

Neonatal exposure to staphylococcal superantigen improves induction of oral tolerance in a mouse model of airway allergy

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The hygiene hypothesis suggests that lack of microbial stimulation in early infancy may lead to allergy, but it has been difficult to identify particular protective microbial exposures. We have observed that infants colonised in the first week(s) of life with *Staphylococcus aureus* have lower risk of developing food allergy. As many *S. aureus* strains produce superantigens with T-cell stimulating properties, we here investigate whether neonatal mucosal exposure to superantigen could influence the capacity to develop oral tolerance and reduce sensitisation and allergy. BALB/c mice were exposed to staphylococcal enterotoxin A (SEA) as neonates and fed with OVA as adults, prior to sensitisation and i.n. OVA challenge. Our results show that SEA pre-treated mice are more efficiently tolerised by OVA feeding, as shown by lower lung-cell infiltration and antigen-specific IgE response in the SEA pre-treated mice, compared with sham-treated mice. This was not due to deletion or anergy of lymphocytes by SEA treatment, because the SEA pre-treated mice that were fed with PBS showed similar inflammatory response as the sham-treated PBS-fed mice. Our results suggest that strong T-cell activation in infancy conditions the mucosal immune system and promotes development of oral tolerance.

Key words: Allergic sensitisation · Mucosal immunity · Staphylococcal enterotoxin A · Tolerance



Supporting Information available online

Introduction

Allergies have increased markedly in Western industrialised countries, where they now afflict every third child. Allergies are linked to wealth, good education and small families,

observations that have given rise to the hygiene hypothesis, according to which the developing immune system should be exposed to appropriate microbial stimulation in early childhood in order to mature correctly and for allergies to be avoided [1, 2].

The hygiene hypothesis is also supported by experimental data. Germ-free animals are less prone to develop oral

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tolerance than animals reared conventionally [3, 4]. Oral tolerance is induced by passage of antigens over the gut mucosa, prevents against both Th1- and Th2-dominated immune responses [5] and includes development of Treg [6, 7]. Natural thymic-dependent Treg ($CD4^+CD25^{hi}$ FoxP3⁺) are fundamental in protection against autoimmunity, gut inflammation and IgE responses. This cell subset has reduced functional capacity in germ-free animals [8].

The strongest T-cell activators known are the “superantigens”, exotoxins produced by certain pathogenic bacteria such as *Staphylococcus aureus*. These include staphylococcal enterotoxin A, B, C, D and E, as well as toxic shock syndrome toxin-1. The superantigen binds to the variable part of the TCR and to the MHC class II molecule, thereby “mimicking” antigen recognition, which leads to T-cell activation [9–12]. As many as 10–30% of all T cells can become activated by a certain superantigen compared with <0.1% for a normal antigen [13]. The enterotoxins induce vomiting and diarrhoea. They are taken up by small intestinal epithelial cells and strongly activate intraepithelial lymphocytes to produce IFN- γ [14]. Toxic shock syndrome toxin-1 does not cause food poisoning due to inactivation in the digestive system and is instead responsible for the toxic shock syndrome caused by growth and toxin production of *S. aureus* in highly absorptive tampons.

S. aureus is foremost a skin bacterium and has previously been incriminated in the pathogenesis of eczema, because *S. aureus* are often found colonising eczematous skin lesions, aggravating the lesion and because IgE antibodies to *S. aureus* superantigen may be found in such cases [15]. However, we have recently observed that *S. aureus* has become a quite common inhabitant of the gut microbiota in Swedish infants [16, 17], probably as a result of decreased competition from “classical” faecal bacteria, whose circulation has decreased strongly in today’s highly hygienic hospitals and homes [18, 19]. Of *S. aureus* strains colonising the neonatal gut, approximately 45% have the capacity to produce a toxin with superantigenic function [16, 17]. We have not noted any untoward effect of such colonisation, as parents report no increased prevalence of gastro-intestinal or other symptoms [17]. Quite unexpectedly, we instead found that infants colonised in the first week(s) of life with *S. aureus* had significantly decreased risk of developing food allergy [20].

Animal experiments indicate that parenteral administration of *S. aureus* superantigen may promote development and functional activity of Tregs [21–23]. The present study is designed to test the hypothesis that mucosal exposure to *S. aureus* superantigen in neonatal life would enhance the capacity to develop oral tolerance to a specific antigen and thereby protect against later hypersensitivity to the same antigen. This hypothesis was tested by exposing newborn mice to staphylococcal enterotoxin A (SEA); they were later fed with OVA and the degree of oral tolerisation was tested in a model of OVA-induced airway allergy.

Results

Induction of oral tolerance protects against development of airway allergy

We first tested whether prior feeding of OVA could tolerise mice for later sensitisation and airway allergy induced by OVA. We tested different feeding doses of OVA and found that airway allergy was reduced in mice given less than 3 mg of OVA (Supporting Information Fig. 1). We selected the optimal OVA dose to 5 mg because this dose induced only partial tolerance and resulted in a reduction of lung eosinophil infiltration by approximately 50% compared with PBS-fed control mice. Partial tolerance enabled us to study a possible improvement in oral tolerance induction. Mice were fed with OVA (5 mg) 9 days prior to OVA sensitisation. One week after sensitisation they were challenged by OVA i.n. (Fig. 1). Sensitisation was measured as serum IgE anti-OVA titres and airway allergy was measured as infiltration of cells into the lung diagnosed by BAL. Figure 2 shows that mice fed with OVA prior to sensitisation and challenge in the model of airway allergy had approximately one-fifth of the concentration of inflammatory cells and 30% lower numbers of eosinophils in the BALF (Fig. 2A) compared with mice not orally tolerised (fed PBS instead of OVA). Lung tissue sections also displayed less inflammatory cell infiltration in OVA-fed mice compared with those fed with PBS (Fig. 2B). In addition, OVA-fed mice showed approximately threefold lower serum levels of OVA-specific IgE and sixfold lower levels of total IgE (Fig. 2C) compared with PBS-fed mice.

Neonatal SEA administration improves development of oral tolerance

To evaluate whether neonatal superantigen treatment could augment the capacity to develop oral tolerance to OVA, we treated newborn pups with SEA via different routes during their first 2 wk of life (Fig. 1). Control mice were sham treated with PBS *per oral* (ly) (p.o.). OVA (or PBS) were fed to the mice at 6 wk of age and the degree of OVA-specific tolerance was evaluated as reduction of OVA-dependent sensitisation and lung-cell infiltration in the airway allergy model.

