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Food allergic infants have impaired regulatory T cell responses following *in vivo* allergen exposure

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ABSTRACT

Background: Regulatory T cells (Tregs) are critical for development of oral tolerance, and studies suggest that dysfunction of Tregs may lead to food allergy. However to date no study has investigated Treg responses following *in vivo* exposure to peanut or egg allergens in humans.

Objectives: To examine changes in peripheral blood CD4⁺CD25⁺FoxP3⁺ Treg populations (total, activated, and naive) in food allergic, food-sensitised but tolerant, and healthy (non-sensitised non-allergic) patients over time following *in vivo* allergen exposure.

Methods: A subset of infants from the HealthNuts study with egg or peanut allergy (n=37), egg or peanut sensitisation (n=35), or who were non-sensitised non-allergic (n=15) were studied. All subjects underwent oral food challenge (OFC) to egg or peanut. PBMC were obtained within 1 hour of OFC (*in vivo* allergen exposure); and Treg populations enumerated *ex vivo* on day 0, and after 2 and 6 days rest *in vitro*.

Results: Non-allergic infants showed stable total Treg frequencies over time; food-sensitised infants had a transient fall in Treg percentage with recovery to baseline by day 6 (6.87% day 0, 5.27% day 2, 6.5% day 6); and food allergic infants showed persistent reduction in Treg (6.85% day 0, 5.4% day 2, 6.2% day 6) following *in vivo* allergen exposure. Furthermore, food allergic infants had a significantly lower ratio of activated Treg:activated T-cells

(10.5±0.77) at day 0 compared to food-sensitised (14.6±1.24) and non-allergic subjects (16.2±1.23).

Conclusion: Our data suggest that the state of allergen sensitisation is associated with depletion of Treg following allergen exposure. Impaired capacity to regenerate the Treg pool following allergen exposure may be an important factor that determines clinical allergy versus sensitisation without allergic reaction.

Key words: Food allergy, peanut allergy, egg allergy, regulatory T cells, Treg, FoxP3, HealthNuts, oral food challenge

Abbreviations

OFC: Oral food challenge

PBMC: Peripheral blood mononuclear cell

Treg: Regulatory T cell

aTreg: Activated regulatory T cell

SPT: Skin prick test

INTRODUCTION

Both regulatory T cell (Treg) deficiency and dysfunction have been found to be associated with development of allergic diseases¹⁻⁴. However, only a limited number of studies have assessed the role of Tregs in IgE-mediated food allergy. The acquisition of tolerance to food allergens, whereby the immune system is rendered hyporesponsive to antigen after oral feeding, has been extensively characterised in animal⁵⁻⁷ models but is poorly understood in humans. It has been postulated that tolerance to food antigens and suppression of the

antigen-specific allergic response arises from Tregs induced in the periphery (iTregs)⁸, and a diminished capacity to produce these cells could result in the development of food allergy.

Upon activation, naïve Treg (CD4⁺CD45RA⁺Foxp3⁺) with less suppressive function can be induced to differentiate into highly suppressive activated Treg (CD4⁺CD45RA⁻Foxp3⁺)⁹⁻¹¹, though activated Tregs are short lived and become anergic after activation *in vitro*^{9, 12-14}.

Only one study (none investigating food allergy) has assessed the kinetics of Treg activity following *in vitro* activation, confirming the short-term susceptibility of CD4⁺CD25⁺ suppressive T-cells to apoptosis after 40 hours in culture following *in vitro* antigen stimulation¹⁵. However, the kinetics of the various Foxp3⁺ sub-populations (total Tregs, activated Tregs, naïve Tregs, and activated T-cells) are yet to be explored in food allergy. To date no study has assessed the effect of acute food allergen exposure on these Treg sub-populations (naïve and activated), and it is likely that food allergic infants have a reduced frequency of activated Tregs, which contributes to the breakdown of oral tolerance.

We postulated that infants who are allergic will have a reduced capacity to generate or maintain activated Treg sub-populations following exposure to food allergen as compared to those who are sensitised while healthy (non-sensitised non-allergic) infants will have a robust induction of activated Treg populations following allergen exposure. In this study, we aimed to characterise differences in Treg sub-populations in food allergic infants compared to both food-sensitised tolerant, and non-sensitised non-allergic infants. We also explored the effects of the Treg response following allergen challenge in these infant groups.

METHODS

Subjects

A total subset of 87 subjects were selected from the HealthNuts cohort for this study¹⁶. The selection criteria was that they were allergic or sensitised tolerant to only a single food: peanut or egg. Median age was 12 and 14 months at the time of SPT and OFC respectively [ranges 11-15 and 11.5-20 months]). OFC was performed according to the protocols published in the HealthNuts study¹⁶ in all subjects, representing *in vivo* allergen exposure. Combined food allergics infants (FA) consisted of peanut allergic (PA, n=18), and egg allergic (EA, n=19) with a positive SPT ≥ 2 mm to peanut or egg respectively and an unequivocal allergic reaction during OFC. Subjects selected for this study were challenged to either egg or peanut at the Royal Children's Hospital, Melbourne. Subjects received increasing doses of the suspected food allergen at 15 minute intervals until they received the equivalent of 2 teaspoons of peanut butter or 60g of egg. The food challenge was stopped if an objective allergic reaction was observed that fulfilled the predetermined stopping criteria from the study protocol i.e. at least one of the following: three or more concurrent non-contact hives (urticarial lesion) lasting for more than 5 min, perioral or periorbital angioedema, vomiting (excluding immediate post-ingestion gag/vomits), or evidence of anaphylaxis as defined by the Australian Society of Clinical Allergy and Immunology (evidence of circulatory or respiratory compromise) within 2 h of the last dose within the food challenge¹⁷. Combined food-sensitised tolerant infants (FST) consisted of peanut (peanut sensitised tolerant (PST) n=18), and (egg sensitised tolerant (EST) n=17) with a positive SPT ≥ 2 mm only to either peanut or egg respectively and a negative OFC. Healthy control infants were non-sensitised, and non-allergic (NA, n=15) with a negative SPT (wheal 0 mm) to peanut, egg, sesame and cow's milk together with a negative peanut or egg challenge outcome.

