

ORIGINAL PAPER

CD4⁺CD25⁺ T cells regulate the intensity of hypersensitivity responses to peanut, but are not decisive in the induction of oral sensitization

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Clinical and Experimental Allergy

Summary

Background Naturally occurring CD4⁺CD25⁺ regulatory T cells (Tregs) play a critical role in the maintenance of self-tolerance and it has been suggested that these Tregs may also be involved in preventing allergic disease.

Objective The precise role of CD4⁺CD25⁺ T cells in the regulation of allergic responses to mucosal antigens remains to be elucidated. In the present study, it was investigated whether CD4⁺CD25⁺ T cells are involved in the induction of oral tolerance and whether they play a role in controlling hypersensitivity responses to food proteins.

Methods CD4⁺CD25⁺ T cells were depleted with PC61 mAb before the induction of low dose oral tolerance to peanut extract (PE). In addition, CD4⁺CD25⁺ T cell depletion was performed during sensitization or before oral challenge, using a C3H/HeOJ mouse model of allergic sensitization to peanut.

Results Oral tolerance to PE could not be induced in CD4⁺CD25⁺ T cell-depleted mice. However, CD4⁺CD25⁺ T cell depletion during long-term exposure to PE alone did not result in allergic sensitization. In sensitized mice, anti-CD25 treatment during oral exposure resulted in higher levels of PE-specific IgE and increased mast cell degranulation upon an oral challenge. In contrast, anti-CD25 treatment of PE-sensitized mice before oral challenges did not affect the level of mast cell degranulation.

Conclusion These results indicate that CD4⁺CD25⁺ Tregs are involved in maintaining tolerance to oral antigens and regulate the intensity of an IgE-mediated food hypersensitivity response, but are not crucial in preventing sensitization. Accordingly, CD4⁺CD25⁺ Tregs may represent a potential tool for the treatment of food allergic disorders.

Keywords allergic sensitization, CD25⁺ regulatory T cells, oral tolerance, peanut allergy

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Introduction

Food allergy has emerged as a major health problem in westernized countries, affecting up to 8% of children under 3 years of age and approximately 2% of the adult population [1, 2] and, at present, no definitive therapy is available. Revealing the underlying immunological mechanisms of sensitization to food proteins may contribute to the development of new therapeutic strategies.

Normally, oral ingestion of food proteins induces active inhibition of the immune response to these antigens, leading to a state of tolerance. Failure to induce oral

tolerance or breakdown of oral tolerance may result in hypersensitivity responses to food antigens [3]. The balance between oral tolerance and active immunity is tightly regulated by different cells of the mucosal immune system and their interactions. Among others, several types of regulatory T cells (Tregs), such as NKT cells, transforming growth factor (TGF)- β -producing Th3 cells, and IL-10-producing Tr1 cells [4] appear to be involved in modulating the immune response to harmless food antigens. Recently, another T cell subset has received attention as a possible mucosal immune regulator i.e. the naturally occurring CD4⁺CD25⁺ T cells, first described by

Sakaguchi et al. [5]. These T cells are characterized by a constitutive high expression of the IL-2 receptor α chain and the selective expression of the transcription factor forkhead box p3 (Foxp3) [6]. Although it was originally suggested that these cells were all thymus-derived, it has become apparent that these cells can also be generated in the periphery [7, 8]. Their immunosuppressive activity is likely mediated by direct cell-cell contact in a process that is dependent on signalling via CTLA-4, as well as cell-bound TGF- β [9]. CD4⁺CD25⁺ Tregs play a critical role in the maintenance of self-tolerance and have been shown to prevent the development of organ-specific autoimmune disease and allograft rejection [10]. Currently, there is considerable interest in whether this subset of Tregs is also involved in prevention of sensitization to allergens or in controlling an established allergic sensitization. It has been demonstrated in mouse models that oral exposure to protein induces activation of functional CD4⁺CD25⁺ T cells in the gut-draining lymph nodes [11, 12]. Furthermore, patients with X-linked autoimmunity-allergic dysregulation syndrome, which is caused by a *FOXP3* mutation, often develop atopic dermatitis, elevated serum IgE levels, and food allergy [13]. Finally, it has recently been shown by Karlsson et al. [14] that children, who outgrew their cow's milk allergy and developed tolerance, have a higher percentage of CD4⁺CD25⁺ T cells with regulatory function in the peripheral blood than allergic children. However, the exact role of CD4⁺CD25⁺ Tregs in controlling mucosal immune responses to food antigens remains to be elucidated.

Of all food hypersensitivities, peanut allergy is among the most serious in terms of severity and persistence. This prompted us to investigate the role of CD4⁺CD25⁺ T cells in the development of oral tolerance and sensitization to peanut proteins. For this purpose, an oral tolerance model and an established model of peanut sensitization were used. In the oral tolerance model, oral pre-treatment with peanut extract (PE) before intraperitoneally (i.p.) immunization with PE plus alum leads to suppressed systemic PE-specific immune responses. In the long-term sensitization model, exposure to PE plus the mucosal adjuvant cholera toxin (CT) induces PE-specific antibody levels and subsequent challenge responses, whereas exposure to PE alone induces a state of non-responsiveness to peanut [15]. In these two different models, CD4⁺CD25⁺ T cells were depleted using a monoclonal antibody (mAb).

We show here that the induction of oral tolerance to PE is impaired in CD4⁺CD25⁺ T cell-depleted animals but that depletion of CD4⁺CD25⁺ T cells is not sufficient to induce allergic sensitization in animals exposed to PE in the absence of the adjuvant CT. Nevertheless, in sensitized mice, depletion of CD4⁺CD25⁺ T cells resulted in increased allergic responses to PE.

