

ORIGINAL ARTICLE

Staphylococcus aureus enterotoxin B facilitates allergic sensitization in experimental asthma

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Summary

Background *Staphylococcus aureus* Enterotoxin B (SEB) has immunomodulatory effects in allergic airway disease. The potential contribution of SEB to the sensitization process to allergens remains obscure.

Objective In order to study the effects of staphylococcal-derived toxins on the sensitization to ovalbumin (OVA) and induction of allergic airway inflammation, we have combined the nasal application of OVA with different toxins.

Methods Nasal applications of OVA and saline, SEA, SEB, toxic shock syndrome toxin (TSST)-1, protein A or lipopolysaccharide (LPS) were performed on alternate days from day 0 till 12. On day 14, mice were killed for the evaluation of OVA-specific IgE, cytokine production by mediastinal lymph node (MLN) cells and bronchial hyperreactivity (BHR) to inhaled metacholine. The effect of SEB on dendritic cell (DC) migration and maturation, and on T cell proliferation was evaluated.

Results Concomitant endonasal application of OVA and SEB resulted in OVA-specific IgE production, whereas this was not found with SEA, TSST-1, protein A, LPS or OVA alone. Increased DC maturation and migration to the draining lymph nodes were observed in OVA/SEB mice, as well as an increased T cell proliferation. Bronchial inflammation with an influx of eosinophils and lymphocytes was demonstrated in OVA/SEB mice, together with hyperresponsiveness and the production of IL-4, IL-5, IL-10 and IL-13 by MLN stimulated with OVA.

Conclusions Our data demonstrate that SEB facilitates sensitization to OVA and consecutive bronchial inflammation with features of allergic asthma. This is likely due to augmentation of DC migration and maturation, as well as the allergen-specific T cell proliferation upon concomitant OVA and SEB application.

Keywords allergic sensitization, asthma, CD4 T cell, *Staphylococcus aureus* enterotoxin B, superantigens

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Introduction

Epidemiological data provide strong evidence for a steady increase in the prevalence of allergic diseases in the western world over the past three decades, reaching epidemic proportions [1–3]. As the reason for the steep rise in the prevalence of allergic rhinitis (AR) and asthma remains speculative, several hypotheses have been put

forward to explain this phenomenon. Among others, environmental factors may interfere with the sensitization process and lead to the development of allergic inflammation [4]. These exogenous factors provide immunostimulatory signals that are necessary to overcome immunological tolerance and induce allergic sensitization [5–12]. Eventually, these immunostimulatory signals result in dendritic cell (DC) activation, leading to functional

DC differentiation and the priming of T-helper type 2 (Th2) effector cells [13].

Enterotoxins of *Staphylococcus aureus* have been implicated in the pathology of chronic airway inflammation, as IgE directed against *Staphylococcus aureus* enterotoxins (SAE) was found in chronic upper airway inflammation [14–16]. These SAE act as superantigens as they potentially activate T cells via the linkage of the β chain of the T cell receptor (TCR) to MHC class II molecules on antigen-presenting cells (APC) outside the peptide-binding groove area [17].

Recent evidence suggests a putative role of these enterotoxins in allergic diseases. In patients suffering from the atopic eczema/dermatitis syndrome (AEDS), colonization with *S. aureus* is found more frequently (80–100%) compared with healthy controls (5–30%) [18], and *S. aureus* isolates secrete identifiable *Staphylococcus aureus* enterotoxins A (SEA), SEB and toxic shock syndrome toxin (TSST)-1. IgE to these toxins was found in the serum of 57% of AEDS patients [19], indicating immune responses against these bacterial products. Beside AEDS, 25% of AR patients have detectable serum IgE levels to SAE, whereas this is only a minority of non-allergic patients (6.3%). In AR, the presence of SAE-specific IgE was associated with the highest titre of total serum IgE [14, 20]. IgE against SEA and SEB was also found in nasal polyps (NP) [21] and levels of SAE-specific IgE in NP correlated with markers of eosinophil activation and recruitment [22, 23]. In mice, Herz et al. [24] reported lymphocyte-dependent bronchial inflammation with an increased bronchial responsiveness (BR) by the repeated nasal application of SEB. Furthermore, epicutaneous sensitization of mice with SEB elicited an immune response with Th2 cell activation, allergic skin inflammation and increased IgE titres in serum [25]. Recently, a new mouse model of food allergy was described, demonstrating that SEB impairs the oral tolerance [26]. Finally, we reported previously the aggravation of experimental allergic asthma by the application of SEB, characterized by higher IL-4 production and allergen-specific IgE [27].

Until now, the contribution of SAE to the sensitization to inhaled allergens remains unclear. Therefore, we took advantage of a mouse model of repeated endonasal applications of enterotoxins of *S. aureus* in order to study which of the enterotoxins alters the sensitization to allergens. Evaluating the mechanisms involved in this model, we investigated the behaviour of DCs and allergen-specific T cells.

Methods

Mice

Male inbred BALB/c mice were obtained from Harlan CBP (Horst, the Netherlands) and kept under conventional

relative pathogen-free conditions. Six- to eight-week-old mice were used in all experiments, with five to six mice per group. Ovalbumin (OVA)-TCR transgenic mice (DO11.10) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA).

Experimental protocol

Nasal application of 50 μ L of pyrogen-free saline, SEB, SEA, TSST-1, protein A (all 10 μ g/mL) or lipopolysaccharide (LPS) (0.1 μ g/mL) combined with OVA at 10 mg/mL was performed by means of placing this volume at the nostrils of spontaneously breathing mice in a supine position. Seven applications were performed on alternate days from day 0 till day 12, as depicted in Fig. 1a. In subsequent experiments, 50 μ L of pyrogen-free saline, SEB 10 μ g/mL, Sigma-Aldrich, St Louis, USA, LPS content: 34.82 μ g/mL and/or OVA (10 mg/mL, grade V, Sigma-Aldrich, LPS content: 0.18 μ g/mL) were endonasally applied in an identical schedule (Fig. 2a).

The dose of SEB was chosen according to dosing experiments in naïve [24] and in sensitized mice [27], where it was shown that 500 ng of SEB potently altered bronchial inflammation without clinical signs of the wasting disease. The same dose was chosen for the other toxins. The chosen dose of LPS has been described to induce Th2 responses in a mouse model of allergic asthma [9]. Mice were killed on day 14, i.e. 48 h after the last application. All experimental procedures were approved by the local Ethical Committee.

