats. Animals were exposed to LPS (0.1 and 1.0 µg) at 4 h prior OVA challenge, and BAL fluid was obtained 24 h thereafter. Control animals were instilled with phosphate-buffered saline (PBS; 0.2 ml) instead of LPS. Panels A and B shows respectively neutrophil and eosinophil counts. Results are mean values ± S.E.M. from 5 to 11 rats for each group. $^{*}p < 0.05$ compared with respective control group.

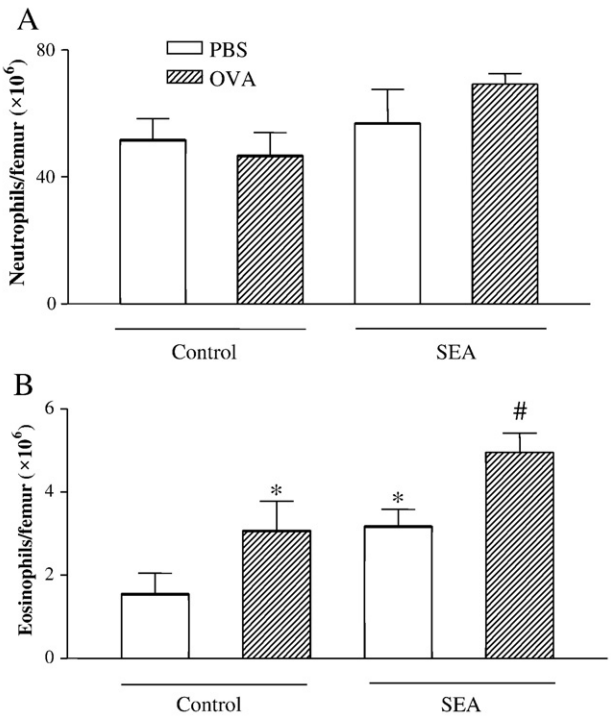

Fig. 4. Effect of rat airways pre-exposure to Staphylococcal enterotoxin A (SEA) in the neutrophil and eosinophil counts in bone marrow of ovalbumin (OVA)-challenged rats. Animals were exposed to SEA (3 ng) at 4 h prior to OVA challenge, and bone marrow obtained 24 h thereafter. Control animals were instilled with phosphate-buffered saline (PBS; 0.2 ml) instead of SEA. Panels A and B shows respectively neutrophil and eosinophil counts. Results are mean values ± S.E.M. from 7 rats for each group. $^{*}p<0.05$ compared with PBS of control group; $^{\#}p<0.05$ compared with all groups.

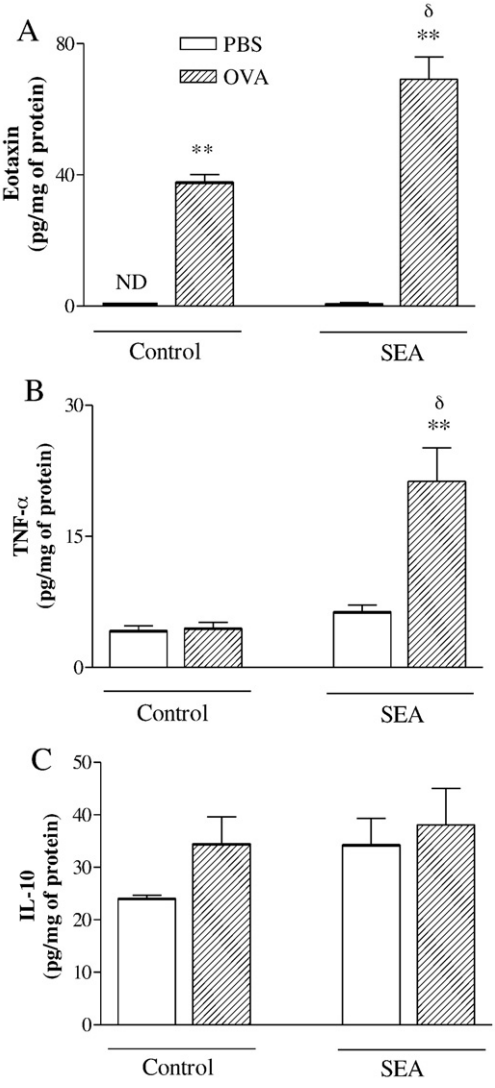

Fig. 5. Levels of eotaxin (panel A), TNF-α (panel B), and IL-10 (panel C) in bronchoalveolar lavage (BAL) fluid of ovalbumin (OVA)-challenged-rat pre-exposed to Staphylococcal enterotoxin A (SEA). Animals were exposed to SEA (3 ng) at 4 h prior to OVA challenge, and BAL obtained 24 h thereafter. Control animals were instilled with phosphate-buffered saline (PBS; 0.2 ml) instead of SEA. Results are mean values ± S.E.M. from 5–7 rats per group. $^{**}p<0.05$ compared with respective control group; $^{\delta}p<0.05$ compared with OVA group in PBS-instilled rats.