Neonatal administration of SEA enhanced the tolerising effect afforded by OVA feeding, as demonstrated in Fig. 3. Thus, neonatal mice treated with SEA p.o. and tolerised when reaching adult age had four times lower number of total cells in the BALF (Fig. 3A) and ten times lower number of eosinophils in the BALF (Fig. 3C) compared with mice that were fed with OVA without having been treated neonatally with SEA (sham-treated mice). Neonatal treatment with SEA via the i.n. or i.p. route also enhanced the capacity to develop tolerance to OVA, but perhaps slightly less efficiently than p.o. exposure (Fig. 3A and C). The effect was dependent on OVA feeding, because neonatal SEA exposure had no effect on OVA-induced airway allergy in the absence of oral tolerisation to OVA (Fig. 3B and D). We saw no

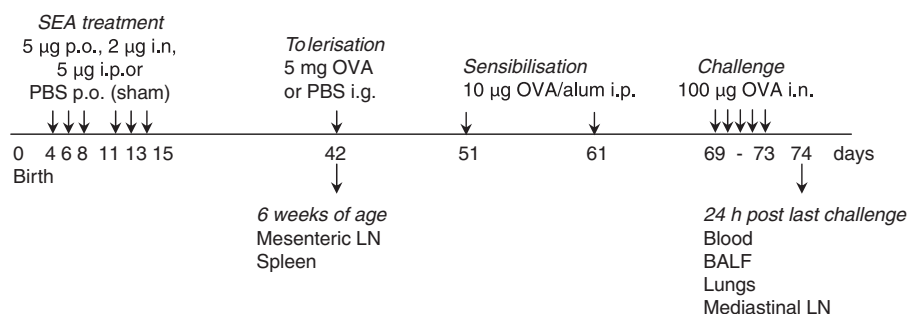


Figure 1. Experimental design. Groups of newborn BALB/c pups ($n = 8–11$) were treated with SEA p.o. (5 µg) or i.n. (2 µg) six times as shown, or i.p. (5 µg) three times (at days 11, 13 and 15 after birth) or sham-treated p.o. At 6 wk of age, the mice were fed with OVA to induce oral tolerance; control groups were fed with PBS. A sub-cohort of mice was sacrificed at 6 wk of age and MLN and spleen were collected for flow cytometric analysis of cell subsets. Airway allergy was induced 1 wk after oral feeding by sensitisation through two i.p. injections of alum-adsorbed OVA, administered 10 days apart, and followed 8 days later by i.n. challenge with OVA on five consecutive days. Mice were sacrificed 24 h after the last challenge and BALF, blood, lungs and mediastinal LN were collected for analysis.

differences in neutrophils or other cell populations in BALF between SEA pre-treated and sham-treated mice (Supporting Information Fig. 2).

Figure 4 also shows that antigen-specific IgE levels were more effectively reduced in mice that had been treated with SEA neonatally. Mice that had been given SEA p.o. as pups and later fed with OVA had five times lower levels of OVA-specific IgE antibodies in serum compared with sham-treated OVA-fed mice (Fig. 4A). i.n. pre-treatment with SEA also significantly reduced OVA-specific IgE production (Fig. 4A). Total IgE levels were unaffected by SEA pre-treatment (Fig. 4C). The effect of neonatal SEA treatment was entirely restricted to augmentation of oral tolerance induction, as it had no effect on IgE production in animals not fed with OVA (Fig. 4B and D).

Lung cells obtained from sensitised and challenged mice were stimulated *in vitro* with OVA and cytokines were measured in the supernatants. Mice that had been fed with OVA prior to sensitisation and challenge produced somewhat lower levels of IL-5 and IL-13 compared with PBS-fed mice (Supporting Information Fig. 3A and B). Neonatal SEA treatment resulted in reduced production of IL-13 after OVA feeding, compared with sham treatment, but the difference was of borderline significance ($p = 0.055$) (Supporting Information Fig. 3B). IL-10 levels were not significantly changed by OVA feeding and neonatal SEA treatment did not seem to affect this variable (Supporting Information Fig. 3C). IFN- γ was not detectable in any of the supernatants from the stimulated lung cells.

We were interested to see whether Treg were involved in the reduction of inflammation seen in the OVA-fed mice and therefore we isolated cells from the mediastinal LN draining the lung after the last i.n. OVA challenge. The cells were stained and examined by flow cytometry. Mice orally tolerised by OVA had a higher proportion of FoxP3⁺CD25⁺ cells among the total CD4⁺ T-cell population (Supporting Information Fig. 4). However, SEA pre-treatment did not further increase the proportion of FoxP3⁺CD25⁺ cells. Thus, the proportion of FoxP3⁺ cells was related to oral tolerisation, but not to SEA pre-treatment.

SEA administration to adult mice does not improve development of oral tolerance

Epidemiological studies strongly support that exposure to “protective factors” against allergy development must be present early in life in order to have an effect. We studied whether pre-treatment with SEA at adult age would enhance the development of oral tolerance as did neonatal exposure to SEA. SEA was administered intragastrically to 6-wk-old mice, followed by oral tolerisation 4 wk later and sensitisation and challenge according to the same protocol as that used for the newborn mice. In contrast to the previous observations in neonatally SEA-treated mice, we saw no improvement of oral tolerance in the adult mice pre-treated with SEA prior to oral tolerisation compared with sham pre-treated mice. The number of eosinophils in BALF and levels of total and OVA-specific IgE are shown in Fig. 5A–C.

Lymphocytes isolated from SEA and sham-treated mice are similar in proportion and activation pattern

Systemic superantigen treatment is known to lead to expansion of T cells followed by deletion of cells with the affected V β receptors [12, 24–27]. In order to investigate whether neonatally SEA primed mice differed from sham-treated mice with respect to lymphocyte or APC availability by the time point for oral tolerisation, we isolated cells from the MLN and spleen at 6 wk of age and analysed them by flow cytometry for the presence of memory, naïve and activated T cells (CD4⁺, CD8⁺ and Treg cells), B cells and DC. We found that spleens and MLN from neonatally SEA- or sham-treated mice had comparable number of total cells (Table 1). After gating of lymphocytes, the numbers of CD4⁺ and CD8⁺ T cells were similar in neonatally SEA- and sham-treated mice both in MLN and in spleen (Table 1). This suggested that, even if the SEA exposed mice had gone through a period of T-cell deletion, the T-cell levels had recovered at the time point for oral tolerisation, which has previously been demonstrated by others [28]. The number of B cells and