Assessment of bronchial responsiveness

Airway responses to inhaled methacholine (MCh) was measured using the forced ventilation technique (Flexi-Vent, SCIREQ, Montreal, Canada) as reported previously [28]. 48 h after the last endonasal application, airway resistance and compliance to incremental doses of MCh were assessed. To this purpose, mice were anaesthetized with sodium pentobarbital (70 mg/kg i.p.). After exposure of the trachea, a tracheotomy was performed and a 19-gauge metal needle inserted into the bronchus. The mice were connected to a computer-controlled small animal ventilator and were quasi-sinusoidally ventilated with a tidal volume of 10 mL/kg at frequency of 150 breaths/minute and a positive end-expiratory pressure of 2 cm H₂O to achieve a mean lung volume close to that during spontaneous breathing. After the measurement of baseline values, each mouse was exposed to an aerosol, generated with an in-line nebulizer and administered directly through the ventilator for 5 s, containing increasing concentrations of MCh (0, 0.625, 1.25, 2.5, 5 and 10 mg/mL). Airway resistance (*R*) was measured using a 'snapshot' protocol each 20 s for 2 min. For each concentration of MCh, the mean of six values of *R* was calculated. For each

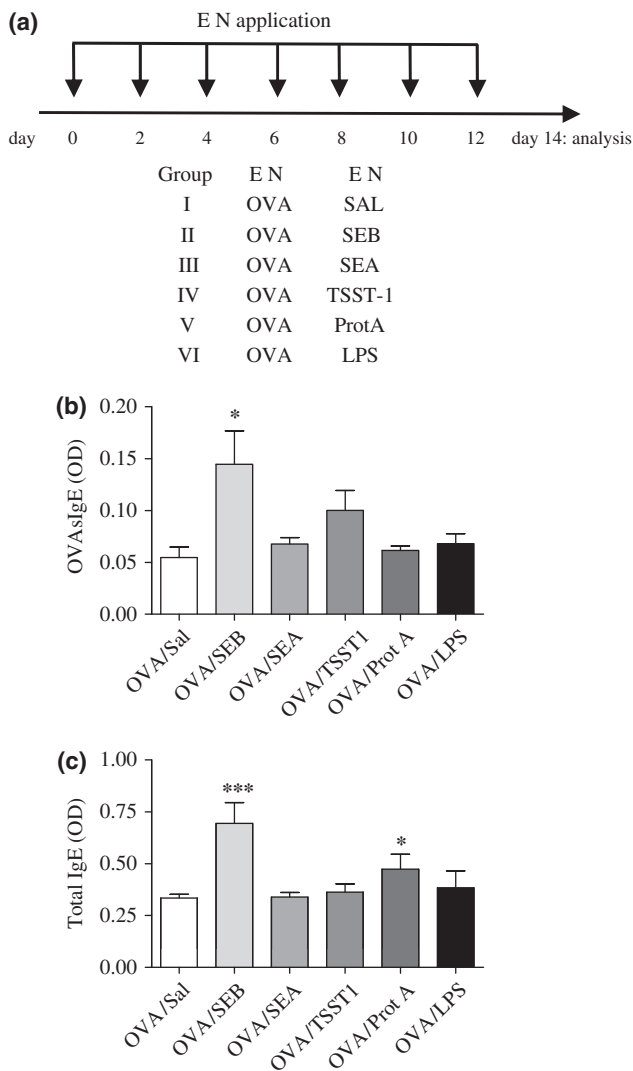


Fig. 1. (a) Nasal application of 50 μ L of saline, SEB, SEA, TSST-1, protein A (all 10 μ g/mL) or LPS (0.1 μ g/mL) combined with ovalbumin (OVA) (10 mg/mL) was performed seven times on alternate days from day 0 till day 12. (b) On day 14, OVA-specific IgE levels in serum were evaluated. Interestingly, only the combination of OVA and SEB resulted in increased serum levels of OVA-specific IgE, whereas this was not the case for other proteins. (c) Moreover, levels of total IgE were also significantly higher in OVA/SEB mice compared with OVA/Sal, and concurrent application of OVA/Prot A also resulted in modestly raised levels of total IgE. * $P < 0.05$, *** $P < 0.001$. SEB, *Staphylococcus aureus* Enterotoxin B; TSST, toxic shock syndrome toxin; LPS, lipopolysaccharide.

mouse, R was plotted against the MCh concentration (from 0 to 10 mg/mL) and the area under the curve (AUC) was calculated.

Measurement of ovalbumin-specific and total immunoglobulin E levels

After anaesthesia with sodium pentobarbital, a retro-orbital bleed was performed on day 14 (i.e. 48 h after the last exposure) and the serum was frozen until analysis. For

measurement of OVA-specific IgG2a, 96-well plates were first coated overnight with rat anti-mouse IgG2a (20 μ g in 100 μ L PBS, PharMingen, San Diego, CA, USA). The remaining binding sites were blocked and plates were incubated with 100 μ L of diluted serum (1:10). After washing, the following substances were sequentially added, incubated and washed: OVA (1 μ g/100 μ L, Sigma), peroxidase-labelled rabbit anti-OVA IgG (240 ng/100 μ L, Rockland, Gilbertsville, PA, USA) and a buffer containing tetramethylbenzidine dihydrochloridhydrate (1 μ L/100 μ L, ACROS, New Jersey, NJ, USA) and H₂O₂ (1 μ L/100 μ L). Then, H₂SO₄ was added and the optical density was measured at 450 nm.

For the measurement of OVA-specific and total IgE, commercially available ELISA kits were purchased and samples were processed according to the manufacturer's guidelines (OVA-specific IgE: MD Biosciences, Zürich, Switzerland – total IgE: Pharmingen). Data were expressed as optical density values at 450 nm.

Analysis of bronchoalveolar lavage fluid

On day 14, mice were lethally anaesthetized with sodium pentobarbital. A polyethylene catheter (0.85 mm) connected to a syringe was gently inserted into the trachea. Then, the bronchoalveolar tree was lavaged five times with 1 mL aliquots of pyrogen-free phosphate-buffered saline (PBS) supplemented with 5% of bovine serum albumin (BSA; Sigma, Bornem, Belgium) at 37 °C through a polyethylene tracheal catheter (0.85 mm). The first lavage was performed with 1 mL PBS/BSA 5%, centrifuged (1400 g, 5 min) and the supernatant was stored at –20 °C until analysis. The cellular pellet was added to the subsequent four lavages, each one performed with 1 mL of PBS. The broncho (BAL) fluid was centrifuged (1400 g, 5 min) and the pellet washed and re-suspended in 100 μ L of PBS. Ten microlitres of cell suspension from BAL fluid were added to 90 μ L of Türk's solution (Merck Diagnostica, Darmstadt, Germany), and the total number of cells was counted in a Bürker–Türk chamber. For differential cell counts, cytopsin preparations were stained according to the May–Grünwald–Giemsa method and 300 cells differentiated into eosinophils, neutrophils, monocytes/macrophages and lymphocytes according to standard morphological criteria.