The eotaxin level was significantly higher ($p<0.05$) in OVA-challenged rats compared with PBS-instilled animal. In rats pre-exposed to SEA alone, no changes in eotaxin levels were found. However, the eotaxin levels markedly increased in rats pre-exposed to SEA and challenged with OVA (Fig. 5A).

The TNF-α levels changed neither in OVA-challenged group nor in SEA group alone. However, a marked increase in this cytokine level was seen in rats pre-exposed to SEA and challenged with OVA (Fig. 5B).

No significant differences for IL-10 levels in any studied group could be detected (Fig. 5C).

3.7. Levels of eotaxin in alveolar macrophages stimulated with SEA in vitro

Alveolar macrophages isolated from BAL fluid of naïve rats were incubated with SEA (1 μg/mL, 2 h, 37 °C), after which eotaxin levels in cell supernatant were measured. The eotaxin level increased by about of 3-fold in SEA samples compared with PBS (1.5 ± 0.04 and 0.5 ± 0.08 pg/mg protein, respectively; $n=8$ rats repeated four times).

4. Discussion

Our present study clearly shows that airways pre-exposure to SEA enhances the eosinophil influx in BAL fluid of OVA-challenged rats. Pre-exposure to the Gram-negative bacterial toxin LPS failed to modify the airways eosinophil influx but rather enhanced the neutrophil influx. Our data suggest that, among the enterotoxins produced by *S. aureus*, SEA contributes to the exacerbation of pulmonary allergic inflammatory responses seen in humans. Staphylococcal enterotoxins B, C and D, as well as toxic shock syndrome toxin (TSST-1) are also produced from human isolates of *S. aureus* [22,23], and no differences in enterotoxin A-D or TSST-1 productions are observed between patients with complicated versus uncomplicated septicaemia [24]. Therefore, whether Staphylococcal enterotoxins other than SEA enhance the pulmonary allergic responses requires further studies.

Mast cells exert critical roles in allergic responses, and have been implicated in the physiopathological responses to staphylococcal enterotoxins. For instance, the skin reactions caused by SEB in monkeys [25] and mice [10] are likely to be triggered by degranulation of cutaneous mast cells. This enterotoxin also releases 5-hydroxytriptamine from rodent mast cell cultures, which is greatly enhanced by bradykinin [26]. Recently, we showed that mast cell amines contribute to SEA and SEB-induced neutrophil influx into the rat airways [27]. Besides stored amines, mast cells release an array of inflammatory mediators including TNF-α, a potent cytokine with critical immunoregulatory activities [28]. In the lung, TNF-α is synthesized and stored mainly in mast cells and alveolar macrophages, and can be released via IgE-dependent mechanisms [29]. Systemic and lung TNF-α levels are increased in allergic animals and patients with bronchial asthma. Our findings showed a marked elevation of TNF-α levels in BAL fluid of rats pre-exposed to SEA and challenged with OVA at 24 h, suggesting an important role for this

pro-inflammatory Th1 cytokine. Of interest, TNF-α has been shown to induce the synthesis of eotaxin in several cell types including dermal fibroblasts [30], human lung epithelial cells [31], monocytic cells [32], epithelial cells [33] and human eosinophils [34].