Preparation of blood samples and cell culture

Up to 7ml of blood was collected into a sodium heparin tube (Sarstedt) 1-2 hours after the final dose of the OFC. Peripheral blood mononuclear cells (PBMC) were separated by density gradient centrifugation within 2 hours of collection and cryopreserved at $10\text{--}20 \times 10^6/\text{ml}$ (mean $\pm$ SEM: $17.2\pm 0.56 \times 10^6$) in RPMI with 15% DMSO in FCS for batched analysis. The median PBMC viability after subsequent thawing determined by trypan blue exclusion was 93% (range 90-97%).

For Treg enumeration, PBMCs were divided into three aliquots – one aliquot of 2×10^6 for immediate flow cytometry staining (day 0), a second aliquot of 6×10^6 was rested in culture for 2 days, and a third aliquot of 5×10^6 was rested in culture for 6 days. Cultured PBMCs were incubated at 37°C at a concentration of 1×10^6 in 1ml of AIM-V serum free medium (Invitrogen) supplemented with 2-mercaptoethanol 4×10^{-5} M (rested PBMC) for 2 or 6 days.

Flow cytometry of regulatory T-cells

Freshly thawed PBMC, or cultured PBMC harvested at 2 or 6 days, were analysed by flow cytometry. The Treg population was characterised by $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+$. Naive Tregs were characterised by $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+\text{CD45RA}^+$ and activated Tregs by $\text{CD4}^+\text{CD25}^+\text{Foxp3}^+\text{CD45RA}^-$. In further analyses, CD27 was used to distinguish Foxp3^+ activated Tregs (CD27^+) from Foxp3^+ activated T-cells (CD27^-) (detailed staining methodology and gating strategy located in online repository).

Statistics

All statistical analyses were performed using STATA software (release 12.0; StataCorp, College Station, Tex). The data is presented as the mean $\pm$ SEM. Comparisons between groups were analysed by Mann–Whitney *U*-test corrected for the number of pairwise

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comparisons between groups by Bonferroni correction. Significance threshold was set to 5%, and after correction, results with a $p < 0.008$ was considered statistically significant. We used 1-way ANOVA to test for differences in the ratio of aTreg to activated T-cells at the 3 time points within each clinical phenotype. Data was plotted using Graphpad Prism 5.03 software.

Ethics

Ethics approval was obtained for the HealthNuts study from the Victorian State Government Office for Children (reference no. CDF/07/492), the Victorian State Government Department of Human Services (reference no. 10/07), and the Royal Children's Hospital Human Research Ethics Committee (reference no. 27047).

RESULTS

Demographics of study cohort

A total of 87 samples from HealthNuts patients were analysed. Our non-sensitised infants were more likely to have a family history of any allergy compared to both egg and peanut sensitised tolerant and allergic infants (Table 1), but at the time of the oral food challenge, they were not sensitised to any of the foods tested. We also observed that more egg and peanut allergic infants had current eczema than both the sensitised tolerant and non-sensitised infants.

Table 1: Demographics of study cohort

Allergy Sensitization	Peanut Allergics (n=18)	Peanut Tolerant (n=18)	Egg Allergics (n=19)	Egg Tolerant (n=17)	Non Sensitised Non Allergic (n=15)
Sex (% of males)	72%	47%	50%	44%	60%
Current Eczema (%)	50%	21%	60%	22%	27%
Family History of Any Allergy (asthma, eczema, hay fever etc) (%)	61%	79%	65%	67%	93%
Family History of Food Allergy (%)	61%	79%	60%	67%	87%

Changes in frequency of the total Foxp3⁺Treg population over 6 days

The study was designed to reveal possible differences in the Treg response to allergen after in vivo exposure in FA, FST and NA infants. We observed that FST infants had a significant reduction in the frequency of Tregs at day 2 compared to day 0 (5.27% vs 6.87%; p=0.003), with subsequent increase back to baseline at day 6 (6.5% day 6 vs 5.27% day 2; p=0.008) following allergen exposure. Although, FA infants had a similar reduction in Treg frequency at day 2 compared to day 0 (5.41% vs 6.85%; p=0.002), there was a more modest recovery with persistently reduced Treg at day 6 (6.28% day 6 vs 5.41% day 2; NS) (Figure 1a). The frequency of Tregs in healthy controls did not change over time from day 0 to day 6.

Comparing the frequencies of total Treg between FA, FST and healthy controls at each time point, there were no differences in the Treg frequencies between the three groups at day 0 or day 6 (total n=87) (Table 2). At 2 days, the FST infants had a lower percentage of Tregs compared to healthy control infants (p<0.05). Although not statistically significant, FA infants showed a modest reduction in the percentage of Tregs on day 2 compared to healthy control infants.

Changes in the frequency of the naïve and activated Foxp3⁺Treg populations over 6 days

We found that in FST infants, the proportion of activated Tregs amongst total Tregs was increased at day 2 compared to day 0 (48.92% vs 60.83% respectively; $p<0.01$) and then returned to baseline on day 6 (day 6 49.66% vs day 0 48.92%) (Figure 1b). In contrast, the naïve Treg population in the FST group was reduced at day 2 compared with day 0 (34.44% vs 43.45% respectively, $p<0.01$), recovering back to baseline day 0 levels on day 6 (46.18%) (Figure 1c).

In comparison, the FA group did not show any changes in the activated Treg proportions across the 6 days (day 0 51.53% vs day 2 49.66 vs day 6 47.41%; NS) (Figure 1b).