These results suggest that CD4⁺CD25⁺ T cells play an important role in both controlling tolerance induction and

regulating the intensity of allergic responses to peanut, but are not exclusively decisive in preventing allergic sensitization.

Methods

Mice and reagents

Five-week-old female specific pathogen-free C3H/HeOJ Ico mice purchased from Charles River (Lyon, France) were maintained under barrier conditions in filter-topped macrolon cages. Drinking water and peanut-free laboratory food pellets were provided *ad libitum*. The experiments were approved by the animal experiments committee of the faculty of veterinary medicine, Utrecht University.

Peanuts from the Runner cultivar (Cargill, Dawson, GA, USA) were kindly provided by Imko Nut Products (Doetinchem, the Netherlands) and PE was prepared as previously described [15, 16].

Chemicals were obtained from Sigma Aldrich (Zwijndrecht, the Netherlands), unless stated otherwise.

In vivo depletion of CD4⁺CD25⁺ T cells

CD4⁺CD25⁺ T cells were depleted *in vivo* by i.p. administration of purified rat anti-CD25 (IL-2R α) mAb produced by hybridoma PC61 (kindly provided by Dr G. Leclercq, University of Ghent, Belgium). Control mice received a rat anti-mouse IgG antibody (Rockland Immunochemicals, Gilbertsville, PA, USA), according to the same schedule.

It has been shown that PC61 selectively depletes CD4⁺CD25⁺ T cells *in vivo* and that the deletion is for short term and is reversible [17, 18]. The efficacy of CD25-depletion in mesenteric lymph nodes (MLN) and spleen was confirmed by FACS analysis (Becton Dickinson, San Jose, CA, USA) using FITC-conjugated anti-CD25 mAb (7D4), PE-conjugated anti-CD4 mAb (L3T4), and Cy-conjugated anti-CD3 ϵ mAb (145-2C11), all obtained from BD Pharmingen (Hamburg, Germany). Two days after anti-CD25-treatment (200 μ g at day -2 and 0), an 85% reduction of CD4⁺CD25⁺ cells was found in the MLN and a 50% reduction in the spleen (data not shown). Maximal depletion lasted for at least 7 days and did not differ between oral treatment groups. These results are consistent with previous observations using the same PC61 mAb [17, 18]. Intracellular staining of Foxp3 [PE-conjugated Foxp3 (FJK-16s), eBioscience, San Diego, California, USA] showed that solely CD25⁺Foxp3⁺ T cells were depleted by anti-CD25 treatment, whereas CD25⁺Foxp3⁻ T cells were unaffected. Further flow cytometric analysis also confirmed that anti-CD25 treatment had no effect on B cell, cytotoxic T cell, CD4⁺CD25⁻ T cell, or dendritic cell (DC) percentages in both spleen and MLN (data not shown).

Treatment protocols (Fig. 1)

CD25 depletion before oral tolerance induction. Mice ($n = 5$) were intragastrically exposed to 1 mg PE (to induce oral tolerance) or phosphate-buffered saline (PBS) for 3 consecutive days (days 1–3). Before oral exposure to PE, mice received an i.p. injection with anti-CD25 mAb or rat anti-mouse IgG control antibody (200 μ g at day –2 and 0). Mice were challenged i.p. with 100 μ g PE in 100 μ L NaCl containing 2.5 mg alum at days 24 and 31 and were killed at day 42.

CD25 depletion during long-term oral exposure. Mice ($n = 5$ –6) were orally exposed to PBS, PE alone (6 mg), or PE (6 mg) plus CT. Oral exposure was performed by intragastric dosing for 3 consecutive days (days 1–3), followed by weekly dosing (days 8, 15 and 22). In the PE plus CT group, CT (10 μ g) was co-administered on days 1, 2, 3, 8, 15, and 22 to induce sensitization. Mice were additionally injected i.p. with 200 μ g anti-CD25 mAb or control antibody on days –2, 0, and 14 during the oral dosing regime. At day 31, mice received an oral challenge with 25 mg PE (without adjuvant) and mice were killed at day 7 or 32.

CD25 depletion before oral challenge. Mice ($n = 8$) were orally exposed to PBS, PE, or PE plus CT for 4 weeks as described in the long-term oral exposure protocol. Subse-

quently, groups were injected i.p. with 200 μ g anti-CD25 mAb or control antibody at days 28 and 30. Finally, mice were orally challenged with 25 mg PE at days 31 and 45 and were killed at day 50.

Measurement of serum immunoglobulin G1, immunoglobulin G2a, and immunoglobulin E antibody levels

Blood samples were collected at various time-points and stored at -20°C until analysis. Levels of PE-specific IgG1 and IgG2a and total IgE Abs were determined by ELISA and levels of PE-specific IgE by sandwich ELISA as previously described [15]. Briefly, plates (highbond 3590; Costar, Cambridge, MA, USA) were coated overnight with 20 μ g/mL PE (for IgG1 and IgG2a detection) or with 1.5 μ g/mL purified rat anti-mouse IgE (BD Pharmingen) in carbonate buffer (pH 9.6), followed by 1 h blocking with PBS–Tween/3% milk powder. Each test serum was titrated (at least eight serial dilutions) starting at 1:8 or 1:16 dilution and incubated for 1 h. For PE-specific Ab levels, a titrated pre-sera pool was used as reference value (start dilution 1:4) and a standard curve of a reference serum (2 μ g/mL IgE) was included to determine total serum IgE levels. For detection of IgG1 and IgG2a and total serum IgE Abs, alkaline phosphatase-conjugated IgG1, IgG2a (both polyclonal goat anti-mouse), and IgE (rat anti-mouse, clone 23G3) Abs (Southern Biotechnology