Eotaxin is a CC-chemokine responsible for selective eosinophil chemotaxis and transendothelial migration in airways of allergic subjects [35–38]. Eotaxin and IL-5 are said to act synergistically to promote eosinophil recruitment into tissues [39–41]. Moreover, the increased number of eosinophils in BAL fluid has been correlated with higher eotaxin levels [42]. In our study, a significant elevation in eotaxin levels in BAL fluid of OVA-challenged rats was found, as expected. The eotaxin levels were further enhanced in rats pre-exposed to SEA. Furthermore, incubation of isolated alveolar macrophages with SEA causes the release of marked amounts of TNF-α [21] and eotaxin (this study). These findings strongly suggest that macrophage-derived TNF-α acts to release eotaxin, which further aggravates the eosinophil influx by SEA pre-exposure in allergic animals. The eotaxin levels in BAL fluid of SEA group alone (non-challenged rats) did not change in comparison with PBS group, which is consistent with the lack of TNF-α in BAL fluid at 24 h. Interestingly, the enhancement of airways eosinophil infiltration by SEA is not seen when this enterotoxin was delivered at 4 h post-OVA challenge. Our previous studies show that exposure of airways to SEA and SEB in non-allergic rats cause an acute lung injury at 4 h as characterized by a marked neutrophil influx, accompanied by the increase of various inflammatory mediators including TNF-α [21,43]. Therefore, a prior (but not a post) contact of airways with SEA may be crucial to allow a pro-inflammatory state with increased TNF-α production that result in an enhanced response to antigen challenge. Several evidences also show that cytokine IL-10 reduces the levels and expression of TNF-α by activated monocytes and/or macrophages, which has been associated with clinical protection and reduction of lung pathology [44]. However, our findings showed that elevations of TNF-α in BAL fluid of OVA-challenged rats pre-exposed to SEA were not accompanied by concomitant elevations in the IL-10 levels, indicating this type of pulmonary allergic inflammation does not undergo downregulation by this cytokine.

It is well-established that bone marrow plays a pivotal role in allergic inflammatory responses [45]. Eosinophils are derived in the bone marrow from myeloid precursors in response to cytokine activation, and following appropriate stimulus they are released into the circulation and recruited to tissues. Evidences indicate that several pathways have been implicated, including stimulation of resident bone marrow cells, release of allergen-induced hematopoietic growth factors and cell trafficking [46]. The cytokines IL-5 and eotaxin, as well as adhesion molecules (VLA-4 and VCAM) play important roles in this process [47,48]. In our study, a significant increase in eosinophil number in bone marrow of OVA-challenged rats was observed at 24 h, confirming previous studies in rats [49], mice [50] and asthmatic subjects after inhaled antigen [45]. In rats exposed to SEA alone, a significant increase in eosinophil number in bone marrow was also observed, and such effect was further enhanced in OVA-challenged animals. Whilst proliferation and differentiation of bone marrow progenitor cells after airway allergen exposure are well-established [51–53], our study is the first to show that SEA itself, given intranasally, promotes eosinopoesis. Therefore, modifications in bone marrow eosinophil pattern may play an important role in determining the exacerbation of airways eosinophil influx by pre-exposure to SEA after OVA challenge in rats. Interestingly, an increased number of eosinophils in bone marrow of SEA-exposed rats alone (SEA + PBS group) was observed. The reasons why eosinophils in such group do not achieve BAL fluid are uncertain. A fundamental feature of asthma associated with allergic sensitization is the ability of the airways to recognize allergens and to generate the so-called Th2 cytokines, which is related to IgE production, eosinopoiesis, and bronchial inflammation. Therefore, the failure of SEA alone to promote eosinophil infiltration into the BAL fluid may reflect the absence of an allergic stimulus.

Epidemiological evidence also suggest that exposure to LPS can influence the development and severity of asthma [54]. Therefore, in our study, the effects of airways pre-exposure to LPS have also been investigated in order to compare with those of SEA. Endotoxin has the potential to either suppress the development of asthma through induction of counter-regulatory Th1 cells or to exacerbate asthma severity, presumably through its pro-inflammatory activities [55]. In our study, pre-exposure to LPS neither modified the eosinophil number in BAL fluid when instilled alone nor when instilled in OVA-challenged rats, which is in agreement with a previous study showing that airways LPS exposure, at low dose (0.3 μg), causes only minimal effects on the recruitment of airways eosinophils in mice; as opposed, at a much higher dose (15–25 μg), LPS markedly enhanced Th2 eosinophilic inflammation [56,57]. Endotoxin is reported to mobilize neutrophils after instillation into the mouse and rat airways [58]. Accordingly, in our study, LPS caused a dose-dependent pulmonary neutrophil influx, which was further enhanced in OVA-challenged rats. Lipopolyssaccharide activates toll-like receptor 4 (TLR-4) causing the mobilization of neutrophils after instillation into the airways [59], an effect largely reduced in TLR4 mutant mice [60]. Whether potentiation by LPS of neutrophil influx in both non-challenged and OVA-challenged rats reflects activation of TLR4 signaling pathway requires further studies.

In conclusion, this study shows that airways pre-exposure to SEA in OVA-challenged rats causes a selective increase in eosinophil number in bone marrow and BAL fluid by mechanisms involving enhancement of TNF-α and eotaxin synthesis.

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