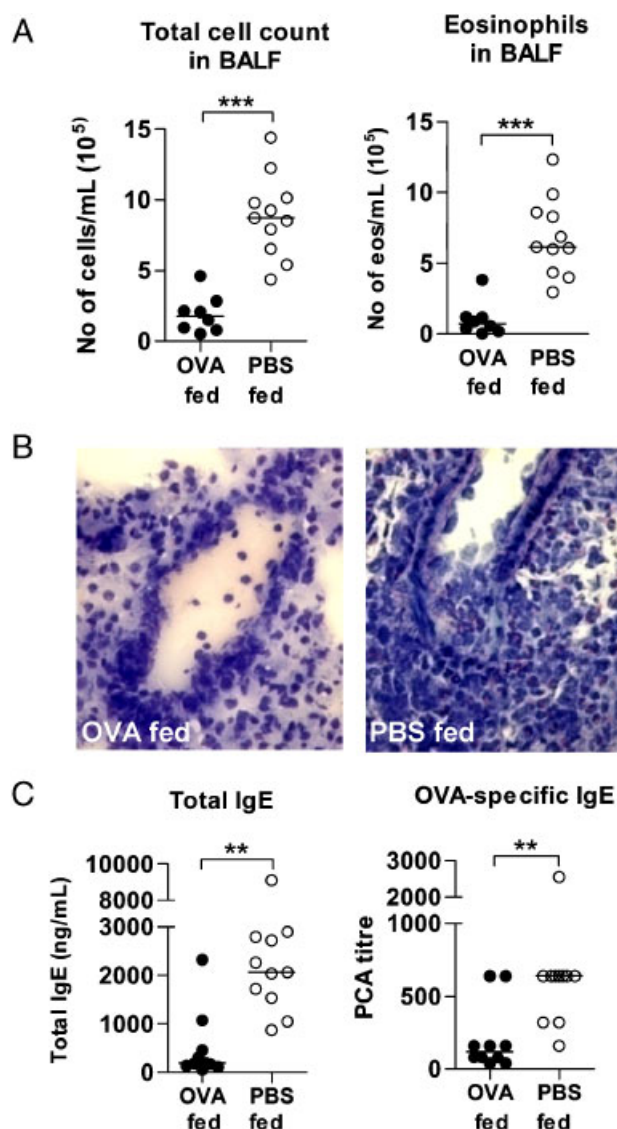


Figure 2. Oral administration of OVA reduces the development of OVA-specific IgE and eosinophil-mediated inflammation. Mice were fed with 5 mg OVA (or PBS) 1 wk before sensitisation and challenge with OVA. This treatment reduced total cell infiltration and the number of eosinophils in the BALF (A), ameliorated inflammation in lung tissue sections (B), reduced serum levels of total and OVA-specific IgE (C). Data are expressed as individual values and median of 8–11 mice and are representative of three independent experiments. Statistical analyses were performed with Mann–Whitney U-test and statistics from OVA-fed mice compared with PBS-fed mice are shown as *** $p < 0.001$ and ** $p < 0.01$.

DC in MLN and spleen did not differ between SEA pre-treated and sham-treated mice (Table 1). The frequency of viable cells as well as early and late apoptotic cells, as estimated by staining cells with 7-AAD and Annexin V, did not differ at the time of oral tolerisation between mice neonatally treated with SEA or PBS (data not shown). In addition, adult SEA pre-treated mice had comparable leukocyte populations as adult sham pre-treated mice during time point for oral tolerisation (data not shown).

When examining the T-cell population at 6 wk of age, we found no difference between the SEA pre-treated and sham-treated mice in the expression of the activation marker CD69 or absence of the naïve T-cell marker CD62L (Table 1). We also stained the cells for different SEA-specific V β regions of the TCR (V β 3 and V β 11) that are expressed by BALB/c mice and found a slightly lower proportion of V β 11⁺ cells in the neonatally SEA-treated mice compared with the sham-treated mice, thus indicating a previous deletion of V β 11⁺ cells owing to the exposure of SEA. We could not detect any V β 3⁺ cells in either SEA- or sham-treated mice (data not shown), which might be due to clonal elimination of the V β 3⁺ cells by endogenous proviruses [29–31].

SEA administration increases the proportion of Treg expressing CCR9

When gating for Treg (FoxP3⁺CD69[−], FoxP3⁺CD25^{hi}, FoxP3⁺CD62L^{low}, CD25^{hi}CTLA-4⁺) among the CD4⁺ population, we found no differences in frequency between SEA- and sham-treated mice (Table 1). There was also no difference in the MFI of CTLA-4 among the CD4⁺CD25^{hi} cells, between the SEA- and sham-treated mice (data not shown). However, a higher proportion of the CD4⁺FoxP3⁺ T cells expressed the gut homing marker CCR9 in SEA pre-treated mice compared with sham-treated mice, thus indicating that SEA pre-treatment might increase the ability of Treg to home to the gut mucosa (Fig. 6).

CD4⁺CD25⁺ Treg isolated from SEA- or sham-treated mice are equally potent suppressors

In order to test the functionality of Treg at the time point for oral tolerisation, we investigated the suppressive ability of CD4⁺CD25⁺ T cells isolated from MLN of mice at 6 wk of age and found that both the CD4⁺CD25⁺ T cells isolated from SEA pre-treated mice and the CD4⁺CD25⁺ T cells isolated from sham pre-treated mice were equally potent in suppressing a polyclonal mitogen stimulation (Supporting Information Fig. 5).

Discussion

Neonatal mucosal exposure to the *S. aureus* superantigen SEA significantly facilitated oral tolerance induction. Tolerance was manifested as reduced production of OVA-specific IgE and OVA-induced eosinophil-mediated airway allergy. Notably, SEA pre-treatment had no effect on airway allergy in general, i.e. in the absence of prior OVA feeding. Thus, neonatal treatment with SEA specifically augmented the capacity to develop oral tolerance and this effect was of long duration as oral tolerisation was performed 4 wk after SEA treatment. Furthermore, the effect was seen only if superantigen was administered neonatally, because SEA pre-treatment of adult mice 4 wk prior to OVA feeding did not enhance their capacity to develop oral tolerance to OVA.

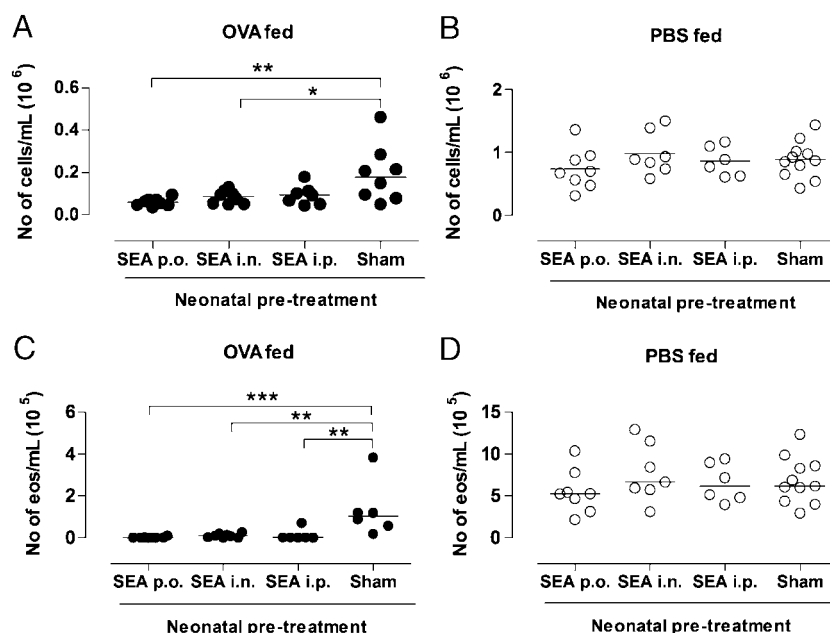


Figure 3. Effects of neonatal administration of SEA on the capacity to develop oral tolerance in a model of OVA-induced airway allergy. Mice that had been primed by SEA neonatally p.o., i.n. or i.p., or sham-treated, were given 5 mg OVA (a dose that induces partial tolerance) by 6 wk of age. The mice were sensitised and challenged with OVA as in the airway allergy model. The total number of cells (A) as well eosinophils (C) in the BALF was reduced in neonatally SEA pre-treated mice. This was not observed in control PBS-fed mice (B and D for number of BALF cells and eosinophils, respectively). Data are shown as individual values and median of 6–11 mice, and are representative of three independent experiments. Statistical analysis was performed with Mann–Whitney U-test. Statistics from SEA pre-treated OVA-fed mice compared with sham-treated OVA-fed mice are shown as * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