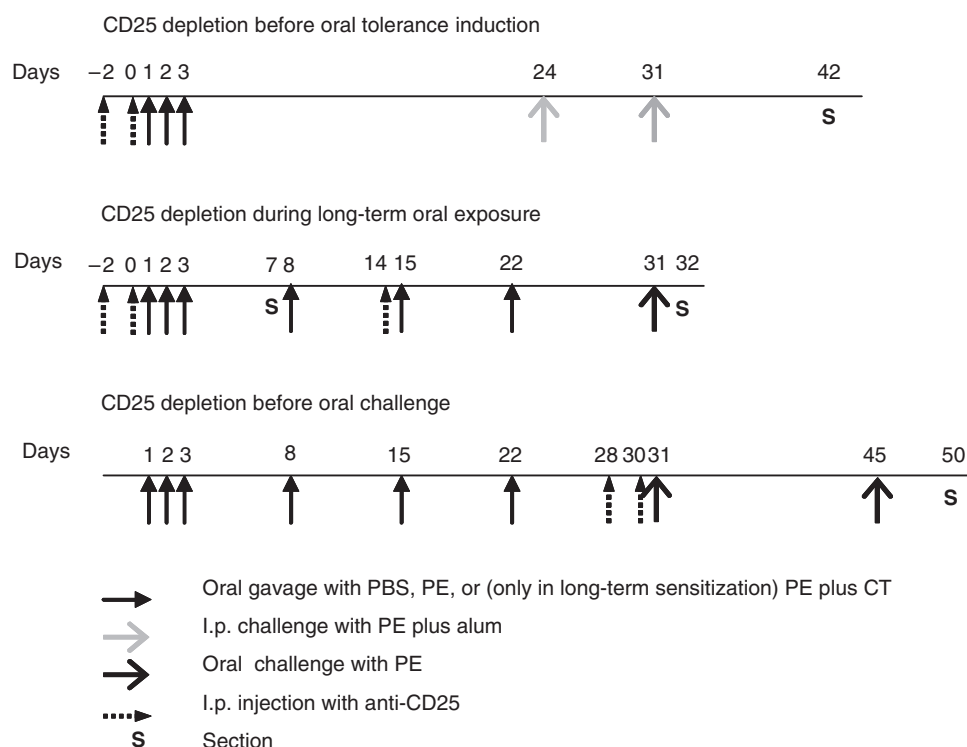


Fig. 1. Treatment protocols.

Associates, Birmingham, AL, USA) were added (1 h at 37 °C). Subsequently, 1 mg/mL *p*-nitrophenylphosphate in diethanolamine buffer was used for the colour reaction, which was stopped with a 10% EDTA solution and absorbance was measured at 405 nm. To measure PE-specific IgE Abs, serum was incubated for 2 h and subsequently a PE-digoxigenin (DIG) conjugate solution was added (1 h at 37 °C). The coupling of DIG to PE was performed according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). After incubation (1 h at 37 °C) with peroxidase-conjugated anti-DIG fragments (Roche Diagnostics, Mannheim, Germany), a tetramethylbenzidine substrate (0.1 mg/mL) solution was added and the colour reaction was stopped with 2 M H₂SO₄. Absorbance was measured at 450 nm. The reciprocal of the highest test serum dilution resulting in an extinction higher than the reference value was read as a titre.

Cell culture and cytokine measurements

Spleen and MLN single cell suspensions [3.75×10^5 cells in 200 µL complete RPMI 1640 (Life Technologies, Paisley, Scotland) containing 10% fetal calf serum (ICN Pharmaceuticals, Costa Mesa, CA)] were incubated in the presence or absence of 200 µg/mL PE in 96-well plates for 96 h at 37 °C, 5% CO₂. In the culture supernatants, levels of IFN-γ, IL-4, IL-5, IL-10, and IL-13 were determined by sandwich ELISA. IFN-γ, IL-4, and IL-5 capture and biotin-conjugated antibodies were obtained from BD PharMingen, and the ELISAs were performed as previously described [15]. The IL-10 (BD Pharmingen) and IL-13 ELISA (Biosource, Camarillo, CA, USA) were performed in accordance with the manufacturer's instructions.

Measurement of serum mmcp-1

Blood was collected before and 45 min after oral challenge with PE and serum levels of mmcp-1 were determined using an ELISA kit (Moredun Scientific Ltd, Midlothian, Scotland). The ELISA was performed according to the manufacturer's instructions.

Statistics

Data were analysed using SigmaStat statistical software package (SPSS, Chicago, IL, USA). The differences between group means were determined by using one-way ANOVA with Bonferroni as *post hoc* test. For cytokine levels and mmcp-1 serum levels, statistical analysis was performed following logarithmic transformation (to achieve normal distribution). A value of $P < 0.05$ was considered statistically significant.

Results

Oral tolerance induction to peanut extract is impaired in the absence of CD4⁺CD25⁺ T cells

In the low dose oral tolerance model, mice were exposed to 1 mg PE or PBS for 3 consecutive days. After 21 days, mice were immunized i.p. with PE plus alum twice within a 1-week interval and killed 2 weeks later (Fig. 1). In this model, oral pre-treatment with PE induces significantly lower levels of Th2-associated cytokines IL-4, IL-5, and IL-13 upon *in vitro* restimulation of splenocytes and lower serum levels of PE-specific IgG1 compared with the PBS-exposed controls. To determine whether oral tolerance to PE can be established in the absence of CD4⁺CD25⁺ T cells, CD4⁺CD25⁺ T cells were depleted before oral exposure to PE. In the anti-CD25-treated group, cytokine (Fig. 2a) and PE-specific IgG1 levels (Fig. 2b) were significantly higher than levels found in the PE-exposed, control antibody-treated group and, although not statistically significant, they were slightly higher than those found in PBS-dosed control mice. These results show that suppression of systemic Th2-associated PE-specific responses, achieved by oral dosing of PE, cannot be established after CD4⁺CD25⁺ T cell depletion.