Similarly, there were no changes in the levels of naïve Tregs (day 0 40.59% vs day 2 46.18% vs day 6 45.98%, NS) (Figure 1c).

There were no significant changes in the frequency of activated Tregs or naïve Tregs in our healthy controls from day 0 to day 6, as was observed for total Treg frequencies in this group (Figure 1b-c). There were no significant changes in the frequency of activated Tregs or naïve Tregs in our healthy controls from day 0 to day 6, as was observed for total Treg frequencies in this group (Figure 1b-c). Comparison of activated and naïve Treg populations between healthy controls, FST, and FA infants showed significant differences in activated and naïve Treg between FST on day 2 compared to FA and NA (Table 2).

Ratio of activated Foxp3⁺Treg to activated T-cell populations (aTreg:activated T-cells)

The ratio of activated Tregs to activated T-cells has been suggested to correlate inversely with airway allergic disease; healthy individual have a higher ratio of Treg:activated T cells¹⁸. We therefore examined the ratio of activated Treg:activated T cells over time to determine if

there were differences in FA, FST and NA infants following allergen exposure. We observed that the ratio of aTreg:activated T-cells was significantly lower in FA infants (10.5 ± 0.77) compared to both FST (14.6 ± 1.24 ; $p < 0.01$) and NA infants (16.2 ± 1.23 ; $p < 0.01$) at baseline on day 0. This reduced ratio in FA infants compared with control infants persisted (although not statistically significant) at day 2 (18.9 ± 2.7 vs 29.2 ± 6.3) and day 6 (22.5 ± 2.8 vs 35.8 ± 11.3) (Figure 2). Interestingly, this reflected a progressive increase in the aTreg:activated T-cell ratio in healthy controls over time ($p < 0.008$), with little change in the ratio for FA infants ($p > 0.05$). Although FST infants had a similar ratio of aTreg:activated T-cells compared with controls infants at day 0 (14.6 ± 1.24 vs 16.2 ± 1.23) and day 2 (30.4 ± 4.8 vs 29.2 ± 6.3), the ratio dropped at day 6 to a level similar to that in FA infants (FST 21.7 ± 2.5 vs FA 22.5 ± 2.8 vs NA 35.8 ± 11.3) (Figure 2).

Frequency of the Foxp3⁺Treg populations in egg and peanut sensitised or allergic infants

We have previously identified that immune markers may be differentially expressed in infants with egg as compared to peanut allergy. We therefore investigated if the differences in Treg frequencies described above were present to a similar extent in infants with egg allergy/sensitisation as compared to infants with peanut allergy/sensitisation. We observed that the frequencies of total Foxp3⁺ Tregs in both PA and PST groups were reduced at day 2 (5.1% and 5.1%, respectively) as compared to day 0 (6.7% and 6.8%, respectively) (Figure 3a). Thereafter, the frequency of Tregs at day 6 returned towards day 0 levels for the PST group (6.2% vs 6.7%, respectively), while the frequency of Treg remained reduced at day 6 compared to day 0 (5.58% vs 6.84%; $p < 0.05$) in the PA group (Figure 3a). In contrast, both the EA and the EST infants demonstrated transient reductions in Treg percentage at day 2 (5.46% and 5.68%, respectively) compared with day 0 (7.04% and 6.63%), with recovery of Treg percentages by day 6 (6.82% EST, 7.02% EA; Figure 3b). Similar to the patterns

observed in the combined groups, the frequency of activated Treg decreased on day 2 whilst naïve Treg increased on day 2 from PA and PST infants (Table 3a) compared to day 0. These observations were not seen in the EA and EST groups (Table 3b).

The ratio of Foxp3⁺ activated Treg to activated T-cell population (aTreg:activated T-cells) in peanut and egg sensitised or allergic infants

Assessment of the ratio of aTregs:activated T-cells in infants sensitised to peanut or egg revealed similar patterns as for our combined food sensitised or allergic cohort, with a significantly lower ratio in PA (9.4; Figure 4a) and EA (11.57; Figure 4b) compared to NA (16.2; $p<0.001$ and $p<0.05$ respectively) at day 0. Furthermore, the ratio of aTregs:activated-T-cells in PA infants was significantly lower compared to PST infants at day 0 (9.4 vs 15.3; $p<0.01$). However, the ratio of aTregs:activated T-cell ratio in EA infants was not significantly lower than the ratio in EST infants, primarily due to a more modest reduction in the ratio amongst EA infants.

Examination of the changes in the ratio of aTreg:activated T-cells over time within each of the PST/EST or PA/EA and NA infants revealed similar findings to the changes observed for the combined FST or FA and NA infants (described above), and no differences between egg as compared to peanut groups. As described earlier, NA infants demonstrated a significant progressive increase in the ratio of aTreg:activated T-cells over the 6 days following in vivo allergen exposure ($p<0.008$). In contrast, there was no significant upward shift in the aTreg:activated T-cell ratio over time in PA or EA infants ($p>0.05$). The aTreg:activated T-cell ratio in both PST and EST infants appeared to rise in parallel with that of control infants from day 0 to day 2, then fall towards that of the allergic infants by day 6.

DISCUSSION

This is the first study to describe time dependent changes in Treg populations in carefully defined clinical food allergy phenotypes and healthy controls following *in vivo* exposure to allergen (in oral food challenge). Although we saw similar frequencies of circulating total CD4⁺CD25⁺Foxp3⁺ Tregs in FA, FST, and NA infants (at day 0), each of the clinical phenotype groups demonstrated changes in the frequency of Treg populations over time after allergen exposure. These observations suggest that food allergy may be associated with an altered Treg response to allergen that may be the result of an impaired ability to regenerate the Treg population following allergen exposure with less pronounced defects in food sensitised infants^{19, 20}.