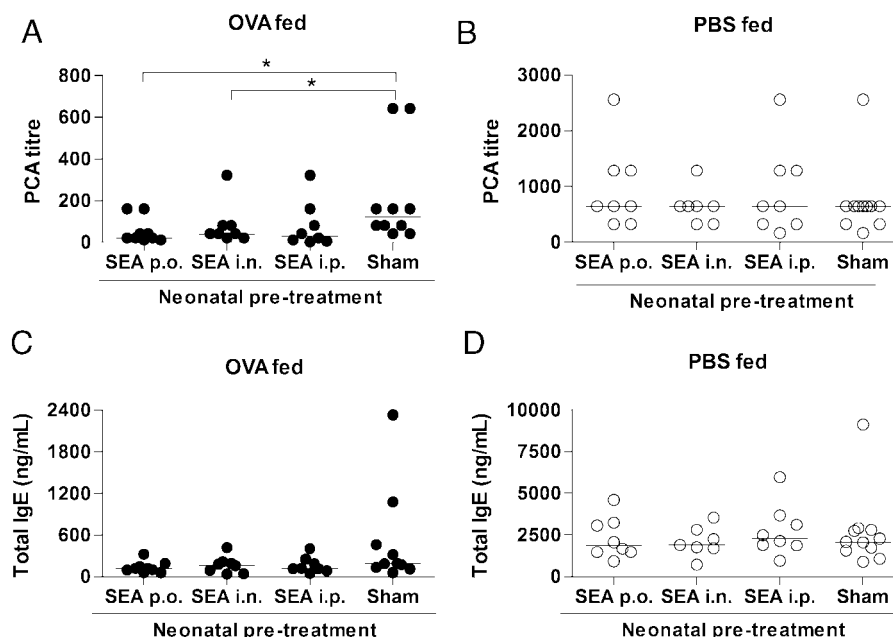


Figure 4. Oral tolerisation of OVA sensitisation is augmented by mucosal neonatal SEA treatment. Mice were pre-treated with SEA p.o., i.n. or i.p., or given PBS p.o. At 6 wk of age, mice were orally tolerised with oral administration of OVA (or PBS as control). OVA-specific and total IgE was measured after sensitisation and challenge as in the airway allergy model. SEA pre-treatment via mucosal route enhanced the tolerising effect of OVA feeding on specific IgE production (A), but had no effect on total IgE levels (C). Neonatal SEA treatment had no effect on production of OVA-specific (B) or total (D) IgE in the airway allergy model in the absence of OVA feeding. Data are shown as individual values and median of 8–11 mice and are representative of three independent experiments. Statistical analysis was performed with Mann–Whitney U-test. Statistics from SEA pre-treated OVA-fed mice as compared with sham-treated OVA-fed mice are shown as * $p < 0.05$.

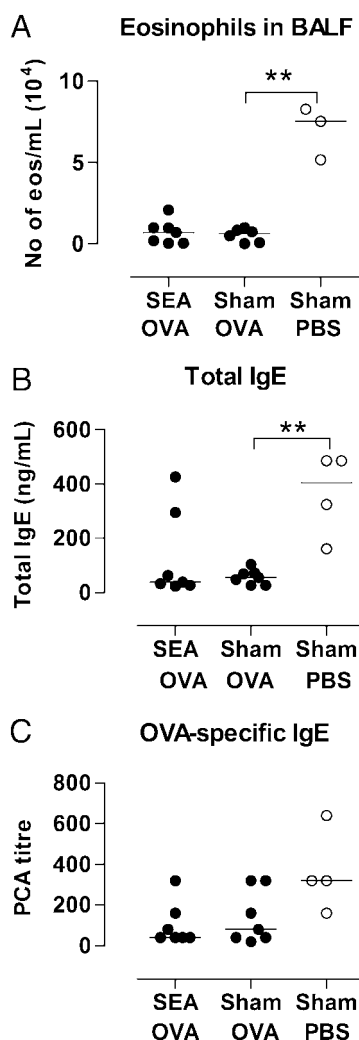


Figure 5. Pre-treatment with SEA in adult mice does not improve oral tolerance induction. Mice were given SEA p.o. or PBS (sham treatment) 4 wk before inducing oral tolerance by feeding OVA, or PBS as a control. Mice were sensitised 1 wk later and challenged with OVA as in the airway allergy model. The number of eosinophils in BALF (A) and levels of total (B) and OVA-specific IgE (C) in serum were reduced in OVA-fed mice compared with PBS-fed mice. Pre-treatment with SEA did not augment the tolerising capacity further. Data are shown as individual values and median of 4–7 mice and are representative of two independent experiments. Statistical analysis was performed with Mann–Whitney U-test. Statistics from sham-treated OVA-fed mice compared with sham-treated PBS-fed mice are shown as ** $p < 0.01$.

SEA is the most potent superantigen [32] and because SEA-producing strains are more common than SEB-producing strains in the gut flora of Swedish children [17], SEA was used in this study. However, we also investigated the effect of SEB pre-treatment in our model of oral tolerance and airway allergy and found that SEB was less effective and less reliable in improving oral tolerance compared with SEA (unpublished data). The greater efficiency of SEA might be due to the fact that SEA can reside in the gut, whereas SEB is more readily transcytosed over the gut epithelium and enters the blood stream [33].

Table 1. In vivo condition in SEA- and sham-treated mice at the time point for oral tolerisation^{a)}

	SEA p.o. Spleen	Sham Spleen	SEA p.o. MLN	Sham MLN
No. of cells(10^6)	37 ± 3.3	48 ± 9.0	12 ± 2.6	13 ± 1.3
No. of cells/g MLN			9.0 ± 1.9	10 ± 1.1
No. of CD4 ⁺ cells	7.9 ± 0.8	8.3 ± 1.6	5.7 ± 1.2	6.5 ± 0.7
No. of CD8 ⁺ cells	3.4 ± 0.4	3.7 ± 0.8	2.2 ± 0.5	2.6 ± 0.2
No. of CD19 ⁺ cells	14 ± 2.0	24 ± 4.8	2.6 ± 0.5	2.5 ± 0.3
No. of CD11c ⁺ cells	2.1 ± 0.2	3.4 ± 0.8	0.2 ± 0.1	0.2 ± 0.1
Proportion (% of CD4 ⁺)				
FoxP3 ⁺ CD25 ^{hi}	7.1 ± 0.3	7.0 ± 0.3	5.1 ± 0.3	5.6 ± 0.2
FoxP3 ⁺ D69 ⁺	10 ± 0.4	10 ± 0.3	6.8 ± 0.4	7.0 ± 0.3
FoxP3 ⁺ CD62L ^{low}	4.6 ± 0.2	4.4 ± 0.3	6.0 ± 0.2	5.4 ± 0.3
CTLA-4 ⁺ CD25 ⁺	10 ± 0.4	9.8 ± 0.7	9.9 ± 1.0	9.0 ± 1.1
CD69 ⁺ of CD4 ⁺	7.0 ± 0.2	7.1 ± 0.1	12 ± 0.2	12 ± 0.4
CD62L ^{low} of CD4 ⁺	20 ± 0.8	21 ± 1.1	53 ± 2.3	50 ± 1.2
CD62L ^{low} of CD8 ⁺	12 ± 0.8	15 ± 2.1	73 ± 0.9	74 ± 0.8

^{a)} Spleen and mesenteric LN cells taken from SEA pre-treated and sham-treated mice at 6 wk of age were analysed by flow cytometry. Data are presented as median ± SEM and are representative of three separate experiments ($n = 3–5$).