Depletion of CD4⁺CD25⁺ T cells enhances peanut extract-specific antibody levels, cytokine levels and challenge response in sensitized mice, but does not abrogate non-responsiveness to peanut extract

Since our data indicate that induction of oral tolerance is impaired in the absence of CD4⁺CD25⁺ T cells, we next investigated whether CD4⁺CD25⁺ T cell depletion results in allergic sensitization to orally administered proteins. To address this question we used repeated oral exposure to PE without adjuvant, which induces non-responsiveness [15]. In addition, the effect of CD4⁺CD25⁺ T cell depletion during an ongoing peanut allergic response (oral exposure to PE plus CT) was investigated. Hence, mice were orally exposed for 4 weeks to PBS, PE alone or PE plus CT, and groups were treated with anti-CD25 depleting mAb or isotype control during the oral dosing regime (Fig. 1).

Groups of mice were killed 7 days after the first exposure in order to determine early cytokine responses. Splenic and MLN single cell suspensions were cultured in the presence or absence of PE and cytokine production was measured in the supernatant. At this time-point (7 days after initial exposure), no cytokines were detected in the MLN cultures of any of the exposure groups following PE restimulation. Furthermore, in agreement with earlier studies, only in spleen cultures of PE plus CT-exposed mice significant IL-4, IL-13, IL-10, and IFN-γ cytokine levels were observed following restimulation with PE (Fig. 3). CD25 depletion before oral exposure to

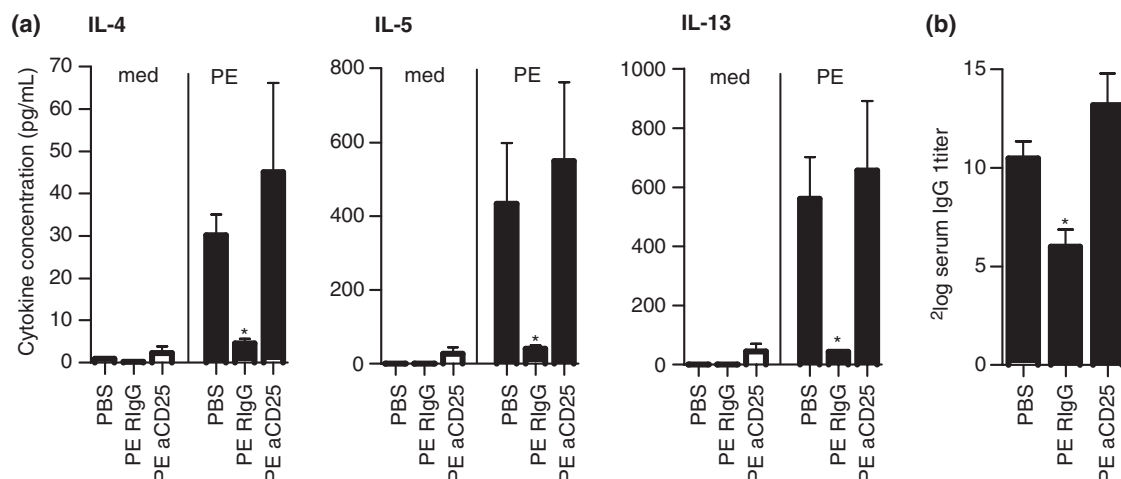


Fig. 2. Effect of anti-CD25 treatment on the induction of oral tolerance to peanut extract (PE). Mice were orally exposed to phosphate-buffered saline (PBS) or PE and challenged intraperitoneally 21 and 28 days after the last oral exposure with PE plus alum. Indicated groups received depleting anti-CD25 mAb (aCD25) or RlgG before oral exposure. Mice were killed 7 days after the last challenge. (a) Cytokine levels in splenocyte culture supernatants. Cell suspensions were cultured in the presence (black bars) or absence (white bars; med = medium only) of PE. Data are presented as the group mean $\pm$ SEM of five mice per group. (b) PE-specific serum IgG1 antibodies. The data are presented as the mean $^2\log$ antibody titre $\pm$ SEM of five mice per group. *Significantly different ($P < 0.05$) from the PBS-exposed group. Data are representative of two independent experiments.