Histologic analysis

After performing BAL, the bronchoalveolar tree was removed for histological examination and fixed overnight in buffered formalin. After dehydration and embedding in paraffin, 3 μ m sections were stained with hematoxylin and eosin (H&E) to demonstrate inflammatory infiltrates, or Congo red to highlight eosinophils. Quantification of eosinophilic influx was performed using Congo red

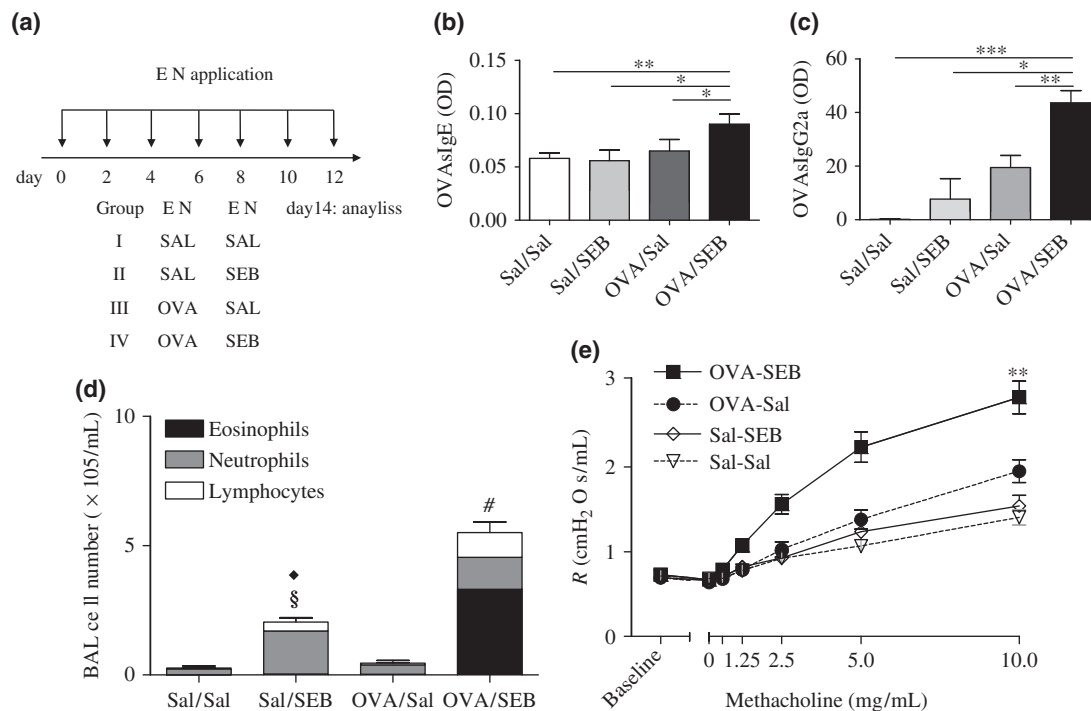


Fig. 2. (b) Concomitant application of OVA and SEB resulted in increased levels of OVA-specific IgE levels, (c) OVA-specific IgG2a levels, (d) BAL eosinophils and lymphocytes and (e) bronchial hyperreactivity measured as resistance (R), compared with the control conditions. One representative experiment out of two is shown. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. # $P < 0.01$ vs. all others (eosinophils) and vs. OVA/Sal and Sal/Sal (lymphocytes). § $P < 0.01$ vs. Sal/Sal (neutrophils). * $P < 0.05$ vs. Sal/Sal (lymphocytes). OVA, ovalbumin; SEB, *Staphylococcus aureus* Enterotoxin B.

staining by counting the number of eosinophils per airway wall at a magnification of $\times 400$, for 10 representative airways with a perimeter of basement membrane around $800 \mu\text{m}$.

Cytokine production by draining lymph node cells

At the time of analysis, submandibular and peribronchial lymph nodes (LN) were dissected and homogenized using a cell strainer (Falcon[®], Becton Dickinson, Franklin Lakes, NJ, USA) and suspended in RPMI (Biowhittaker, Walkersville, MD, USA) supplemented with FCS (5%). The homogenates were washed twice with PBS/FCS and centrifuged at 428 g for 10 min. In parallel, spleen homogenates were incubated for 1 min with 5 mL of lysis buffer containing NH_4Cl , KHCO_3 and Na_2EDTA , and then washed with PBS/FCS. Cells were counted using a Coulter Counter (Analys, Ghent, Belgium).

For the analysis of cytokine production, 1×10^6 LN cells were incubated with splenocytes from naïve mice, as a source of APC, in the presence of OVA, BSA (as negative control) or SEB in 1 mL of culture medium (RPMI with 10% FCS supplemented with penicillin, streptomycin, L-glutamine and 0.1% of 2-mercaptoethanol). Pilot studies showed that 1 ng/mL and $10 \mu\text{g/mL}$ were the optimal doses for SEB and OVA/BSA respectively for further experiments (data not shown). Cells were incu-

bated for 5 days at 37°C . The supernatants were stored at -20°C until cytokine measurement.

To determine levels of IL-4, IL-5, IL-13, IL-10, IL-12 and IFN- γ , a Cytometric Bead Array Flex set (BD Biosciences, Eremodegem, Belgium) was used. IL-17 (R & D, Minneapolis, MN, USA) and TGF- β (BD Biosciences) were measured by ELISA according to the manufacturer's specifications.

Anti-CD4 monoclonal antibodies treatment

To study the role of CD4^+ cells in the SEB-mediated effects on sensitization to OVA, $50 \mu\text{g}$ of GK1.5 rat anti-mouse CD4 (Biolegend, San Diego, CA, USA) was administered i.p. in a volume of 0.5 mL on day 0 and day 6, which is during the phase of exposure of mice to OVA and SEB from day 0 until 12. Rat IgG ($50 \mu\text{g}$ in 0.5 mL, Rockland, Gilbertsville, PA, USA) was injected i.p. on day 0 and 6 as the control. The antibody preparations were centrifuged to remove aggregates ($30\,000 \text{ g}$, 20 min) as reported previously [29]. Groups consisted of 5 mice and were exposed endonasally to both OVA and SEB from day 0 until 12 on alternate days.