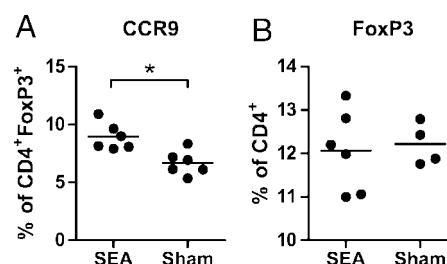


Figure 6. Neonatal treatment with SEA increase the proportion of CD4⁺FoxP3⁺ T cells expressing the gut homing marker CCR9. Mice were pre-treated with SEA p.o. or given PBS p.o. (sham) and MLN cells were taken from the mice 4 wk after SEA/sham treatment and were analysed by flow cytometry. Mice pre-treated with SEA had more CD4⁺FoxP3⁺ cells expressing CCR9 compared with sham-treated mice (A). There was no difference in the frequency of CD4⁺ cells expressing FoxP3 between both the groups (B). Data are shown as individual values and median of six mice and are representative of two independent experiments. Statistical analysis was performed with Mann–Whitney U-test and visualised as * $p < 0.05$.

Extensive variability between individual mice in the response to antigen challenge characterised the orally tolerised sham pre-treated mice, whereas SEA pre-treated mice were remarkably tolerant with a minimal spread among individual animals. It seems as though SEA pre-treatment maximises the capacity to develop tolerance after feeding, whereas the process is more variable in efficiency in control mice, probably due to individual differences regarding conditions prevailing in the gut at the time point for tolerisation.

Studies have shown that i.v. administration of superantigen causes activation of T cells with the corresponding V β chain,

followed by deletion of these cells both in neonatal [12] and in adult mice [24–27]. However, the T-cell levels are restored to normal within a few weeks [28]. In the present study, SEA pretreated mice had equal numbers and proportions of lymphocytes as the sham-treated mice at the time point for oral tolerisation, thus ruling out the possibility that the observed differences in oral tolerance induction between SEA and sham pre-treated mice was due to an elimination of a substantial proportion of the Th cells in the SEA pre-treated mice. However, we found a slight reduction in the frequency of T cells expressing V β 11 in the group of mice that had been treated with SEA, which points towards a previous deletion of these cells.

Moreover, we found no differences between mice pre-treated with SEA or sham-treated mice in the activation state of the T cells or the proportion of naïve cells in the spleens or MLN 4 wk after SEA pre-treatment, also ruling out the possibility that SEA-treated mice had an ongoing inflammation, which in turn could deviate the immune response and affect the inflammatory outcome in the model of airway allergy. This possibility is further refuted because SEA pre-treated mice were equally allergic as sham-treated mice in the absence of OVA feeding. We conclude that neonatal SEA treatment in some way alters the way an oral antigen is handled by the gut-associated immune system.

Oral tolerisation requires that the antigen passes through the intestinal epithelial cell, a process called tolerogenic processing [34]. This process does not function in the absence of T cells [35], is reduced in efficiency in germ-free mice [4, 36] and is dependent on the expression of MHC class II molecules in the intestinal epithelium [37]. SEA feeding induces a massive secretion of IFN- γ from IEL, leading to up-regulation of heat shock proteins and MHC class II on the intestinal epithelium [38]. Later, infiltration of leukocytes into the lamina propria and an increase in IEL are seen.

One possibility in which neonatal SEA treatment could regulate tolerance induction is by a profound activation and expansion of the IEL population and the intestinal epithelium, which in turn would facilitate the tolerogenic processing and presentation of a novel antigen much later in life. Tolerogenic processing involves coupling of dietary antigen peptides to MHC class II molecules within the intestinal epithelial cell and export of the complex on membranous vesicles called tolerosomes [37, 39]. The tolerosomes can be isolated from the blood shortly after feeding of an antigen and induce tolerance upon transfer into naïve recipients [40]. Interestingly, we have found that tolerosomes isolated from mice that were p.o.-treated with SEA induce a better tolerance as compared with tolerosomes isolated from non-SEA-treated mice (Hultkrantz *et al.*, manuscript in preparation).

Another possibility is that neonatal SEA treatment might expand the population of putative Treg in mice. Data from animal experiments indicate that exposure to *S. aureus* superantigen favours development and functional activity of Treg. Thus, repeated i.v. injections of SEB to mice result in

development of T cells with regulatory function [21, 22] and increased *in vitro* suppressive capacity of isolated Treg [23] as well as increased serum levels of the cytokine IL-10 [22], which is known to down-regulate T-cell activation. CD4⁺ T cells from mice injected with SEA are about threefold more potent suppressors of SEA-induced T-cell proliferation and IL-2 production compared with natural CD4⁺CD25⁺ Treg from untreated mice [23]. However, in the present study, we could not detect any increase in the number of T cells with regulatory phenotype in mice pre-treated with SEA, at least not 4 wk after SEA treatment. Interestingly, we observed that SEA pre-treated mice had an increased expression of the chemokine receptor CCR9 on their Treg population in MLN at 6 wk of age. Expression of CCR9 is imprinted on T cells primed in the Peyer's patches and the gut draining MLN, enabling migration to the gut [41–43]. This finding implies that the observed improvement in oral tolerance might be due to an increased recruitment of Treg to the gut.

We also have some preliminary data demonstrating that SEA pre-treated mice have a decreased expression of activation markers on their DC in the MLN at the time point for oral tolerisation (unpublished data). Studies have shown that APC residing both in the intestinal lamina propria and in the MLN have distinct tolerogenic properties and that their antigen presenting ability favours development of Treg [44]. A tolerogenic APC lacks the complete range of co-stimulatory molecules necessary for full T-cell activation and APC with a tolerogenic potential has the capacity to present an antigen to a naïve T cell in such a manner that it is converted to a Treg [45].

In conclusion, we show here that mice neonatally pretreated with *S. aureus* superantigen (SEA) improve their oral tolerance mechanism. Further studies are needed to dissect the involved mechanisms behind the remarkably long-lasting effect of neonatal SEA administration, which act in favour of oral tolerance. Interestingly, the data obtained here correspond to our finding of reduced incidence of food allergy in infants who were neonatally colonised by *S. aureus* in the gut [20].

Materials and methods

Animals

Pre-mated female BALB/c mice, male BALB/c mice (6-wk old) and male Sprague-Dawley rats (12-wk old) were purchased from Scanbur BK (Sollentuna, Sweden). The animals were maintained under conventional housing conditions and provided with food and water *ad libitum*. Within a week, the pregnant mice gave birth to pups, which were used in the experiment. Permission was granted by the regional Ethics Committee, University of Gothenburg, permission number 238-2006.

Administration of superantigen

Neonatal pups were treated with SEA (Sigma, St. Louis, MO) (Fig. 1). Superantigen was administered p.o. (5 µg in 5 µL of PBS) or i.n. (2 µg in 2 µL of PBS) six times over the first 2 wk of life, starting when the pups were 4–5 days old. i.p. administration (5 µg in 25 µL of PBS) was performed three times over 1 wk, starting when the pups were 11 days old. Sham-treated mice were given p.o. PBS. Adult mice (6-week old) were given 5 µg SEA in 300 µL of PBS, or PBS alone, via gastric intubation through a feeding needle.