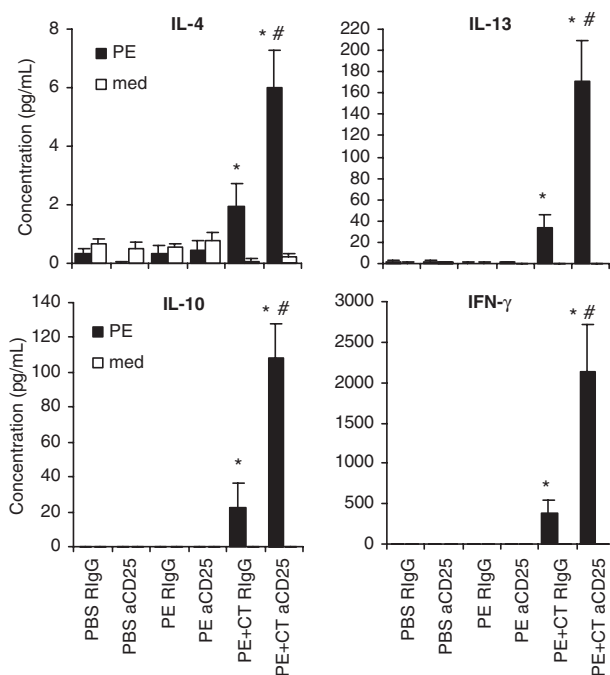


Fig. 3. Effect of anti-CD25 treatment on cytokine levels in splenocyte culture supernatants. Mice were orally exposed to (phosphate-buffered saline) or peanut extract (PE) with or without cholera toxin (CT). Indicated groups received depleting anti-CD25 mAb (aCD25) or RlgG before oral exposure. Splens were removed 7 days after the initial oral exposure and cells were cultured in the presence (black bars) or absence (white bars; med = medium only) of PE. Data (representative of two independent experiments) are presented as the group mean $\pm$ SEM of five mice per group. *Significantly different ($P < 0.05$) from the corresponding control antibody (RlgG)-treated group. #Significantly different ($P < 0.05$) from the corresponding non-CT-treated group.

PE plus CT resulted in a further increase in cytokine production. This elevation appeared to be PE-specific since no cytokine production was observed in cultures with medium alone. Cell cultures derived from PBS- or PE-exposed groups showed no cytokine production and this was not altered by anti-CD25 treatment.

Accordingly, mice exposed to PE plus CT for 4 weeks developed PE-specific IgG1, IgG2a, and IgE serum antibodies (Fig. 4a). In this group, depletion of CD4⁺CD25⁺ T cells during sensitization resulted in significantly higher levels of both Th2-associated (IgG1 and IgE) and Th1-associated (IgG2a) antibodies. Mice exposed to PE alone showed low levels of PE-specific IgG1 and IgG2a and no IgE. In this exposure group, anti-CD25 treatment had no effect on the antibody levels confirming that the non-responsiveness to peanut induced by oral exposure to PE without adjuvant is not abrogated after CD4⁺CD25⁺ T cell depletion. Interestingly, in contrast to PE-specific IgE levels, anti-CD25 treatment clearly augmented total IgE production in all exposure groups (Fig. 4b).

Anti-CD25 treatment also increased the production of IL-4, IL-13, and IFN- γ in spleen cultures, irrespective of the oral exposure (PBS, PE, or PE plus CT) and culture conditions (with or without PE restimulation) (Fig. 5). However, cytokine levels in PE-restimulated cultures from PE plus CT-exposed mice were markedly elevated when compared with cultures from PBS- or PE-exposed mice. In RlgG-treated groups, significant cytokine production was only found in PE-restimulated spleen cultures of the PE plus CT-exposed group. In MLN cell cultures of PBS- or PE-exposed mice, anti-CD25 treatment only slightly increased cytokine production compared with RlgG-treatment, irrespective of antigen restimulation. In contrast,