Major strengths of this study are that longitudinal analyses of Treg populations were performed following a physiological *in vivo* exposure to food allergen, and secondly that studies were conducted in clearly defined populations of NA, FST and FA infants, as determined by both a SPT and OFC. Additionally, our study explored potential differences in subjects who were single sensitised to peanut or egg, revealing notable differences. True population negative controls were also included for comparison. There are a number of limitations that need to be considered. While the results of this study provide us with some insight into what may be occurring *in vivo*, our *in vitro* assay approach is not able to account for other factors which may be involved in the maintenance of the Treg pool *in vivo*, and this is a limitation of the current study. The results observed in this study are only an indication of what may be occurring in the complex *in vivo* environment where other unknown factors may affect the kinetics of the inflammatory response. It has previously been shown that expression of the Foxp3 gene is not limited to Treg cells, and is also transiently upregulated in activated effector T-cells²¹. Unlike the suppressive Treg cells, the unstable Foxp3 expression in non-Treg activated T-cells occurs as a result of incomplete demethylation of the Foxp3 gene¹⁰ that

is required for its suppressive function²¹. To address this, we sought to distinguish Treg cells from activated T-cells that also express Foxp3 by examining co-expression of CD27. While studies investigating the novel CD27 marker are rare, two studies have shown that CD27⁺ Foxp3⁺ Tregs are highly suppressive whereas CD27⁻ Foxp3⁺ T-cells are likely to represent IL-17 producing activated T-cells^{22, 23}. Consistent with the importance of Tregs in the generation of food tolerance, Shreffler *et al* demonstrated that CD25⁺CD27⁺Foxp3⁺ Tregs isolated from cow's milk sensitised children were capable of suppressing the proliferation of allergen specific effector T-cells¹⁹. Our present findings support the suggestion that food allergy coincides with a reduced frequency of CD25⁺CD27⁺Foxp3⁺ aTregs. Moreover, our findings suggest that a less prominent deficiency of Treg may also contribute to the clinical phenotype of food sensitisation without associated clinical allergy.

In contrast to our and others' findings^{19, 20, 24}, Karlsson *et al* conducted the only previous study to report a reduced Treg frequency in cow's milk allergic children compared to those who tolerated milk²⁵. Our study found no difference in Treg frequencies between the clinical phenotypes of allergy, sensitisation and healthy controls at day 0, which is similar to the study by Shreffler *et al*, who reported no difference in the frequencies of circulating Tregs between children with cow's milk allergic and non-allergic children¹⁹; this suggests that it may be difficult to identify abnormalities of Treg in food allergic subjects by measuring the frequency of circulating Tregs in the resting state. However, by enumerating Tregs after *in vivo* allergen exposure, we revealed significantly decreased frequencies of Tregs at 2 days in both FA and FST infants compared to NA infants, with recovery by day 6 in FST but not FA infants. Initially believed to be a state of anergy, it has since been shown that CD25⁺Foxp3⁺ Tregs are highly susceptible to apoptosis following activation to a suppressive state^{9, 28}. The reduction in total Tregs at day 2 in sensitised tolerant and allergic infants likely reflects activation of Treg following *in vivo* exposure to allergen in those who are sensitised, with

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this activation then rendering Treg susceptible to apoptosis 40 hours after allergen stimulation¹⁵. This is then followed by recovery of Treg numbers in sensitised tolerant infants which may contribute to suppression of IgE mediated inflammatory responses to allergen in this group. Conversely, the impaired ability to regenerate a sufficient number of allergen-specific activated Tregs by day 6 in allergic infants may contribute to clinical manifestation of allergic symptoms. The concept of Treg cell instability has been demonstrated in other disease models such as autoimmune encephalomyelitis²⁹, and is postulated to contribute to disease development³⁰. This is the first time data has been presented on the instability of Treg in food allergy.

Studies have shown that after *in vitro* stimulation, naive Tregs become highly resistant to apoptosis and are capable of differentiating into activated, suppressive Foxp3⁺ Tregs⁹. We observed a depletion of naive Treg in FST infants at day 2, which could be consistent with differentiation into aTreg. In contrast, there were no changes in the overall naive Treg population in FA infants throughout the 6 days; we postulate that a reduced ability for naive Treg to differentiate to aTreg after allergen stimulation may be one reason that allergic subjects are unable to suppress the allergic response. The precise mechanisms responsible for the Treg differences we have shown require further study.

Assessing the balance of Treg to activated T-cells may provide a more sensitive measure of reduced Treg expression than enumeration of circulating Treg populations. We found a reduced aTreg:activated T-cell ratio in the food allergic infants compared to both healthy controls and food sensitised infants, consistent with the findings by Akdis *et al.* in aeroallergen allergic subjects¹⁸. The reduced ratio of aTreg:activated T-cells could be a key factor in determining the development of allergy. Furthermore, healthy subjects showed a clear and robust increase in this ratio following allergen exposure which fits with the paradigm that the predominant response in healthy subjects is a Treg response, compared to a

transient increase in ratio for sensitised infants, and an attenuated and delayed Treg response in food allergic infants.

Similar to our previous investigations revealing different immunological profiles between egg and peanut sensitisation³¹, we found differences in Treg profiles between peanut and egg allergic / sensitised infants. Treg defects appeared to be more prominent for peanut compared to egg groups, which may contribute to the different natural history of disease^{32, 33}. A more severe defect of Treg may contribute to the persistence of peanut allergy; whereas a less severe defect may be associated with greater likelihood of resolution of egg allergy through maturation and recovery of Treg responses during childhood. Our findings also suggest that while the clinical phenotype of food allergy is similar for all types of foods¹⁷, the underlying mechanisms behind egg and peanut allergy may be different.