Reduction of CD4^+ cells was assessed in blood and draining lymph nodes. To this purpose, blood was collected in 0.5 M EDTA by a retro-orbital bleed. Subsequently, draining lymph nodes were taken and put

through a cell strainer. Samples were incubated with antibodies against CD3 (rat anti-mouse clone RM4-5), CD8 (rat anti-mouse Ly-2 Clone 53-67) and CD4 (rat anti-mouse clone 17A2, all BD Biosciences, San Diego, CA, USA). The red blood cells were lysed and all cells were fixed with 4% paraformaldehyde. 10×10^3 lymphocytes were analysed using a FACScan flow cytometer (BD Biosciences, San Diego, CA, USA). Data were analysed using CellQuest Software.

Analysis of dendritic cell and T cell behavior

DC migration was evaluated *in vivo* 24 h after intratracheal (i.t.) application of 500 µg OVA-FITC. For this, mice were slightly anaesthetized with isoflurane. Mediastinal lymph node (MLN) cells were harvested and stained with antibodies against CD11c and MHC class II. DAPI was used to exclude dead cells. Cells were analysed by FACS Aria II (BD Biosciences, San Diego, CA, USA).

DC maturation was investigated in bone marrow - derived DC cultures from naïve WT BALB/c mice. Bone marrow cells were collected, depleted of red blood cells using RBC-lysisbuffer (0.15 M NH_4Cl , 1 mM KHCO_3 , 0.1 mM Na_2EDTA), and grown in RPMI culture medium containing 5% FCS and GM-CSF for 8 days. At day 8, DCs were pulsed *in vitro* with TCM, OVA (100 µg/mL), SEB (100 ng/mL) or OVA/SEB. After 24 h, cells were collected and stained for MHC class II, CD11c, CD86 or isotype control before analysis by FACS Aria II.

To study T cell proliferation *in vivo*, cell suspensions of pooled peripheral lymph nodes and spleen from naïve OVA TCR transgenic DO11.10 mice were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, Merelbeke, Belgium) [30]. Then, 5×10^6 CFSE+T cells were transferred intravenously (i.v.) into naïve WT mice. After 24 h, mice received OVA (100 µg)^{+/-} SEB (500 ng) in 55 µL PBS i.t. Seventy-two hours after i.t. injection, MLN were analysed for the proliferation of CFSE-labelled OVA-specific TCR Tg T cells. As a control, non-lung-draining LNs were used. Transferred OVA-specific TCR transgenic CD4⁺ T cells were recognized as CD4⁺CFSE⁺ cells expressing the clonotypic TCR. DAPI was used to exclude dead cells. T cell divisions were measured by flow cytometry. The percentage of cells recruited into each cell division was calculated by dividing the number of individual cells by CFSE content, after correction for the multiplying effect of division, as described previously [30].

Statistical analysis

Statistical analysis was performed with Medcalc software 9.2.0.1 (F. Schoonjans, Belgium; <http://www.medcalc.be>). Data are expressed as mean with error bars expressing the standard error of the mean. All outcome variables were compared using non-parametrical tests (Kruskal-Wallis;

Mann-Whitney *U* test for unpaired data). The significance level was set at $\alpha = 0.05$.

Results

Effects of different toxins on ovalbumin-specific and total immunoglobulin E levels

In order to study the effects of staphylococcal-derived toxins on the sensitization to OVA and the induction of allergic airway inflammation, we have combined the nasal application of OVA with different toxins. Interestingly, only the combination of OVA and SEB resulted in increased serum levels of OVA-specific IgE compared with OVA/Sal, whereas this was not the case for other proteins (Fig. 1b). Moreover, levels of total IgE were also significantly higher in OVA/SEB mice compared with OVA/Sal (Fig. 1c). Concurrent application of OVA/Prot A also resulted in modestly raised levels of total IgE (Fig. 1c). The combined application of OVA and LPS did not result in raised OVA-specific or total IgE levels in our model.

Consequently, the same protocol was used to study the effect of SEB on the immune response to OVA (Fig. 2a), and we confirmed that repeated and concomitant exposure to OVA and SEB resulted in significantly higher titres of allergen-specific IgE, compared with the three control groups Sal/Sal, Sal/SEB or OVA/Sal (Fig. 2b). In addition, OVA-specific IgG2a levels were also significantly higher in the OVA/SEB group compared with all other groups (Fig. 2c).

Effects of Staphylococcus aureus Enterotoxin B on the ovalbumin-induced bronchial allergic inflammation

Mice that were sensitized to OVA following OVA/SEB application developed features of allergic asthma. A significant influx of eosinophils and lymphocytes counts in the broncho-alveolar lumen was seen in mice that were simultaneously exposed to OVA and SEB (Fig. 2d). Interestingly, neither Sal/SEB nor OVA/Sal exposure caused any bronchial influx of eosinophils. Sal/SEB application resulted in raised BAL lymphocyte and neutrophil number compared with controls (Fig. 2d). The total number of cells in the BAL fluid did not differ between groups (data not shown).

Simultaneous application of OVA and SEB resulted in a significant increase in BR) to incremental doses of nebulized methacholine (Fig. 2e) compared with all other conditions, while OVA or SEB application alone did not cause any bronchial hyperreactivity (BHR) compared with the control mice.

The BAL fluid findings were in line with the histological analyses, revealing that a concomitant application of OVA and SEB induced bronchial inflammation in the OVA/SEB group with minor inflammation around bronchi in the three control groups (Fig. 3a-d). Detailed analysis of the cellular infiltrate revealed that a simultaneous application