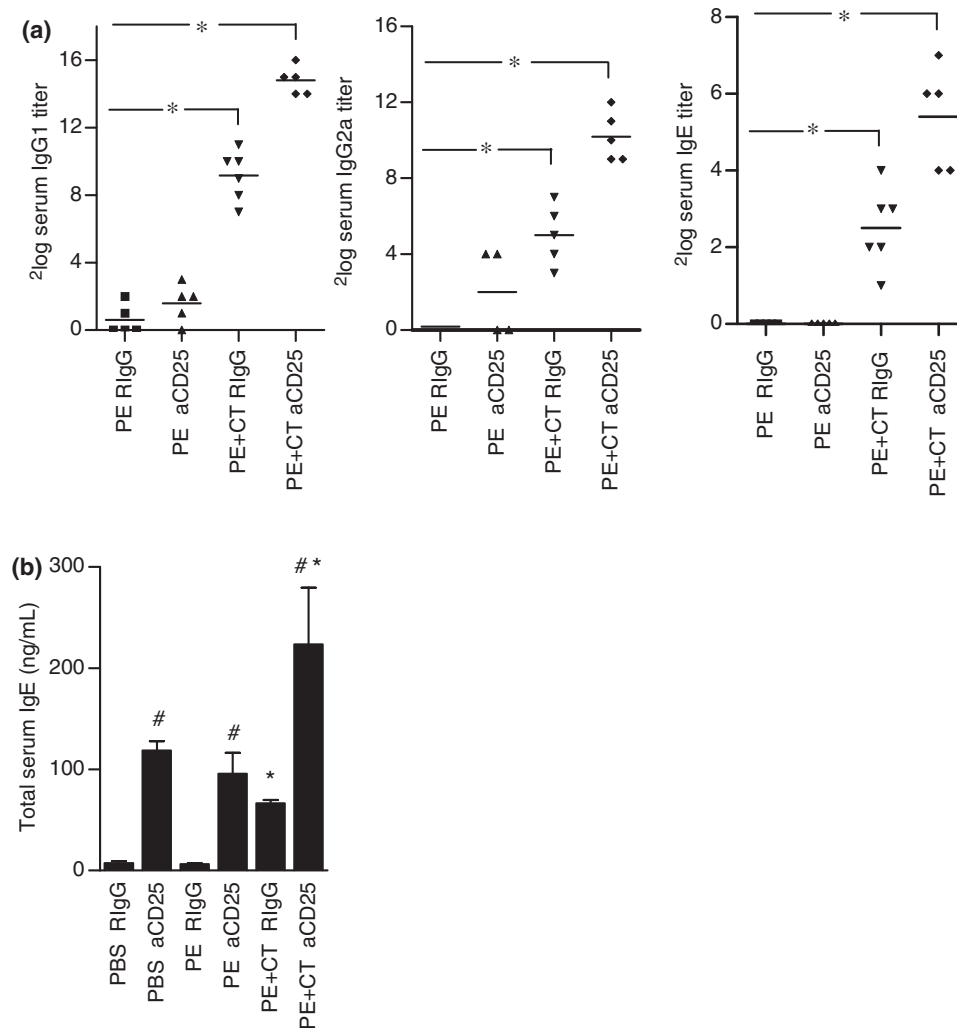


Fig. 4. Effect of anti-CD25 treatment on antibody production. Mice were orally exposed to phosphate-buffered saline, or peanut extract (PE) with or without cholera toxin (CT). Indicated groups received depleting anti-CD25 mAb (aCD25) or RlgG during the oral exposure protocol. (a) PE-specific Th1-associated (IgG2a) and Th2-associated (IgG1 and IgE) serum antibody levels in end serum. The data are presented as the mean ²log antibody titre of five to six mice per group (symbols indicate individual animals). *Significantly different ($P < 0.05$) from groups covered by the horizontal line. (b) Total IgE serum levels. The data (representative of two independent experiments) are presented as the group mean $\pm$ SEM of five mice per group. #Significantly different ($P < 0.01$) from the corresponding control antibody (RlgG)-treated group. *Significantly different ($P < 0.05$) from the corresponding non-CT-exposed group.

cytokine levels in MLN cultures from PE plus CT-exposed mice were significantly increased when cells were restimulated with PE.

After the 4-week oral exposure regime, all mice received an oral challenge with PE and the serum concentration of mmcp-1 45 min after challenge was used as a read-out for the challenge response. In accordance with PE-specific IgE levels, mast cell degranulation upon an oral challenge was only found in the PE plus CT-exposed mice (Fig. 6). In this group, treatment with anti-CD25 during sensitization resulted in a higher level of mmcp-1 than treatment with the isotype control. Exposure to PE alone induced no significant mast cell degranulation compared with the control group, irrespective of anti-CD25 treatment.

Depletion of CD4⁺CD25⁺ T cells before oral challenge does not result in enhanced mast cell degranulation

It has been hypothesized that Tregs may directly or indirectly affect effector cells such as mast cells and basophils [19, 20]. Therefore, it was examined whether depletion of CD4⁺CD25⁺ T cells before oral challenges would also affect the challenge response. For this purpose, mice were orally exposed for 4 weeks to PBS, or PE with or without CT. Subsequently, mice were treated with anti-CD25 mAb or isotype control and orally challenged with PE, 1 day and 2 weeks after the last antibody treatment (Fig. 1). Only mice exposed to PE plus CT showed a significant increase in mmcp-1 serum levels after the first and second oral challenge (Fig. 7). Anti-CD25 treatment

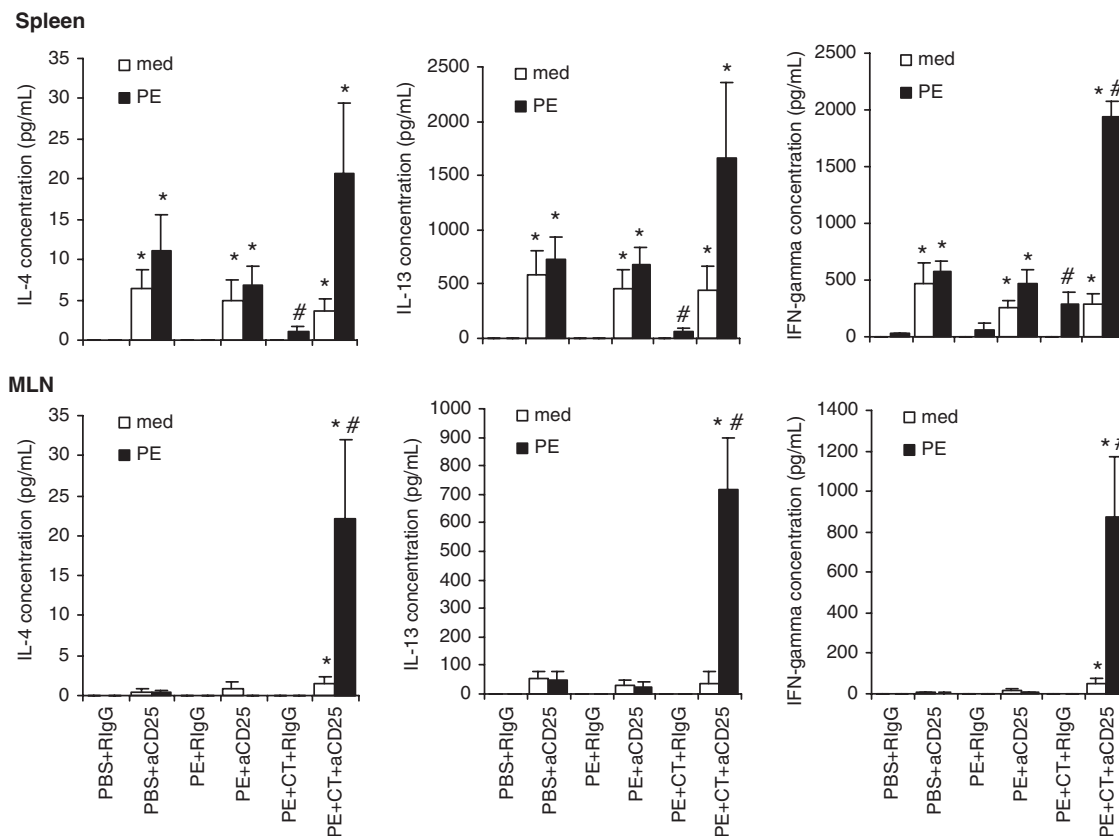


Fig. 5. Cytokine levels in the spleen and mesenteric lymph nodes (MLN), 4 weeks after the initial oral exposure. Mice were orally exposed to phosphate-buffered saline or peanut extract (PE) with or without cholera toxin (CT). Indicated groups received depleting anti-CD25 mAb (aCD25) or RlgG during the oral exposure protocol. Spleen and MLN single cell suspensions were cultured for 96 h in the presence (black bars) or absence (white bars; med = medium only) of PE. Data are presented as the group mean $\pm$ SEM of five to six mice per group. *Significantly different ($P < 0.05$) from the corresponding RlgG-treated group. #Significantly different ($P < 0.05$) from the corresponding non-CT-treated group.

before the oral challenges did not affect the extent of mast cell degranulation in any of the exposure groups suggesting that $CD4^+CD25^+$ T cells are not directly involved in controlling the challenge response to peanut.