Taken together, we have found that infants with food allergy have a prolonged reduction in the frequency of total Foxp3⁺ Treg following *in vivo* allergen challenge, compared to sensitised tolerant infants who had a transient reduction in Treg, and healthy infants who had a stable frequency of Treg. Healthy infants also displayed a rapid and robust increase in the aTreg:activated T-cell ratio following allergen exposure; whereas, there was only a transient increase in the aTreg:activated T-cell ratio in food sensitised infants, and an attenuated as well as delayed increase in this ratio in food allergic infants. Our data suggest that the state of allergen sensitisation is associated with depletion of Treg following allergen exposure, and the impaired capacity to regenerate the Treg pool following allergen exposure may be an important factor that determines clinical allergy versus a sensitised tolerant state.

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Figure Legends

Figure 1a-c: The frequency of a) total CD4⁺CD25⁺Foxp3⁺Treg; b) CD4⁺CD25⁺Foxp3⁺CD45RA⁻ Activated Treg; and c) CD4⁺CD25⁺Foxp3⁺CD45RA⁺ Naive Treg frequencies in the three clinical phenotype groups following *in vivo* allergen exposure. Data expressed as the mean $\pm$ SEM % of total Tregs (calculated as % of total CD4⁺ cells) cultured *in vitro* following *in vivo* peanut or egg exposure (oral food challenge) respectively in controls (n=15), food sensitised tolerant (n=35), and food allergic infants (n=37). Comparisons of the changes in Treg frequency over time were performed using the Mann-Whitney test. * p-value <0.05, ** p-value<0.01

Figure 2: Comparison of the ratio of CD27⁺ activated Treg: CD27⁻ activated T cells between the clinical phenotype groups at different times following *in vivo* allergen exposure. Data expressed as the mean $\pm$ SEM % of total Tregs cultured (*in vitro*) following (*in vivo*) peanut or egg exposure (oral food challenge) respectively over the course of 6 days in controls (n=15), food sensitised (n=35), and food allergic infants (n=37). Comparisons between groups were performed using the Mann-Whitney test. * = p-value <0.05, ** = p-value<0.01, *** = p-value<0.001

Figure 3a-b: The changes in total CD4⁺CD25⁺Foxp3⁺Treg population in a) peanut sensitised or allergic and healthy control infants and b) egg sensitised or allergic and healthy control infants. Data expressed as the mean $\pm$ SEM % of total Tregs (calculated as % of total CD4⁺ cells) cultured (*in vitro*) following (*in vivo*) peanut or egg exposure (oral food challenge) respectively from non allergic (n=15), peanut or egg sensitised tolerant (n=18 or n=17), and peanut or egg allergic infants (n=18 or n=19). Comparisons of changes in Treg frequency over time were performed using the Mann-Whitney test. * = p-value <0.05, ** = p-value<0.01

Figure 4a-b: Comparison of the ratio of CD27⁺ activated Treg/CD27⁻ activated T cells in a) peanut sensitised, peanut allergic and non-sensitised non-allergic infants; and b) egg sensitised, egg allergic and non-sensitised non-allergic infants. Data expressed as the mean $\pm$ SEM % of total Tregs cultured (*in vitro*) following (*in vivo*) peanut or egg exposure (oral food challenge) respectively over the course of 6 days in non allergic (n=15), peanut/egg sensitised (n=18/17), and peanut/egg

allergic infants (n=18/19). Comparisons between groups were performed using the Mann-Whitney test. * = p-value <0.05, ** = p-value<0.01, *** = p-value<0.001

Table 2. Comparison of Treg populations between groups at day 0, 2 and 6

Frequency of Tregs	Time point (day)	Controls (n=15)	Sensitised Tolerant (n=35)	Food Allergic (n=37)	Controls vs FST (p-value)	Controls vs FA (p-value)	FST vs FA (p-value)
Total Treg [#] (mean±SEM)	0	7.55 ± 0.58	6.87 ± 0.25	6.85 ± 0.29	0.29	0.29	0.96
	2	6.41 ± 0.45	5.28 ± 0.28	5.41 ± 0.26	0.04*	0.06	0.74
	6	6.93 ± 0.55	6.52 ± 0.29	6.28 ± 0.39	0.51	0.34	0.63
Activated Treg [^] (mean±SEM)	0	55.27 ± 2.26	53.01 ± 1.74	51.53 ± 1.35	0.43	0.17	0.51
	2	48.92 ± 3.81	60.83 ± 2.57	49.66 ± 3.00	0.01*	0.88	0.007**
	6	45.38 ± 5.39	46.43 ± 1.74	47.41 ± 2.01	0.86	0.73	0.72
Naive Treg [^] (mean±SEM)	0	39.04 ± 1.77	39.69 ± 1.68	40.59 ± 1.36	0.79	0.49	0.68
	2	43.45 ± 4.37	34.44 ± 3.04	46.18 ± 2.97	0.10	0.61	0.008**
	6	48.79 ± 3.96	46.38 ± 1.98	45.98 ± 2.37	0.59	0.55	0.90

[#] Total Tregs presented as percentage of total CD4⁺ cells.

[^] Activated and Naïve Treg subsets presented as percentage of Total Tregs

Table 3a. Comparison of Treg populations between peanut sensitised groups at day 0, 2 and 6.

Frequency of Tregs	Time point (day)	Controls (n=15)	Peanut Sensitised Tolerant (n=18)	Peanut Allergic (n=18)	Controls vs PST (p-value)	Controls vs PA (p-value)	PST vs PA (p-value)
Total Treg [#] (mean±SEM)	0	7.55 ± 0.58	6.70 ± 0.30	6.9 ± 0.43	0.20	0.34	0.77
	2	6.41 ± 0.45	5.11 ± 0.33	5.15 ± 0.34	0.03*	0.04*	0.94
	6	6.93 ± 0.55	6.21 ± 0.41	5.59 ± 0.32	0.30	0.052	0.25
Activated Treg [^] (mean±SEM)	0	55.27 ± 2.23	56.82 ± 2.48	50.45 ± 1.99	0.65	0.12	0.06
	2	48.92 ± 3.81	62.45 ± 3.90	49.05 ± 5.00	0.02*	0.98	0.05*
	6	45.38 ± 5.38	43.32 ± 1.65	46.56 ± 2.77	0.72	0.85	0.32
Naïve Treg [^] (mean±SEM)	0	39.04 ± 2.32	36.47 ± 2.32	42.68 ± 1.92	0.39	0.17	0.05*
	2	43.45 ± 4.37	30.84 ± 4.48	46.86 ± 4.92	0.06	0.61	0.03*
	6	48.79 ± 3.96	50.16 ± 2.45	47.63 ± 3.08	0.77	0.82	0.52

Total Tregs presented as percentage of total CD4⁺ cells.