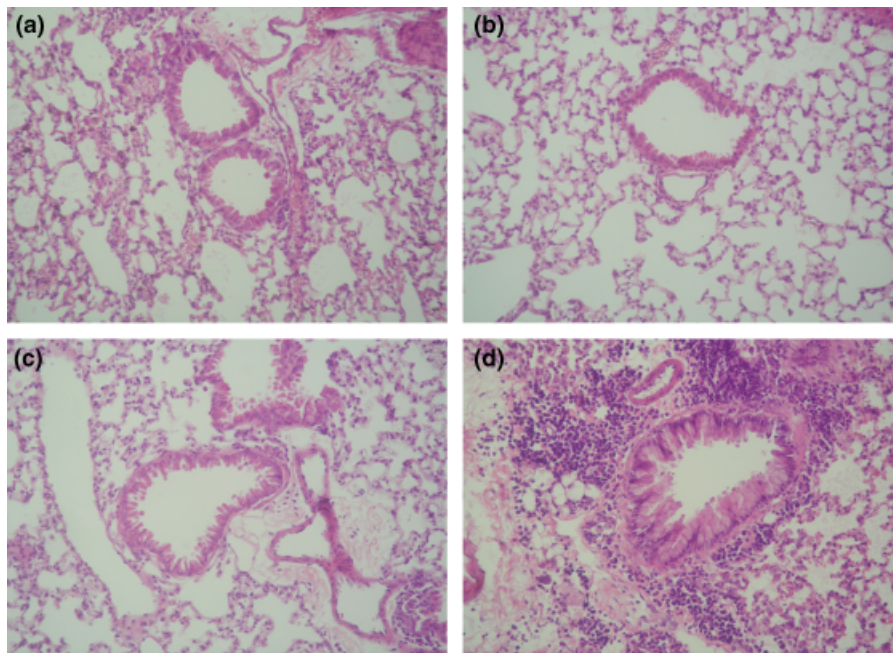


Fig. 3. Histological analysis revealed that a concomitant application of OVA and SEB induced bronchial inflammation primarily in the OVA/SEB group (d) with minor inflammation around bronchi in the three control groups: Sal/Sal (a), Sal/SEB (b) and OVA/Sal (c). H&E, $\times 200$. Representative bronchial sections are presented. OVA, ovalbumin; SEB, *Staphylococcus aureus* Enterotoxin B.

of OVA and SEB caused a significantly higher number of eosinophils in the lung parenchyma of OVA/SEB (2.825 ± 0.66) mice compared with the 3 other groups (0.32 ± 0.06 , 0.43 ± 0.24 and 0.87 ± 0.49 , $P < 0.05$), as was shown by Congo red staining.

Cytokine profile analysis of Staphylococcus aureus Enterotoxin B-mediated effects on ovalbumin sensitization

Submandibular and peribronchial draining lymph nodes were harvested for IL-4, IL-5, IL-10, IL-12, IL-13, IFN- γ , TGF- β and IL-17 cytokine production, after stimulation with OVA, BSA or SEB. LN cells of OVA/SEB mice showed an increase in IL-4, IL-5, IL-13 and IL-10 production upon stimulation with OVA compared with BSA (Fig. 4a–d). *In vitro* stimulation of OVA/SEB LN cells with OVA induced higher IL-5 production compared with OVA stimulation of control LN cells (Fig. 4b). This trend was also seen for IL-4, IL-10 and IL-13, although significance was not attained for these cytokines. In contrast to the Th2 cytokines, the production of IFN- γ , IL-12, TGF- β or IL-17 was not altered in the OVA/SEB group upon stimulation with OVA (data not shown).

Effects of anti-CD4 monoclonal antibodies on the Staphylococcus aureus Enterotoxin B-mediated sensitization to ovalbumin

Intraperitoneal (i.p.) injection of anti-CD4 monoclonal antibodies (mAb) significantly altered the relative

CD3⁺CD4⁺ and CD3⁺CD8⁺ cell percentages in blood and in draining lymph nodes (Fig. 5a). Furthermore, anti-CD4 mAb caused a significant reduction in OVA-specific IgE production in OVA/SEB mice compared with control IgG-treated OVA/SEB mice (Fig. 5b), as well as a reduction of total IgE levels (Fig. 5c). Anti-CD4-treated OVA/SEB mice showed a reduced number of eosinophils and lymphocytes in the BAL fluid compared with the control IgG-treated OVA/SEB mice (Fig. 5d). In addition, levels of BHR were reduced to baseline values in anti-CD4-treated mice (Fig. 5e), reaching levels of naïve (Sal/Sal) mice (Fig. 2e). This was not the case in the control group where BHR consistently developed upon OVA/SEB administration (Fig. 5e).

Staphylococcus aureus Enterotoxin B augments ovalbumin-induced dendritic cell migration, maturation and T cell proliferation

After antigen recognition, lung DCs are known to migrate to the MLN. We evaluated the DC migration process *in vivo* 24 h after i.t. OVA-FITC^{+/−} SEB administration. As shown in Fig. 6a, the number of OVA-FITC⁺ DCs in the MLN is augmented in mice receiving both SEB and OVA-FITC, compared with mice receiving only OVA-FITC. Subsequently, we investigated the DC maturation in this model, and demonstrated that DCs exposed to both OVA and SEB had a higher expression of the CD86 maturation marker *in vitro*, compared with control conditions (Fig. 6b), which is consistent with their involvement in the induction of allergic sensitization in this model.

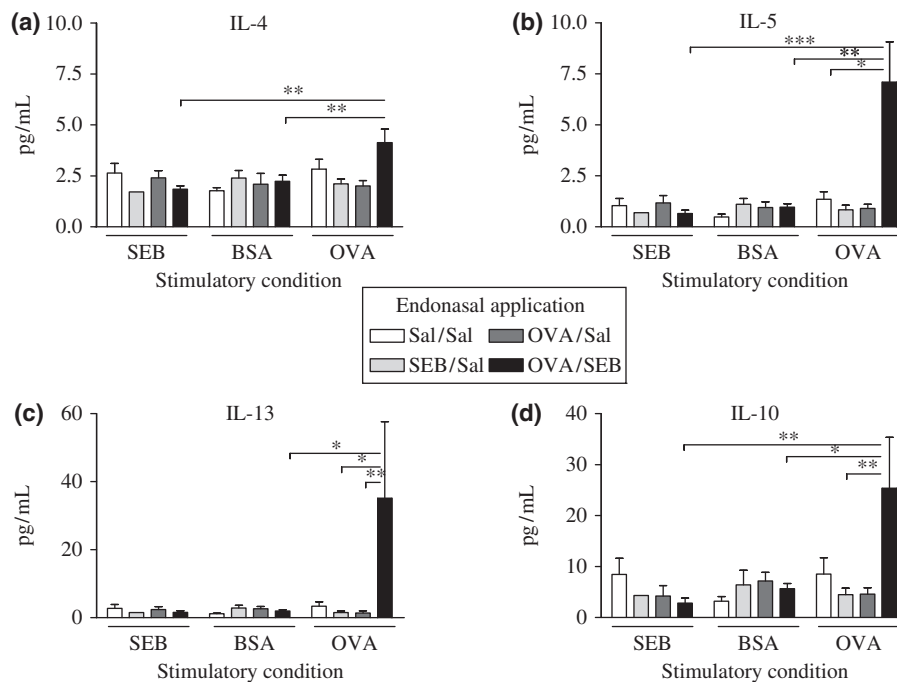


Fig. 4. (a–d) LN cells of OVA/SEB mice showed a significant increase in IL-4, IL-5, IL-13 and IL-10 production upon stimulation with OVA compared with BSA, whereas this is not the case for other mice. (b) *In vitro* stimulation of OVA/SEB LN cells with OVA induced higher IL-5 production compared with OVA stimulation of control LN cells. (c–d) This trend was also seen for IL-4, IL-10 and IL-13, although significance was not attained for these cytokines. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. OVA, ovalbumin; SEB, *Staphylococcus aureus* Enterotoxin B; LN, lymph node.