Discussion

$CD4^+CD25^+$ T cells have been described to be important in maintaining self-tolerance [21] but they also appear to play a role in the induction of tolerance to exogenous antigens [22]. It has been shown that oral exposure to ovalbumin (OVA) in OVA TCR transgenic mice induces proliferation of $CD4^+CD25^+$ T cells that are able to suppress proliferation of $CD4^+CD25^-$ T cells *in vitro* [11, 12] and to transfer tolerance to wild-type mice [11, 23]. However, it remains to be elucidated whether $CD4^+CD25^+$ T cells are involved in controlling tolerance induction to orally administered antigen in non-transgenic mice. In the present study, we have demonstrated that $CD4^+CD25^+$ T cells are crucial for the induction of oral tolerance to PE. In $CD4^+CD25^+$ T cell-depleted animals, PE-specific IgG1 antibodies and PE-induced cytokine production were no longer suppressed by oral pre-treatment with a low dose

of PE. In line with our results, $CD4^+CD25^+$ T cells were recently shown to be involved in the induction of low dose oral tolerance to OVA [24].

While it is clear that Th2 responses play a pivotal role in the development of allergic responses, it remains unclear which mechanisms are involved in controlling and/or preventing allergic sensitization. Therefore, we additionally examined the role of $CD4^+CD25^+$ T cells in the regulation of allergic sensitization in an established model of peanut hypersensitivity [15]. In the present study we demonstrate that depletion of $CD4^+CD25^+$ T cells during allergic sensitization to peanut (exposure to PE in the presence of the adjuvant CT) results in increased PE-induced cytokine production, PE-specific IgE levels, and challenge responses. In support of a role for $CD4^+CD25^+$ T cells in controlling Th2 responses, it has been shown that infusion of mouse $CD4^+CD25^+$ T cells prevents high serum IgE levels in transgenic mice [25] and that $CD4^+CD25^+$ T cells suppress the *in vitro* differentiation of Th2 cells [26]. In healthy individuals $CD4^+CD25^+$ T cells have been shown to control allergen-specific T cell responses [27, 28] and Karlsson et al. [14] have demonstrated that when allergy to cow's milk is outgrown, this is

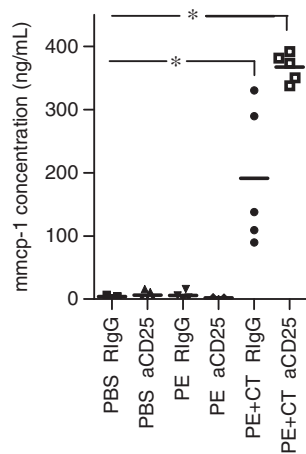


Fig. 6. Mmcp-1 serum levels upon an oral challenge with peanut extract (PE). Mice were orally exposed to phosphate-buffered saline or PE with or without cholera toxin (CT) and challenged at day 31. Indicated groups received depleting anti-CD25 mAb (aCD25) or RlgG during the oral exposure protocol. Levels of mmcp-1 were determined in blood samples collected before and 45 min after the oral challenge and the increase in mmcp-1 after challenge is shown. The data (representative of two independent experiments) are presented as the group mean (symbols indicate individual animals) of five to six mice per group. *Significantly different ($P < 0.05$) from groups covered by the horizontal line.

associated with the development of CD4⁺CD25⁺ T cells that are capable of suppressing allergen-specific effector cells. Additionally, it has been recently reported that CD4⁺CD25⁺ T cell depletion before sensitization enhances the asthma phenotype in an experimentally induced asthma model [29]. Similarly, our data indicate that CD4⁺CD25⁺ T cells play an important role in controlling the intensity of allergic sensitization to orally administered antigens.

Data presented in this study additionally revealed that mast cell degranulation upon oral challenge was not affected when CD4⁺CD25⁺ T cells were depleted before oral challenge, whereas depletion during sensitization did enhance the challenge response. Hence, CD4⁺CD25⁺ T cells appear to be mainly involved in the induction phase of an allergic response and do not play a major role in the challenge response, when sensitization has already been established.

The increase in Th2-associated responses induced by anti-CD25 treatment was accompanied by an enhancement of the Th1-associated response (IFN- γ production and serum IgG2a levels). These data demonstrate that CD4⁺CD25⁺ T cells control both Th1 and Th2 to orally administered proteins.