Oral tolerisation protocol

Four weeks after the last dose of SEA (or PBS), 5 mg of OVA (grade V; Sigma) in 300 µL of PBS, or PBS alone, was given intragastrically (Fig. 1). The dose was selected to induce partial oral tolerance.

To investigate the immune status and potential effect of SEA pre-treatment, a sub-cohort of mice were sacrificed at 6 wk of age, which is the time point for oral tolerisation. Single-cell suspensions were prepared from spleens and MLN. After incubation of spleen cells with ammonium chloride buffer (pH 7.3) for 5 min at 37°C to lyse red blood cells, 0.5×10^6 cells were stained for flow cytometric analysis (FACSCanto; BD Biosciences).

Airway allergy protocol

The degree of OVA tolerisation was tested in the OVA-induced airway allergy model (Fig. 1). On day 9 and 19 after oral tolerisation, mice were immunised i.p. with 10 µg of OVA adsorbed onto 2 mg of Al(OH)₃ gel (Alum; Sigma) in order to trigger an IgE response. Studies suggest that alum adjuvanticity is due to an increased antigen uptake by APC, as well as local APC recruitment and migration of APC to draining LN. In addition, alum seems to induce activation of Nlrp3 (Nalp3) inflammasome complex, leading to secretion of pro-inflammatory cytokines. These findings have recently been discussed and commented [46]. Eight days after the second immunisation, the animals were briefly anaesthetised (Isofluran, Baxter Medical AB, Kista, Sweden) and challenged by i.n. administration of 100 µg of OVA in 25 µL of PBS. Challenge was repeated on five consecutive days.

Twenty-four hours after the last OVA challenge, BALF, serum, mediastinal LN and lungs were collected (Fig. 1). The mice were deeply anaesthetised by i.p. injection of a mixture of xylazine (130 mg/kg) and ketamine (670 mg/kg). The chest was opened, blood was drawn by heart puncture and serum was collected after clotting and centrifugation (3000g) for 15 min. BAL was performed by instilling 0.4 mL of PBS through the trachea followed by gentle aspiration and then repeated a second time with 0.4 mL of PBS. Cells from the BALF were counted in a haematocytometer and the proportion of eosinophils was evaluated in cytopsin preparations stained with May–Grünwald/Giemsa.

The left lung was taken from sets of mice that did not undergo BAL lavage. The lung was embedded in freeze medium (Tissue-Tek O.C.T compound; Sakura, Zoeterwoude, The Netherlands) and instantly frozen in isopentane cooled with liquid nitrogen and then stored at –70°C. Tissue sections (6 µm) were prepared using a cryostat. The sections were dried at 37°C for 1 h before staining with May–Grünwald/Giemsa and examination in the microscope (Leica DMR).

Flow cytometry

The following mAb with matching isotype control were used on single-cell suspensions from spleen and mediastinal and MLN: FcγR-blocking mAb, PerCP anti-mouse CD25, APC anti-mouse CD4, PerCP anti-mouse CD8α, FITC hamster anti-mouse CD69, PE anti-mouse CD62L (L-selectin), PE anti-mouse Vβ3 TCR, PE anti-mouse Vβ11 TCR, PerCP anti-mouse CD19, APC anti-mouse CD11c and PE anti-mouse CD152 (CTLA-4). These were purchased from BD Biosciences Pharmingen. PE anti-mouse CCR9 was purchased from R&D systems (Minneapolis, MN) and FITC anti-mouse/rat FoxP3 was purchased from eBioscience (San Diego, CA). To examine the expression of cell surface molecules, cells were first pre-incubated with FcγR-blocking mAb and then stained with antibodies towards the different surface molecules. For intracellular staining with FoxP3-PE and CTLA-4-PE, the cells were fixed and permeabilised with Fix/Perm (eBioscience) for 30 min followed by staining. To determine the proportion of viable cells, the cells were stained with 7-AAD (BD Biosciences Pharmingen) and APC-conjugated Annexin V (BD Biosciences Pharmingen). Cells were acquired using FACSCanto (BD Biosciences) and analysed with FlowJo software (Treestar, Ashland, OR).

Determination of specific and total IgE

OVA-specific IgE levels in serum were assayed by passive cutaneous anaphylaxis in Sprague-Dawley rats. Rats were anaesthetised by i.p. injection of 8 mg/kg xylazine and 40 mg/kg ketamine. Test mouse sera were serially diluted in twofold steps with PBS, and 50 µL of each dilution was injected intradermally into shaved dorsal skin of rats. After 72 h, the rats were challenged i.v. with 5 mg of OVA in 1 mL of PBS with 1% Evan's blue (Sigma). Half an hour later, the rats were sacrificed and the skin was examined for the appearance of blue spots. The passive cutaneous anaphylaxis titres were defined as the reciprocal of the highest dilution of serum giving a spot of ≥5 mm in diameter.

The concentration of serum IgE was determined by sandwich ELISA. Costar-plates were coated with capture antibody (1 µg/mL of mouse anti-IgE antibody; BD Biosciences Pharmingen). Biotinylated anti-mouse IgE (2 µg/mL; BD Biosciences Pharmingen) was used as the detection antibody. The limit of detection was 6 ng/mL.

Statistical analysis

Data are expressed as individual values and medians, and statistical analysis was conducted using non-parametric Mann–Whitney *U*-test; *p* values <0.05 were considered significant.

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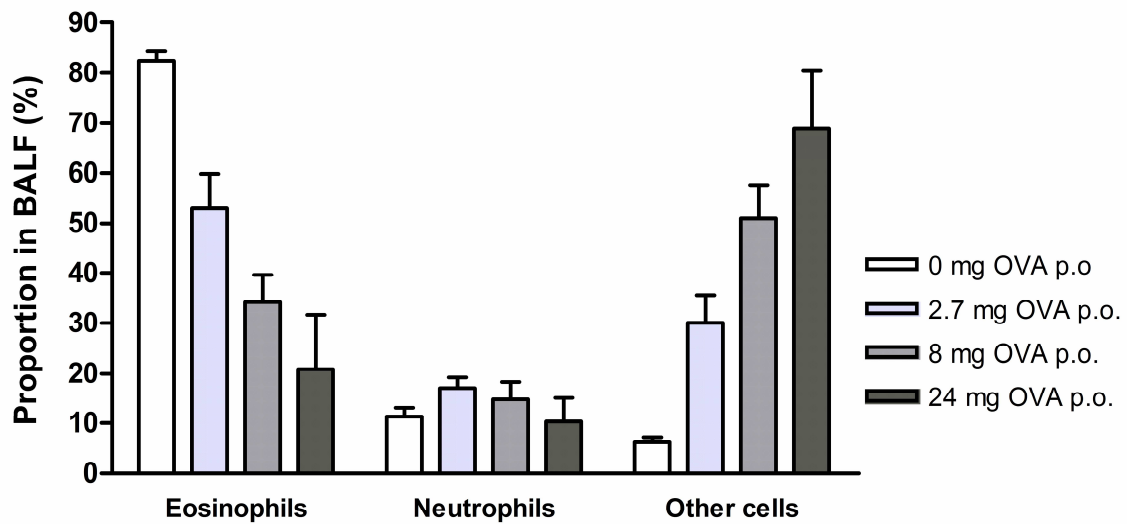
Abbreviations: p.o.: per oral(ly) · SEA: staphylococcal enterotoxin A