In addition to the effect of SEB on DCs, we also investigated its effects on T cell proliferation *in vivo*, through an adoptive transfer of CFSE-labelled DO11.10 OVA-specific T cells into WT BALB/c mice. 24 h after an i.v. injection of DO11.10 T cells, mice were i.t. challenged with OVA^{+/−} SEB or SEB. Figure 7 demonstrates the significant increase in the proliferation index of DO11.10 OVA-specific T cells in the MLN of mice that were concomitantly exposed to OVA and SEB, compared with mice exposed to OVA ($P < 0.001$) or SEB ($P < 0.05$) alone.

Discussion

We demonstrate here that SEB facilitates the sensitization of CD4⁺ cells to nasally applied allergen resulting in the subsequent development of experimental asthma. Concomitant endonasal application of OVA and enterotoxin B resulted in an immune response to an otherwise inert allergen, characterized by the production of allergen-specific IgE, increased production of IL-4, IL-5 and IL-13, bronchial influx of eosinophils and development of BHR. Moreover, these findings can be explained by the increase in DC migration and maturation observed in these mice, which occur in parallel to the SEB-mediated augmentation of allergen-specific T cell proliferation.

Mouse models of allergic asthma classically use artificial Th2-inducing stimuli or adjuvants such as aluminium hydroxide acting mainly on DC activation [31], to obtain

allergic sensitization. Alternatively, additional danger signals can be used to induce Th2 priming. Interestingly, we could induce the allergic inflammation in this model by applying the allergen and the enterotoxin endonasally, instead of using i.p. injections. Moreover, the combination of both was critical to induce asthma, as neither substance could do so itself. Asthmatic features in this model consisted among others of a marked bronchial influx of eosinophils. This influx may largely be attributed to the increased IL-5 production by T lymphocytes within the lung-draining lymph nodes. Besides eosinophils, SEB also induced a minor increase in neutrophils and lymphocytes in the BAL fluid. The increase in neutrophils in Sal/SEB and OVA/SEB mice may be induced by the stimulation of macrophages, which in response release chemotactic proteins for neutrophils [32]. In addition, SEB-induced IL-8 secretion by endothelial cells has also been described in *in vitro* studies [33]. A similar increase in SEB-treated mice was seen in lymphocyte counts, likely due to the superantigen activity of this enterotoxin, activating large numbers of lymphocytes.

We demonstrate a clear increase of total serum IgE in OVA/SEB mice, indicating the activation of B lymphocytes by SEB. Besides the induction of total IgE production, SEB also induced the production of OVA-specific IgE, suggestive of an important role for SEB in the process of sensitization to allergens. Of note, the facilitating effects on OVA sensitization appear to be mouse strain independent, as we