[^] Activated and Naïve Treg subsets presented as percentage of Total Tregs

Table 3b. Comparison of Treg populations between egg sensitised groups at day 0, 2 and 6

Frequency Tregs	Time point (day)	Controls (n=15)	Egg Sensitised Tolerant (n=17)	Egg Allergic (n=19)	Controls vs EST (p-value)	Controls vs EA (p-value)	EST vs EA (p-value)
Total Treg [#] (mean±SEM)	0	7.55 ± 0.58	7.05 ± 0.41	6.85 ± 0.39	0.49	0.33	0.73
	2	6.41 ± 0.45	5.47 ± 0.48	5.69 ± 0.39	0.17	0.24	0.72
	6	6.93 ± 0.55	6.82 ± 0.41	7.02 ± 0.68	0.87	0.93	0.8
Activated Treg [^] (mean±SEM)	0	55.27 ± 2.26	48.98 ± 2.08	52.56 ± 1.84	0.05	0.36	0.21
	2	48.92 ± 3.81	59.05 ± 3.39	50.36 ± 3.13	0.06	0.77	0.07
	6	45.38 ± 5.38	49.55 ± 2.92	48.32 ± 3.24	0.50	0.64	0.78
Naïve Treg [^] (mean±SEM)	0	39.04 ± 1.78	43.09 ± 0.28	38.61 ± 1.86	0.16	0.87	0.13
	2	43.45 ± 4.37	38.37 ± 3.94	45.34 ± 3.35	0.40	0.72	0.19
	6	48.79 ± 3.96	42.60 ± 2.73	44.23 ± 3.68	0.21	0.41	0.73

Total Tregs presented as percentage of total CD4⁺ cells.

[^] Activated and Naïve Treg subsets presented as percentage of Total Tregs

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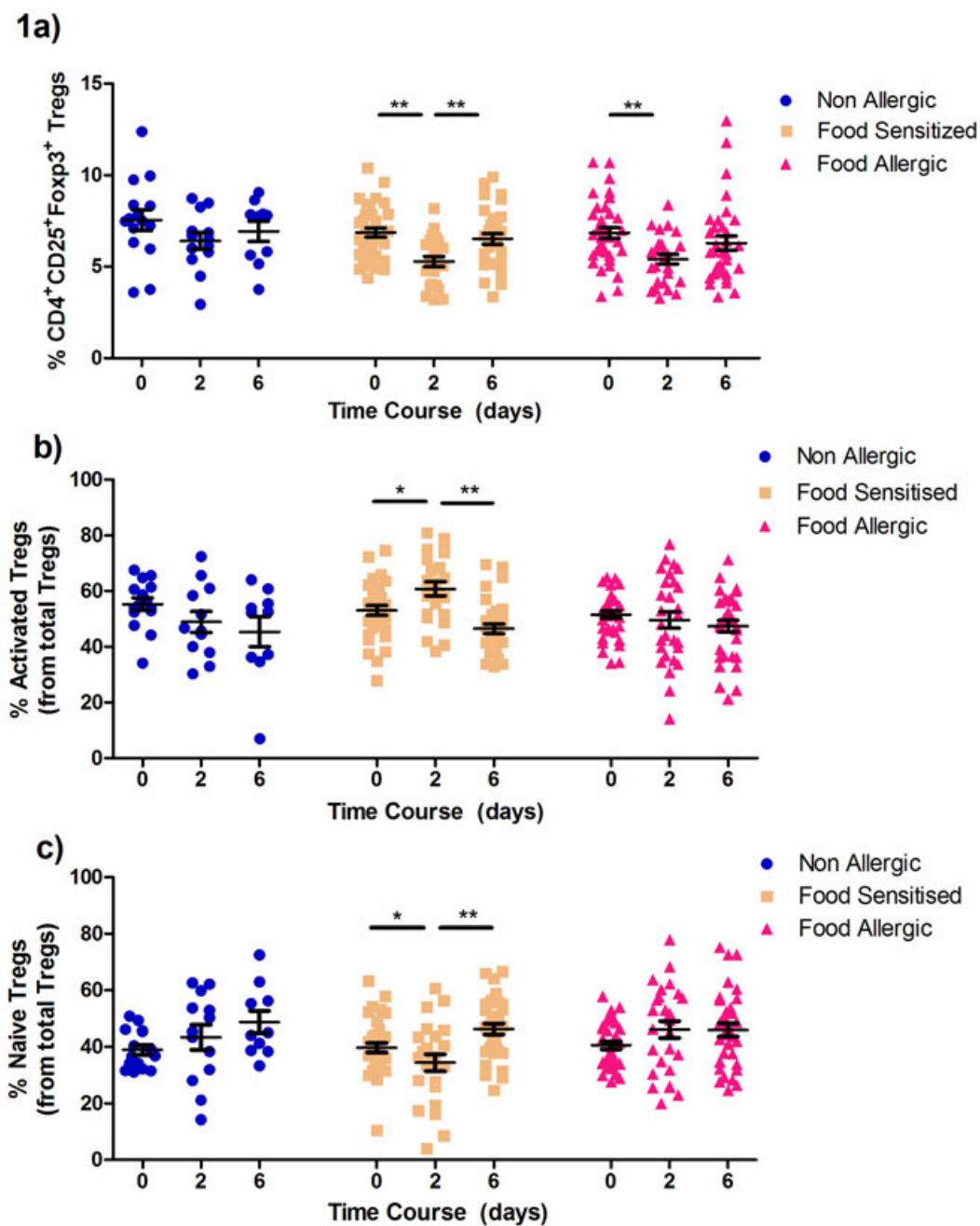
S1. Flow cytometric staining of Tregs