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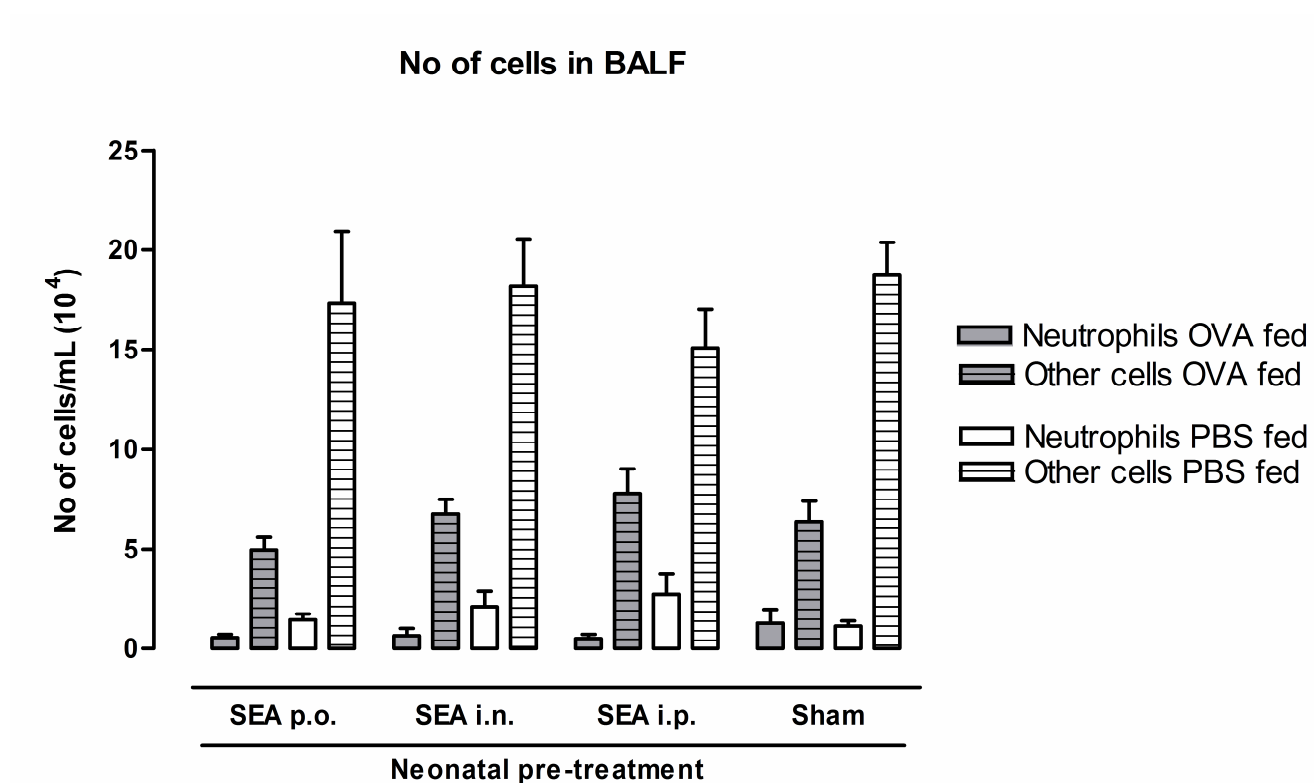
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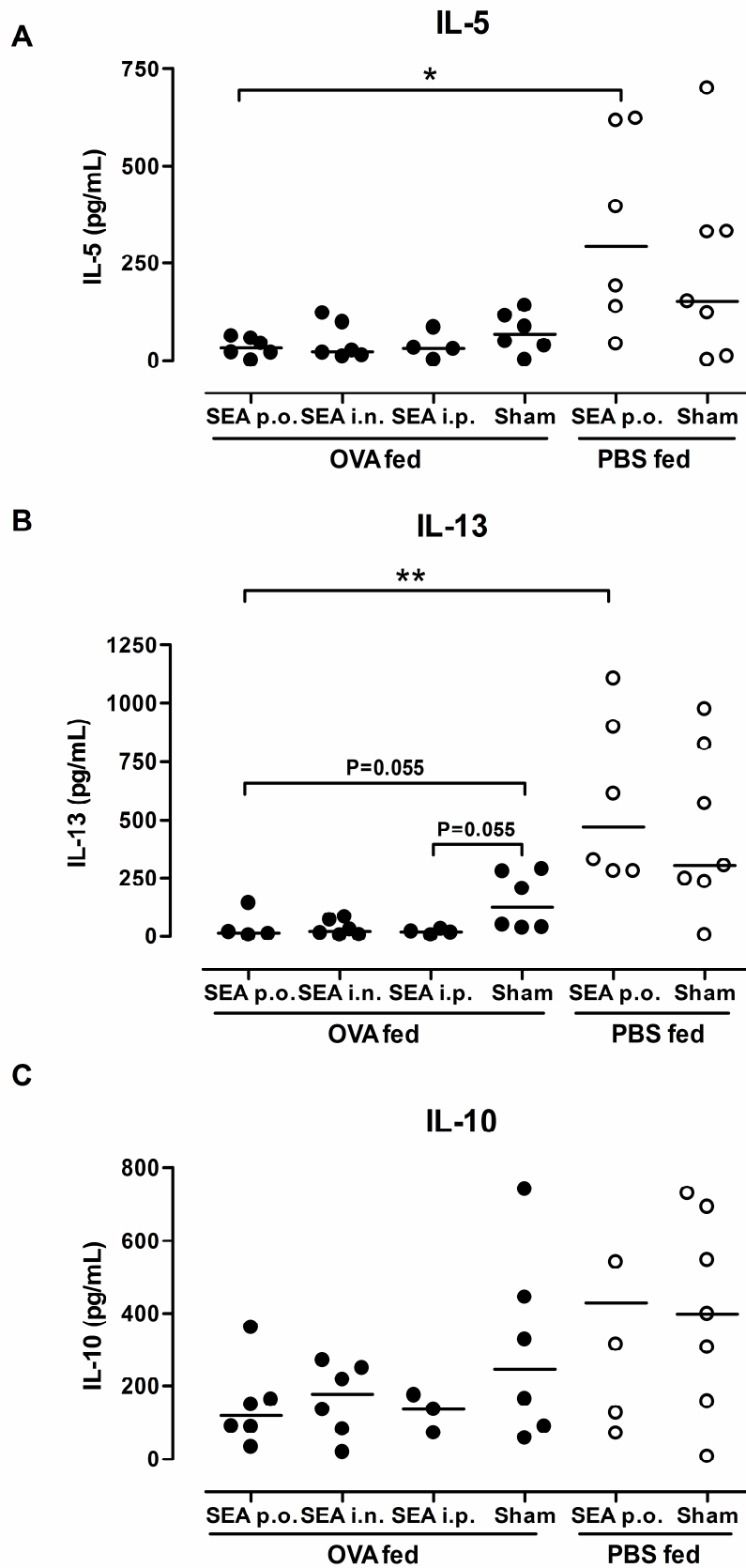
Supporting information S1



Supporting information S1. Optimizing the feeding dose of OVA in order to induce partial tolerance. Mice were fed different doses of OVA (or PBS as control) one week prior to sensitisation and challenge in the OVA-induced airway allergy model. This feeding reduced the proportion of eosinophils in the BALF to different degrees and a dose lowering the proportion of eosinophils to approximately 50% compared to PBS fed animals (5 mg) were chosen for further experiments.