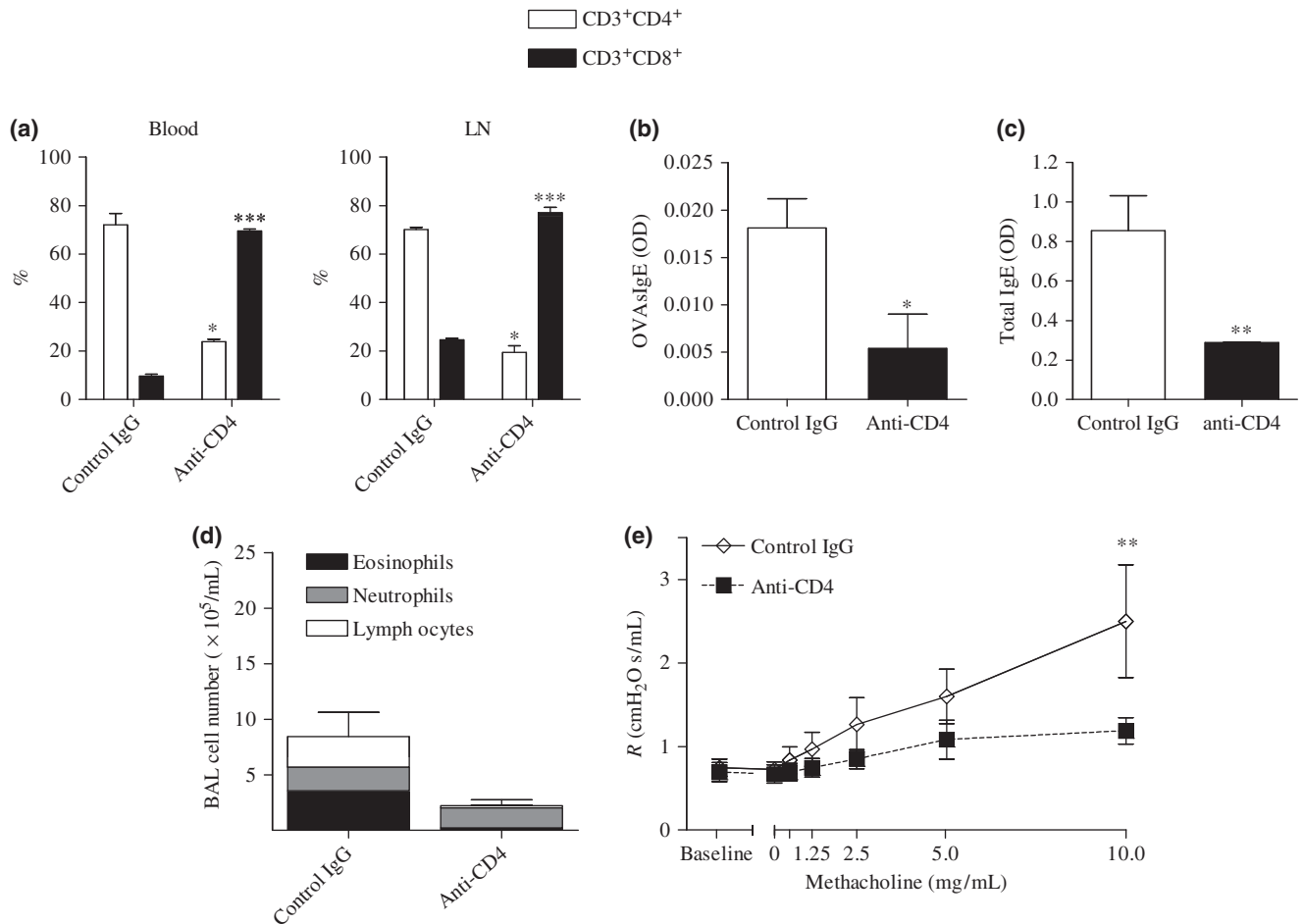


Fig. 5. (a) Change in the relative CD3⁺CD4⁺ and CD3⁺CD8⁺ cell percentages in blood and in draining lymph nodes by i.p. injection of anti-CD4 mAb on day 0 and 6 of the experimental protocol. This has resulted in decreased levels of (b) OVA-specific IgE and (c) total IgE, (d) decreased BAL eosinophils and lymphocytes and (e) bronchial hyperreactivity measured as resistance (R), compared with control IgG treatment. All mice received OVA/SEB endonasally. **P* < 0.05, ***P* < 0.01, ****P* < 0.001 vs. Control IgG. OVA, ovalbumin; SEB, *Staphylococcus aureus* Enterotoxin B; mAb, monoclonal antibodies.

have observed a similar influx of BAL eosinophils and induction of OVA-specific IgE in C57BL/6 mice using the same experimental protocol (unpublished observations). In our model, OVA or SEB application alone did not primarily alter the OVA-specific IgE production, whereas concomitant exposure of the nasal mucosa to OVA and SEB did lead to OVA-specific IgE production. We suspect that the enhanced production of IL-4 and/or IL-13 at the level of the LNs may be responsible for these observations. Among the enterotoxins studied here in BALB/c mice, only SEB influenced the sensitization process to OVA. The fact that SEA, TSST-1 or Prot A did not induce sensitization to OVA may be related to the V β chain profile of the TCR. It has been shown that SEB particularly has a high affinity for V β 8⁺ compared with V β 6⁺ T cells, and mucosal contact with SEB selectively induces the infiltration of V β 8⁺ T cells, which can be followed by a sustained influx of nonspecific T cells [24–26]. In this model, we hypothesize that the V β repertoire of the BALB/c mice appears to have

the largest affinity for SEB. The fact that we could not repeat previous reported data on mucosal airway exposure to SEB [24] or on the enhancing effects of LPS on OVA sensitization [9], might be due to differences in experimental protocol related to the route of administration, usage of anaesthesia, doses of allergen and toxin used and/or genetic background of mice used in the experiments.

To demonstrate the Th2 priming by concomitant mucosal contact with OVA/SEB, cytokines IL-4, IL-5 and IL-13 were measured in LN cell cultures after stimulation with OVA or BSA. We used splenocytes as APC, derived from a pool of spleens from 3 naïve WT mice, and co-cultured them with LN cells from Sal/Sal, Sal/SEB, OVA/Sal or OVA/SEB mice. The differences in cytokine production observed must therefore originate from the LN cell production. As expected, we demonstrated a rise in levels of IL-4, consistent with the increase in total and OVA-specific IgE observed in OVA/SEB mice [34, 35]. Moreover, OVA stimulation in OVA/SEB mice caused a significant