There has been some debate on the mechanism of suppression by CD4⁺CD25⁺ T cells. *In vitro* studies have shown that CD4⁺CD25⁺ T cells exert their regulatory activity on other T cells via a cell-contact-dependent mechanism mediated by either CTLA-4 signalling [30] or membrane-bound TGF- β [31]. *In vivo* studies have addi-

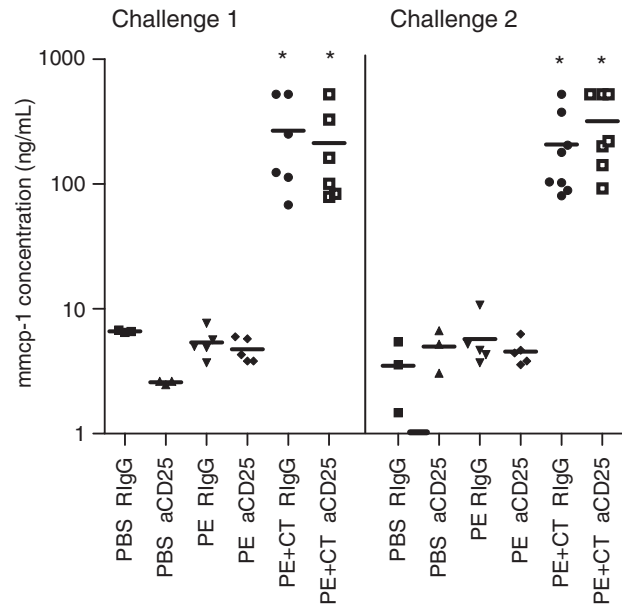


Fig. 7. Mmcp-1 serum levels upon oral challenges with peanut extract (PE), 1 day and 2 weeks after CD4⁺CD25⁺ T cell depletion. Mice were orally exposed to phosphate-buffered saline or PE with or without cholera toxin (CT) and challenged at day 31 and 45. Indicated groups received depleting anti-CD25 mAb (aCD25) or RlgG 1 day before the first oral challenge. Levels of mmcp-1 were determined in blood samples collected 45 min after the oral challenge. The data are presented on a log scale as the group mean (symbols indicate individual animals) of five to eight mice per group. *Significantly different ($P < 0.01$) from all non-CT-exposed group.

tionally shown that certain forms of immunity can be suppressed by IL-10 secreting CD4⁺CD25⁺ T cells [32, 33]. Our unpublished data suggest that, in the present sensitization model, neutralization of TGF- β and/or IL-10 does not have a major influence on PE-induced immune responses. However, in a previous study we have shown that blockade of the CTLA-4-signalling pathway results in enhanced allergic sensitization to peanut [15]. Hence, in the present model, the suppressive function of CD4⁺CD25⁺ T cells might be mediated by CTLA-4-dependent interaction of these Tregs with other T cells or antigen-presenting cell. Additionally, CD4⁺CD25⁺ T cells may alter DC phenotype and function. Lewkowich et al. [29] have shown that CD4⁺CD25⁺ T cell depletion in a murine asthma model induces increased CD80 and CD86 expression on pulmonary DCs following allergen challenge. Accordingly, in our model anti-CD25 treatment during oral exposure induced a significant increase in CD80 and CD86 expression on MLN and spleen DCs (data not shown). However, this effect was only observed in the sensitized (PE plus CT-exposed) group and but also the PE- or PBS-exposed groups. Together, it is likely that *in vivo* CD4⁺CD25⁺ T cells suppress immunity by several distinct mechanisms that may depend on the microenvironment in which these cells are activated and further

research is needed to delineate the mechanisms of CD4⁺CD25⁺ T cell-mediated suppression upon oral antigen administration.

Failure to induce or breakdown of oral tolerance may be one of the processes that precede the induction of allergic sensitization. Interestingly, although CD4⁺CD25⁺ T cells appear essential for the induction of oral tolerance to PE, our data show that depletion of CD4⁺CD25⁺ T cells is not sufficient to induce allergic sensitization after long-term exposure to PE without adjuvant. Data on the early-phase of sensitization (day 7) demonstrated that anti-CD25 treatment increased cytokine production solely in sensitized mice and also IgE production and challenge responses were only affected in sensitized mice. These results suggest that, in the absence of CD4⁺CD25⁺ T cells, an additional stimulus (such as the adjuvant activity of CT) is required to induce allergic sensitization to an orally administered protein. We speculate that, in the absence of CD4⁺CD25⁺ T cells, other local regulatory factors in the gut such as specialized subsets of DCs and other subsets of Tregs create a suppressive environment, which prevents the development and function of effector T cells and subsequent allergic sensitization to oral antigens. The local suppression in the gut of orally induced responses may then coexist with impaired peripheral tolerance to the same antigen. This is confirmed by the observation that despite the failure to induce PE-specific IgE levels, total IgE levels were increased in PE plus anti-CD25-treated mice compared to PE-treated mice. Furthermore, depletion of CD4⁺CD25⁺ T cells led to significantly increased cytokine responses in the spleen but not in the MLN of PBS and PE-treated mice. This proposes a more extensive regulation of immune responses to orally administered antigens than to systemic (presumably self-) antigens. In line with these results, humans that have a mutation in the *FOXP3* gene do not always develop food allergy [34], which supports the necessity for an additional trigger for the induction of sensitization. Although it cannot be ruled out that complete depletion of CD25⁺ Tregs could lead to allergic sensitization to oral antigens, our data suggest that allergic sensitization via the oral route is not solely regulated by CD25⁺ Tregs.

In conclusion, data presented in this manuscript provide new insights into the role of CD4⁺CD25⁺ T cells in the regulation of immune responses to orally administered proteins. The results suggest that CD4⁺CD25⁺ T cells are crucial for the induction of tolerance to peanut proteins. Furthermore, although CD4⁺CD25⁺ T cells appear not to be exclusively decisive in the prevention of allergic sensitization, they are involved in regulating hypersensitivity responses to peanut especially in an early stage of sensitization. Accordingly, CD4⁺CD25⁺ T cells may represent a potential tool for the treatment of food allergic disorders or even for the re-establishment of tolerance.

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