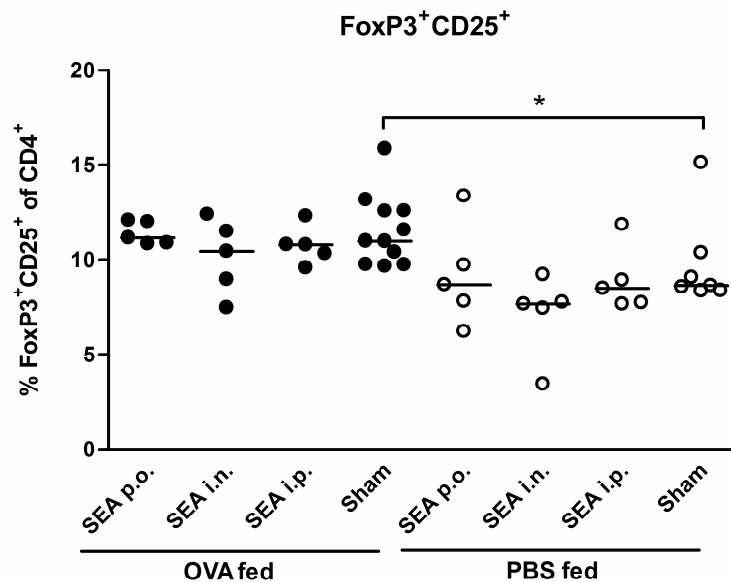


Supporting information S2. Mice were pre-treated with SEA p.o., i.n. or i.p., or given PBS p.o. (sham). At 5-6 weeks of age they were orally tolerised by OVA (or fed PBS) and thereafter sensitised and challenged in the airway allergy model. The number of neutrophils and other cells (macrophages, lymphocytes) in BALF was estimated by differential counts.



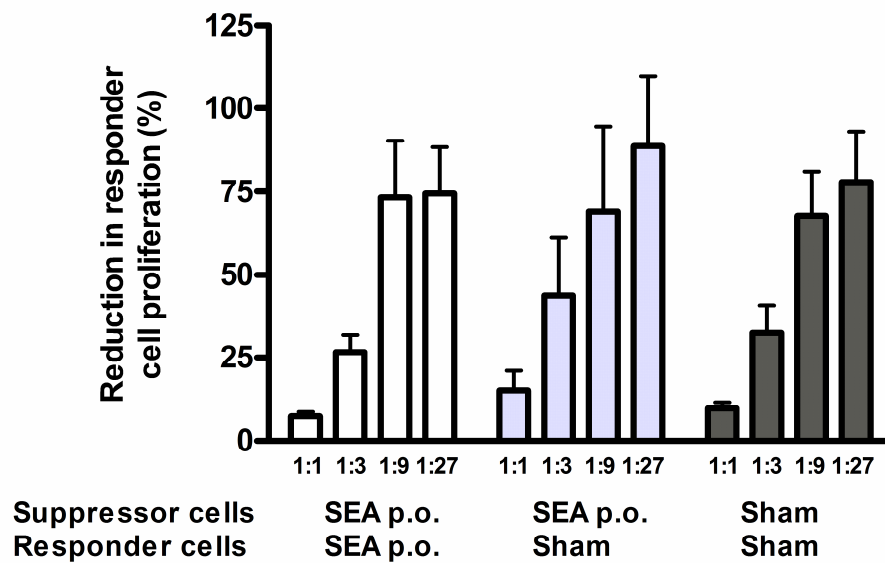
Supporting information S3. Levels of cytokines (IL-5, IL-13 and IL-10) in 48 h

supernatants of lung cells stimulated *in vitro* with OVA. The lungs was cut into small pieces, incubated with collagenase (10 mg/mL, type IV; Sigma) and deoxyribonuclease I from bovine pancreas (0.1 mg/mL; Sigma) for 20 min at 37°C, and gently mashed through a cell strainer (70µm). The cells were washed and cultured in round-bottom 96-well plates (0.5 x 10⁶/well) in Iscove's medium with 2mM L-glutamine (Sigma), 40µM mercaptoethanol (Sigma) and 50 µg/mL gentamicin (Sigma) and OVA (0.5 mg/mL). Supernatants were collected after 48 h of culture and assayed for interleukin (IL)-5, IL-10 and IL-13 by cytometric bead array (CBA flex sets; BD Biosciences Pharmingen) performed according to the manufacturer's instructions. The limits of detection were 4.8 pg/mL (IL-5), 16 pg/mL (IL-10) and 7.3 pg/mL (IL-13). Data are shown as individual values and median of 4-7 mice. Statistical analysis was performed with Mann-Whitney U test. Statistics from staphylococcal enterotoxin A (SEA) pre-treated OVA fed mice compared to SEA pre-treated PBS fed mice are visualized as * $p < 0.05$ and ** $p < 0.01$.



Supporting information S4. OVA feeding increase the proportion of FoxP3⁺CD25⁺ cells in lung draining LN after sensitisation and challenge in the OVA-induced airway allergy model. Twenty-four hours after the last OVA challenge, mediastinal LN were collected and single-cell suspensions were prepared. The proportion of FoxP3⁺CD25⁺ cells among CD4⁺ cells was examined by flow cytometric analysis. Statistical analysis was performed with Mann-Whitney U test. Statistics from sham treated OVA fed mice compared to sham treated PBS fed mice are shown as visualized as * $p < 0.05$.

Supporting information S5



Supporting information S5. Similar suppressive capacity of $CD4^+CD25^+$ cells in mesenteric LN from SEA pre-treated and sham treated mice at the time point for oral tolerization. For the *in vitro* suppression assay, mesenteric LN $CD4^+$ cells were fractionated into $CD25^+$ and $CD25^-$ subpopulations using magnetic beads (Miltenyi Biotec, Bergish Gladbach, Germany) according to the manufacturer's instructions and APCs were isolated with $CD11c^+$ beads (Miltenyi Biotec). $CD4^+CD25^+$ T cells from SEA pre-treated or sham treated mice were serially diluted in threefold steps and co-cultured together with a fix number of $CD4^+CD25^-$ responder T cells. The responder cells were either from the $CD4^+CD25^+$ T cell donor or from a pool of responder cells isolated from sham treated mice, giving different ratios of $CD4^+CD25^+ : CD4^+CD25^-$ (1:1, 1:3, 1:9 etc.). The cells were cultured for seven days together with $CD11c^+$ APCs and stimulated with 0.5 μ g Concavalin A (ConA, Sigma). Proliferation was assessed by [^{3} H]thymidine (Amersham Biosciences) uptake during the last 16 h of culture.