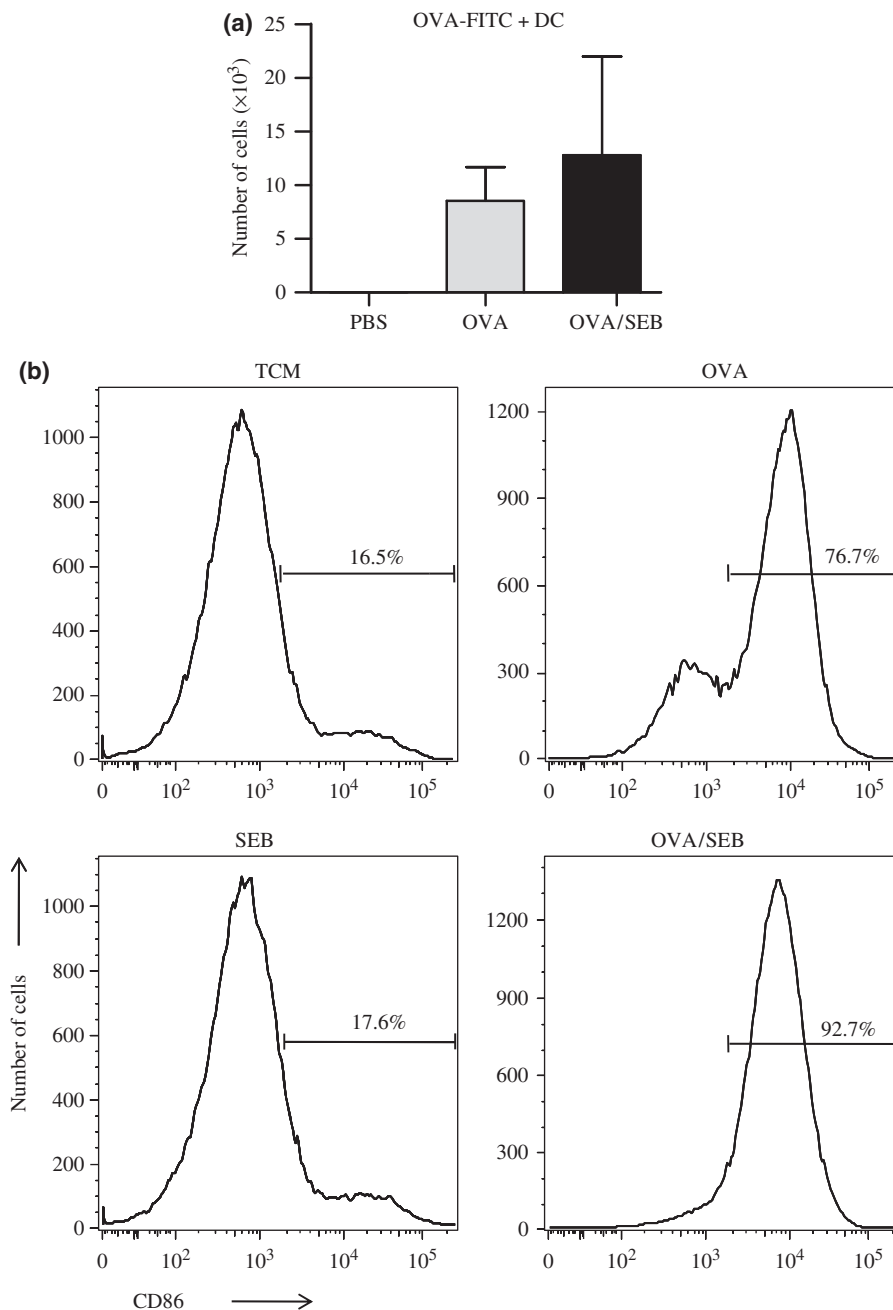


Fig. 6. (a) Twenty-four hours after OVA-FITC⁺/SEB application, the number of OVA-FITC⁺ DCs in the MLN is augmented in mice receiving both SEB and OVA-FITC, compared with mice receiving only OVA-FITC. (b) DCs exposed to both OVA and SEB had a higher expression of the CD86 maturation marker compared with the control conditions, which is consistent with their involvement in the induction of allergic sensitization in this model. One out of two representative experiments is shown. OVA, ovalbumin; SEB, *Staphylococcus aureus* Enterotoxin B; DC, dendritic cell; MLN, mediastinal lymph node.

increase in IL-5 production compared with OVA stimulation in naïve (Sal/Sal) mice, explaining the observed BAL eosinophilia in this group [36]. However, increased titres of OVA-specific IgG2a in OVA/SEB mice suggest a B cell expansion involving both IgE and IgG2a production [37, 38]. The Th2 priming in the draining lymph nodes described did not alter Th1 cytokine production (IFN- γ and IL-12), as allergen stimulation did not result in increased levels compared with BSA stimulation.

In this model, we could induce one of the major characteristics of human asthma, namely bronchial hyperresponsiveness. The increase in BR to metacholine as observed in the OVA/SEB group may be attributed to activation of different cell types among which are CD4⁺ cells. Moreover, BHR in these mice may be explained by the increased production of IL-13 and IL-10 by these T lymphocytes in OVA/SEB mice, as both cytokines have been associated with BHR in experimental asthma

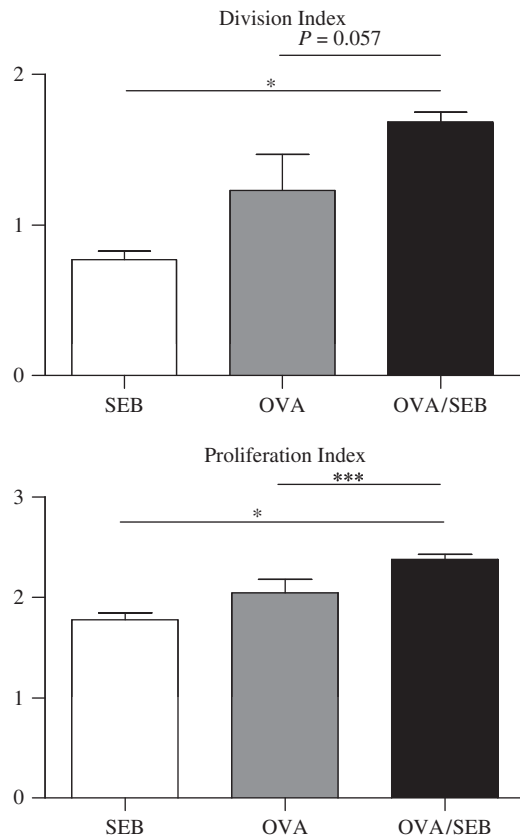


Fig. 7. Twenty-four hours after i.v. injection of CFSE-labelled DO11.10 T cells, mice were i.t. challenged with OVA^{+/−} SEB or SEB. After 3 days, MLN were harvested and analysed for the proliferation of OVA-specific TCR Tg T cells. Proliferation index was calculated, and appeared to be significantly higher in OVA/SEB mice compared with mice receiving OVA or SEB alone. One out of two representative experiments is shown. * $P < 0.05$, *** $P < 0.001$. OVA, ovalbumin; SEB, *Staphylococcus aureus* Enterotoxin B; MLN, mediastinal lymph node; TCR, T cell receptor.

[39, 40]. Interestingly, the secretion of the regulatory cytokine IL-10 was also augmented in OVA/SEB mice upon allergen stimulation *in vitro*, probably representing an inhibitory signal for the further rise in Th2 activity [41].

Treatment with anti-CD4 mAb abrogated the development of allergic disease in OVA/SEB mice, indicating the critical involvement of CD4⁺ cells in the SEB-mediated effects. Prevention of SEB-induced T cell activation by anti-CD4 mAb averted the production of OVA-specific and total IgE, the development of BHR and the influx of eosinophils in the BAL fluid. As CD4⁺ cells are primarily responsible for the different features of asthma through their cytokine profile [42–44], it is no surprise to find that a reduction of IL-4, IL-5 and IL-13 production by anti-CD4 mAb treatment resulted in abrogation of the development of experimental asthma.

Mechanistically, we were interested whether and how DCs were involved in the facilitation of sensitization in

this model, as DCs are pivotal in the induction of Th2 sensitization. Upon contact with antigens, and in the presence of some form of activation, DCs evolve towards their mature state and migrate out of the antigen-exposed site towards the draining LNs. There, antigen-specific naïve T lymphocytes are activated by DCs for proliferation and differentiation. We observed an increased migration of OVA-FITC loaded DCs towards the draining lymph nodes upon concomitant SEB application. Moreover, DCs from OVA/SEB mice appeared to express higher levels of the CD86 maturation marker. As DC migration and maturation are pivotal in the induction of Th2 sensitization, these findings can explain the induction of allergic sensitization observed. In parallel, we demonstrated the SEB-driven significant increase in allergen-specific T cell proliferation, possibly due to the superantigen activity of this enterotoxin, contributing to the described features of allergic asthma in this mouse model. Altogether, these findings provide arguments for a direct effect of SEB on DCs. However, SEB might also activate DCs via TLRs on epithelial cells producing chemokines and DC growth/differentiation factors [13]. In addition, these processes might be reinforced by the polyclonal T cell stimulation of the SEB superantigen.

In conclusion, we show that SEB facilitates the sensitization process to allergens like ovalbumin, thereby inducing allergic airway disease. The SEB-mediated effects on sensitization to OVA and the consecutive bronchial inflammation and hyperreactivity appeared to be CD4⁺ cell dependent, and rely on increased DC migration and maturation upon SEB exposure in the presence of allergen, in parallel to augmented allergen-specific T cell proliferation. These combined pathways result in the allergic asthma phenotype in this mouse model of nasal applied allergen and enterotoxin.

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