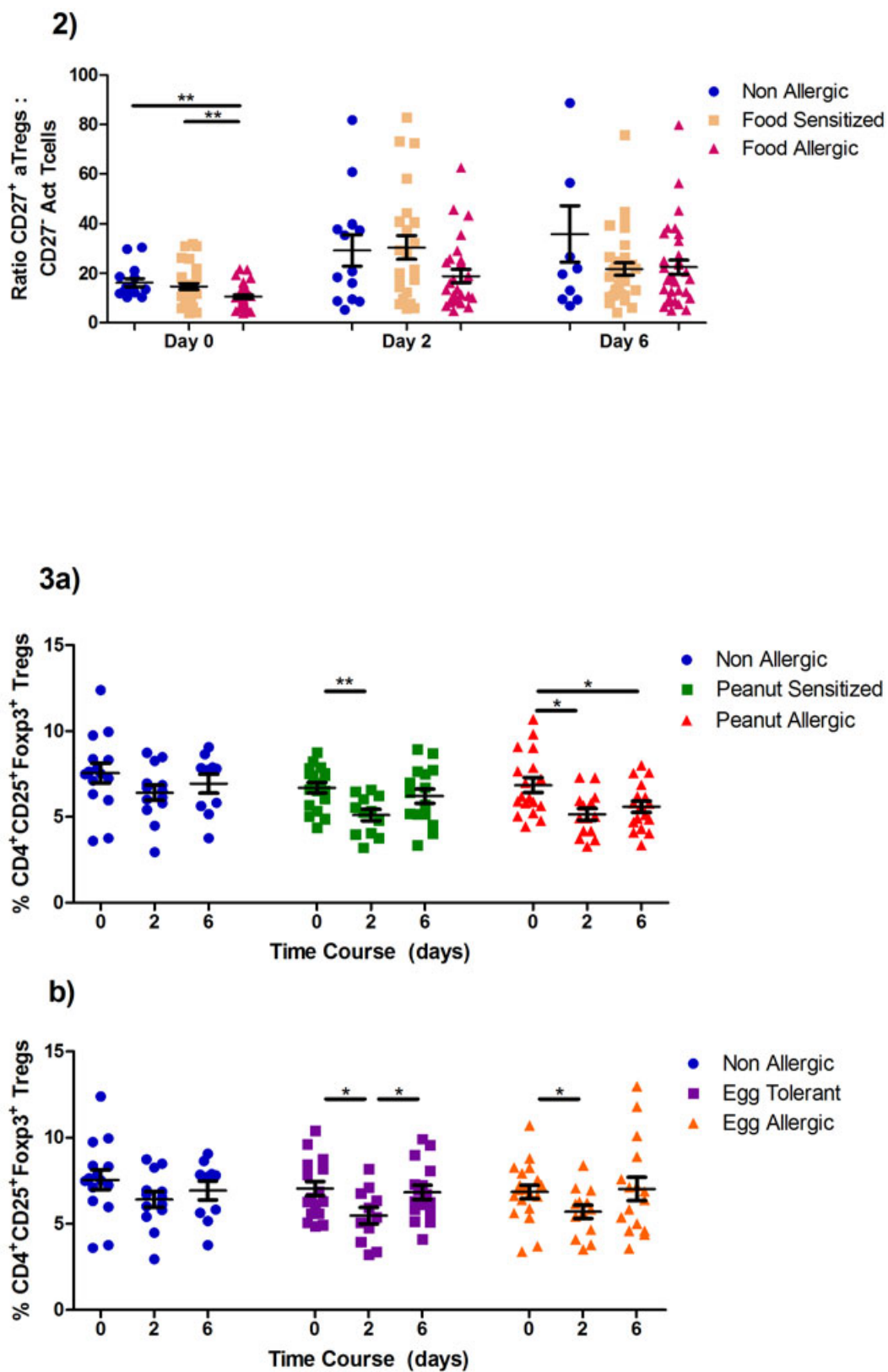
Cells were washed once with PBS and stained with fluorochrome-conjugated monoclonal or isotype control antibodies in 100 μ l staining volumes for 30 minutes at room temperature.

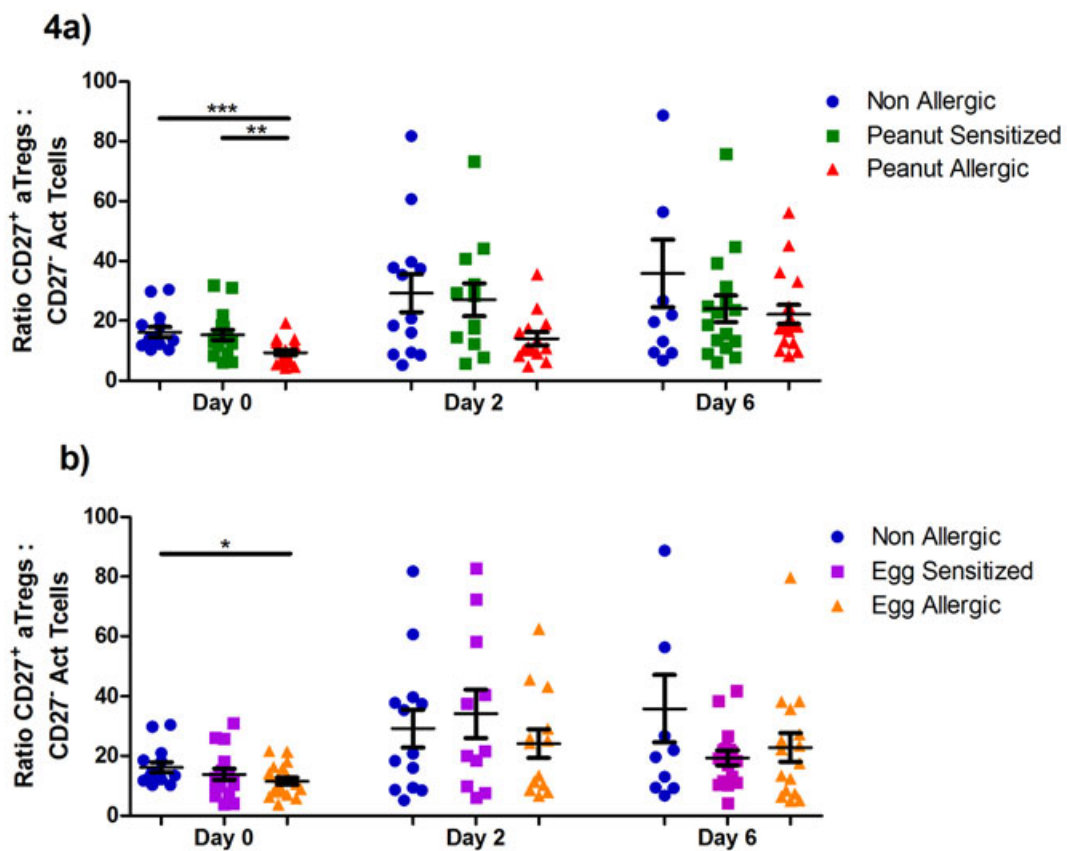
Cells were first stained with the following surface stains: anti-CD4 (-PerCP, Clone SK3), anti-CD25 (-PE-Cy7, Clone M-A251), anti-CD27 (-V450, Clone M-T271), and anti-CD45RA (-APC, Clone HI100) all from Becton Dickinson (BD, San Jose, CA). For isotype staining, antibodies used were PerCP (Clone X56), APC (Clone MOPC-21), and V450 (Clone 2D1) mouse IgG₁ isotype antibodies (BD, San Jose, CA). For intracellular staining cells were subsequently permeabilised, fixed and stained with anti-Foxp3 (-PE, Clone 259D/C7 from BD) antibody according to the manufacturer's instructions (BD, San Jose, CA). All flow cytometric data were acquired on a 10-colour LSR II (BD, San Jose, CA) and analysed with FACS Diva v8.2 software using well defined gating strategies. Compensation experiments were performed using positive and negative control beads (BD, San Jose, CA). The same compensation settings were used for each flow cytometric analysis. Data was captured from $\geq 200,000$ events to obtain $\geq 75,000$ CD4⁺ lymphocyte events.

Gating on total, activated, and naïve Treg populations

The total Treg population is characterised by CD4⁺CD25^{hi}Foxp3⁺ and the frequency is calculated as a percentage of CD4⁺ cells (Figure S2a). Naïve Tregs were characterised by CD4⁺CD25^{hi}Foxp3⁺CD45RA⁺ and activated Tregs by CD4⁺CD25^{hi}Foxp3⁺CD45RA⁻. In further analyses, CD27 was used to distinguish Foxp3⁺ activated Tregs (CD27⁺) from Foxp3⁺ activated T-cells (CD27⁻) (Figure S1a-b).







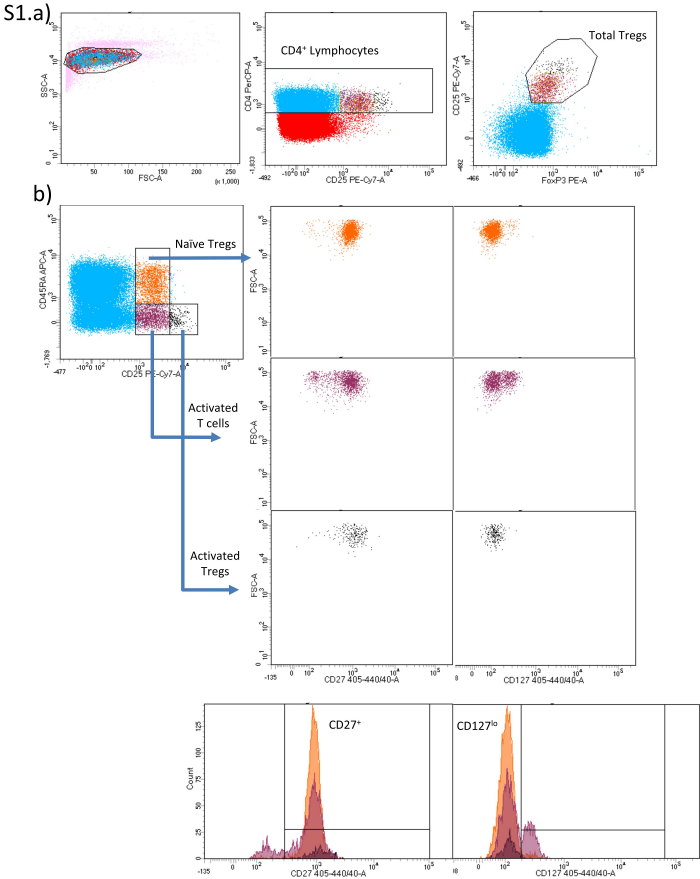


Figure S1.a-b: Gating on total, naïve, and activated Tregs. a) Representative staining on Total Tregs; b) Representative staining of Treg subpopulations of naïve Tregs, activated Tregs, and activated T cells.